ABSTRACT

Title of dissertation: **DIFFERENTIATION AND REGULATION OF BOVINE TH2 CELLS**

Anmol Kandel, Doctor of Philosophy, 2024

Professor Dr. Zhengguo Xiao
University of Maryland Department of Animal and Avian sciences

Memory CD4+ T cells, specifically type-2 (Th2) cells, are pivotal in defending against infections caused by extracellular pathogens, including several economically important parasites. However, whether interleukin-4 (IL4) expression is a signature feature of bovine Th2 cells likewise in mice and humans is unclear. Pasture-raised cattle, routinely exposed to extracellular parasites such as *Ostertagia ostertagi* (OO), are likely to develop a typical Th2 memory response. Therefore, using cytokine induction assay, we evaluated the circulatory memory bovine T cell profile of these cattle and also analyzed if expression of presumptuous memory marker, CD45RO, is reliable in identifying memory bovine T cells. Surprisingly, the majority of the memory CD4+ T cells dominantly produced interferon-gamma (IFNγ), with only a small fraction co-expressing IL4, and memory bovine T cell identification did not correlate with CD45RO expression. Results suggested that cattle naturally exposed to extracellular parasites do not develop typically IL4 dominant Th2 response. To further investigate these results, resting CD4+ T cells isolated from healthy cattle blood were cultured under simple in vitro Th2 culture. Analysis of differentiated cells through

flow cytometry revealed limited IL4 protein detection, which was in line with the lack of upregulation of IL4 and its master regulator GATA3 transcripts shown by the quantitative polymerase chain reaction (qPCR) assay. To validate whether differentiated cells were actually Th2, unbiased proteomic analysis was conducted. Based on differentially expressed 397 proteins between differentiated cells and naïve phenotype, bovine Th2 differentiation was validated; nonetheless, the process was not found to be associated with IL4 induction. Moreover, despite using published strategies from mice and humans, such as reducing T cell receptor (TCR) stimulation strength and adding exogenous recombinant bovine IL4, expression of IL4 could not be significantly enhanced. Interestingly, differentiated bovine Th2 cells proliferated in the presence of OO antigens, suggesting that extracellular parasites could influence bovine Th2 differentiation, at least in vitro. To validate the results from pathogen-infected tissues and in vitro culture, a panel of anti-parasitic CD4+ single T cell clones was established from five pasture-raised cattle that were infected with OO. Evaluation of memory responses exhibited by the anti-parasitic CD4+ single T cell clones strongly supported IFNγ dominant memory response, and only 20% of them co-expressed IL4 through a small subset of IFN γ + cells. All the data pointed out that bovine CD4+ T cell differentiation is partially distinct from those in mice and humans, and IL4 expression is not a hallmark feature of the bovine Th2 cells.

DIFFERENTIATION AND REGULATION OF BOVINE Th2 CELLS

by

Anmol Kandel,
Department of Animal and Avian Sciences,
University of Maryland, College Park,

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Spring 2024

Advisory Committee:

Professor Dr. Zhengguo Xiao, Chair
Professor Dr. Daniel Nelson
Professor Dr. George Belov
Professor Dr. Debabrata Biswas
Associate professor Dr. Nishant Sunny

Table of Contents

Dedication

To the boundless creative energy that courses through the universe and within each of us, guiding our paths and illuminating our journeys.

Acknowledgements

I am profoundly grateful to my PhD advisor Dr. Zhengguo Xiao, whose unwavering guidance and mentorship have been the cornerstone of my doctoral journey. Without his creative suggestions and support it would have been impossible to achieve this insurmountable goal. His constant attention and suggestions were keys to my success. Dr. Wenbin Tuo, a distinguished USDA scientist, offered invaluable insights and shared resources that significantly enriched the fruition of my endeavors. I extend heartfelt appreciation to Dr. Daniel Nelson for coordinating an enlightening series of lectures on genomics and proteomics, which has broadened my understanding of biological science. Dr. George Belov, had coordinated a journal club, which was one of the enjoyable science discussion forums that I experienced during attending his lectures on recombinant viral vectors class. Dr. Debabrata Biswas has always become my resource person in solving professional confusions, and his advice were really helpful in formulating the plans for my dissertation defense. Dr. Nishant Sunny provided significant insights through his critical feedback, which deserve my heartful appreciations.

The support of my colleagues, particularly Lei Li and Akanksha Hada, falls short on words: their consistent assistance, collaborative spirit, and insightful suggestions were instrumental in shaping this journey to the end. Also, I express my gratitude to Dr. Carol Keefer, whose role as a program director provided unwavering support and inspiration. I also extend appreciation to individuals leading their respective facilities, including Ken Class, director of the Flow Cytometry Core, and Betre Legesse, manager of the DLAR Imaging Core. Special thanks are due to all the members of the Wye Angus farm for their continuous assistance.

Specially, I dedicate this work to my beloved wife, Arya Niraula, whose unwavering trust has been my anchor, and to my dear mother, Nirmala Koirala, whose boundless love has been my constant source of strength. I extend heartfelt gratitude to my maternal uncle, Dhruva Koirala, and Sadhu ram Koirala, for their unwavering support, and to my cherished grandparents, Hari Prasad Koirala and Savitri Koirala, whose profound influence has shaped my life in ways beyond measure. I would also like to remember several family members including my father in law and mother in law, whose contributions are extremely valuable on the unfoldment of my life and career. I would also dedicate this dissertation to each individual, who supported my growth as a person until this moment including revered teachers, saints, scientists, musicians, poets, philosophers and all the inspiring insurmountable personalities in their respective fields.

List of Figures

List of Tables

List of Abbreviations

Th1	Type-1 helper CD4+ T cells
Th2	Type-2 helper CD4+ T cells
IFNγ	Interferon gamma
IL4	Interleukin-4
IgG1	Immunoglobulin γ 1
IgG2	Immunoglobulin γ 2
TCR	T cell receptor
MHC	Major histocompatibility complex
APC	Antigen presenting cells
ILC	Innate lymphoid cells
PBMC	Peripheral blood mononuclear cells
DLN	Draining lymph node
IGLN	Inguinal lymph node
SP	Spleen
IL2	Interleukin-2
Tbx21	T-box transcription factor TBX21
GATA3	GATA binding protein 3
STAT-6	Signal transducer and activator of transcription 6
OO	Ostertagia ostertagi

Chapter:1 CD4+ T cell responses to pathogens in cattle

Anmol Kandel, Magdalena Masello, & Zhengguo Xiao

1.1 Abstract:

Helper CD4+ T cells are essential in shaping effective antibody response and cytotoxic T cell response against pathogen invasion. There are two subtypes of pathogen-specific helper T cells in mice and humans; type 1 (Th1) and type 2 (Th2), with Th1 producing interferon-gamma (IFNγ) and Th2 producing interleukin-4 (IL-4). While effective Th1 controls intracellular pathogens like viruses, efficient Th2 controls extracellular pathogens like most parasites. However, the most predominant CD4+ T cell subtype in cattle is Th0, which produces both IFNγ and IL4, and only exists in small amounts in mice and humans. Moreover, in many bovine infections, both IFNγ and IL4 were detected in the blood and both antigen-specific IgG2 (Th1 associated bovine antibody) and antigen-specific IgG1 (Th2 associated bovine antibody) were upregulated in the serum, suggesting bovine CD4+ T cell responses may vary from those in mice and humans. How bovine CD4+ T cell differentiation differs from that in mice and humans and how some critical bovine pathogens regulate immunity to establish chronic infections are largely unknown. This chapter summarizes current literature and identifies the knowledge gaps to provide insights into future research in the field.

Keywords: bovine, CD4+ T cell differentiation, antigen-specific clones, Th0 responses, pathogens, chronic infections

1.2 Introduction

CD4+ T cells, also called helper T cells, are important regulators of adaptive immune responses, which are antigen-specific and critical in protecting animals from pathogen

infections. The control of intracellular pathogens, such as viruses, primarily depends on antigen-specific CD8+ T cell response, whereas antibodies (produced by B cells) or humoral immune responses is mostly responsible for the control of extracellular pathogens such as most bacteria and parasites. CD4+ T cells are the lynchpin in shaping both CD8+ T cell and antibody responses [4, 5].

Common lymphoid progenitor cells migrate from the bone marrow into the thymus for further development and maturation into T cells. Inside the thymus, these progenitor cells proliferate into a large pool of T cells, with each expressing a unique T cell receptor (TCR) through a genetic recombination process. After TCR recombination, T cells must go through two selection processes, and only a fraction of them pass through these selections to become either CD4+ or CD8+ T cells [6]. Surviving CD4+ T cells then exit the thymus as naïve CD4+ T cells but without the ability to help CD8+ T cells and B cells. To become fully functional, naïve CD4+ T cells need to become activated and differentiated into specialized effector subtypes; helper type 1 (Th1) to facilitate CD8+ T cell responses, and helper type 2 (Th2) to facilitate antibody responses [7]. Naïve CD4+ T cells constantly survey secondary lymphoid tissues to detect pathogens through their antigen-specific TCRs [8]. As opposed to antibodies, which bind directly to pathogens or their derivatives, TCRs can only recognize short chains of amino acids (derived from pathogens) that are presented by major histocompatibility-II (MHC-II) expressed on antigen presenting cells (APCs) [5]. This recognition process provides the 1st signal required to activate naïve CD4+ T cells. Along with the 1st signal, APCs also offer co-stimulation as the 2nd signal and cytokine signaling, as the 3rd signal, to the naïve CD4+ T cell. Combined, these three signals coordinate CD4+ T cell differentiation into distinct effector subtypes with different helper functions [5].

Studies in humans and mice have identified numerous helper subtypes, including: Th1, Th2, Th3, Th9, Th17, Treg, and Tr1 [5, 9]. Among these, Th1 and Th2 are considered to play major roles in defending the host from pathogen invasion [10-12]. Th1 cells help CD8+ T cells to gain killing functions, which leads to apoptosis of infected cells and induces Interferon gamma (IFNγ) mediated immunity [13-16]. On the other hand, Th2 cells help B cells differentiate into plasma cells, which produce pathogen-specific antibodies [17]. Antibodies or humoral immunity contribute to the control of extracellular pathogens by mechanisms like neutralizing toxins, preventing bacterial attachment to the host cell, and stimulating basophil and mast cells to release toxic chemicals that induce the expulsion of large gastrointestinal parasites [18, 19]. Although antibodies are mostly responsible for controlling extracellular pathogens, they can also play important roles in cell-mediated killing of intracellular pathogens [20]. For instance, during intracellular infections in mice, Th1 cells help B cells become plasma cells that secrete antigen- specific immunoglobulin subtype G2a (IgG2a), which in turn can help killing infected cells through antibody dependent cytotoxicity (ADCC) [21, 22]. In short, Th1 is responsible for control of intracellular pathogens mostly through shaping CD8+ T cell responses and Th2 is for control of extracellular pathogens through antibody responses. In addition, antibodies can be involved in both Th1 and Th2 responses, but with unique subtypes, such as IgG2 for Th1, and IgG1 for Th2 in cattle. This will be discussed further in section 2.

There are many similarities in the immune system across species. Therefore, knowledge generated from the research in mice and humans has been extensively applicable to study immune responses in cattle [23-26]. In the past several decades, however, unique features have been discovered in the bovine immune system that are not shared with that of

mice and humans, such as high prevalence of circulating γδ T cells [27], production of IL-10 by γδ T cells [28], regulation of CD4+ T cell activation by neutrophils [29], which are able to secrete IL-10 , and high prevalence of hybrid helper T cells (*i.e.,* co-express both Th1 and Th2 cytokines), which is relatively low in humans and mice [25, 30, 31].

Cattle industry suffers billions of dollar's losses annually due to infections, and many of the commercially available vaccines for cattle are not fully effective [32-35]. Understanding the mechanisms underlying bovine CD4+ T cell differentiation, which seems to be partially different from that of mice and humans, is critical to identify novel strategies to achieve more effective immunity after vaccinations, such as through generating strong Th1 responses against intracellular pathogens and Th2 responses against extracellular pathogens. In this chapter, we will summarize the current knowledge and key findings on bovine CD4+ T cell responses, highlight the existing knowledge gaps, and provide some insights on future directions.

1.3 CD4+ T cells regulate adaptive immunity:

Naive CD4+ T cells exit the thymus and search for pathogen-derived antigens presented by APCs in secondary lymphoid tissues (*e.g.*, lymph nodes and the spleen). During infections, pathogens break through barriers (Physical, chemical etc) of the host to establish infection in the local tissues [36]. As a result, the immune system in the host initiates an inflammatory response through recruitment of immune cells such as neutrophils to the site of infection, which secretes inflammatory cytokines and chemokines [37, 38]. These chemokines provide signals for further recruitment of APCs to the site of infection. APCs constantly search for invading pathogens through recognizing pathogen associated molecular patterns (PAMPS) on pathogens by their pattern recognition receptors (PRRs)[39]. For example, Toll-like

receptor-4 (TLR-4) on APCs can recognize the lipopolysaccharide (LPS) present on the cell membranes of gram-negative bacteria [40]. After recognition, APCs engulf the pathogen, break it down into small peptides, and finally present the peptides to CD4+ T cells in the secondary lymphoid tissue. Recognition of this peptide-MHC-II complex by the TCRs on the naïve CD4+ T cells provides the 1st activation signal, as shown in **Figure 1.1** [41]. At the same time, co-stimulatory molecules on the CD4+ T cell surface (*e.g.*, CD28) recognize their corresponding ligands on the APC surface (*e.g.*, CD80 or CD86), which provides the 2nd activation signal [42]. The final and 3rd signal, which occurs simultaneously with antigen stimulation and co-stimulation, is provided by cytokines such as Interleukin-12 (IL-12) or Interleukin-4 (IL-4) that not only enhance the activation process, but also drive CD4+ T cell differentiation into a specific subtype (*e.g.*, Th1 or Th2) [43]. Therefore, APCs can provide all 3 signals to naïve CD4+ T cells, which facilitates their activation and differentiation **(Figure 1.1)**. Pathogens can regulate host helper T cell response through targeting any of the three signals directly or indirectly, which will be discussed in section 5. Recently, we have reported that bovine CD4+ T cells respond to three signals in a way similar to that in humans and mice [44]. Furthermore, IL-12 and neutrophils can work on bovine CD4+ T cells synergistically to enhance their production of IFNγ [44].

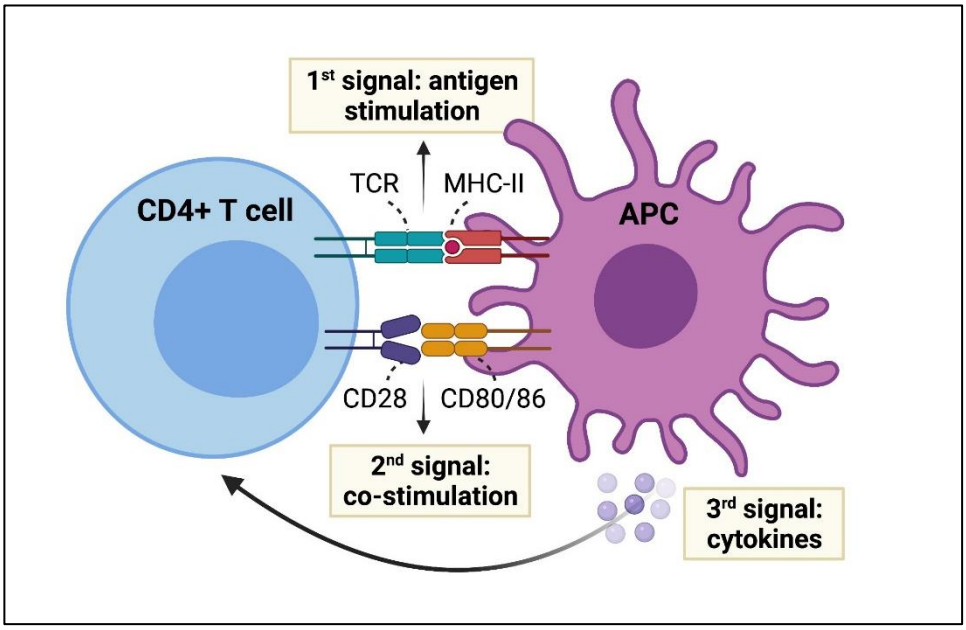

Figure 1.1: Three-signal model for CD4+T cell activation. The 1st signal is provided when TCRs recognize the peptide-MHC-II complex presented by APC; the 2nd signal is initiated when CD28 on CD4+ T cells interacts with CD80/86 on APCs, and the 3rd signal is triggered by cytokines released from the APCs and other cells. CD28/CD80/CD86 interaction is used as an example. This figure was adapted from previous reviews [45-47], and was animated in Bio Render software.

1.3.1 Th1 cells coordinate CD8+ T cell response to intracellular pathogens

During the infection, the host responds to the intracellular pathogens by inducing cytokines such as IFNγ and IL-12 from APCs like macrophages and dendritic cells (DCs), which further leads to the polarization of CD4+ T cells into a Th1 subtype. IFNγ and IL-12 enhance the expression of transcription factor Tbet, which directs Th1 differentiation in the activated naïve CD4+ T cells (**Figure 1.2A**) [48, 49]. More specifically, when bound to their receptors on naïve CD4+ T cells, these cytokines induce the activation of transcription factor STAT-1 or STAT-4 respectively, which in turn causes Tbet upregulation [50]. Subsequently, Tbet induces histone modification and binds to the promoter region of Th1-specific cytokine genes, which leads to enhanced expression of IFNγ [48, 49]. In addition, Tbet also inhibits Th2 differentiation by repressing the transcription of Th2 specific genes, such as *GATA3*, which is the transcription factor responsible for IL4 expression [48, 51]. Thus, IFNγ and IL-

12 induce Th1 differentiation, which leads to IFNγ production and suppression of Th2 differentiation.

One key functions of differentiated Th1 cells is to facilitate the activation of CD8+ T cells by "conditioning" dendritic cells; a process that induces dendritic cell (DC) maturation by modifying their cytoskeletal structure, upregulating co-stimulatory molecules, and by enhancing their migration to secondary lymphoid tissues [52-54]. Once conditioned, these DCs can induce CD8+ T cell activation as shown in **Figure 1.2B**. Although these two processes, conditioning of DCs and activation of CD8+ T cells, might occur simultaneously, some researchers argue that this process may occur in two sequential steps: conditioning DC first, followed by CD8+ T cell activation [53, 55, 56]. Activated CD8+ T cell secretes cytotoxicity-related proteins such as perforin and granzyme-B. While perforin forms pores at the cell membrane, granzyme enters through these pores and cause apoptosis of the infected cell [57]. Additionally, antigen-specific CD8+ T cells can kill infected cells through caspase mediated pathway, when Fas molecules expressed on the infected cells interact with Fas Ligand expressed on the antigen-specific CD8+ T cells [58].

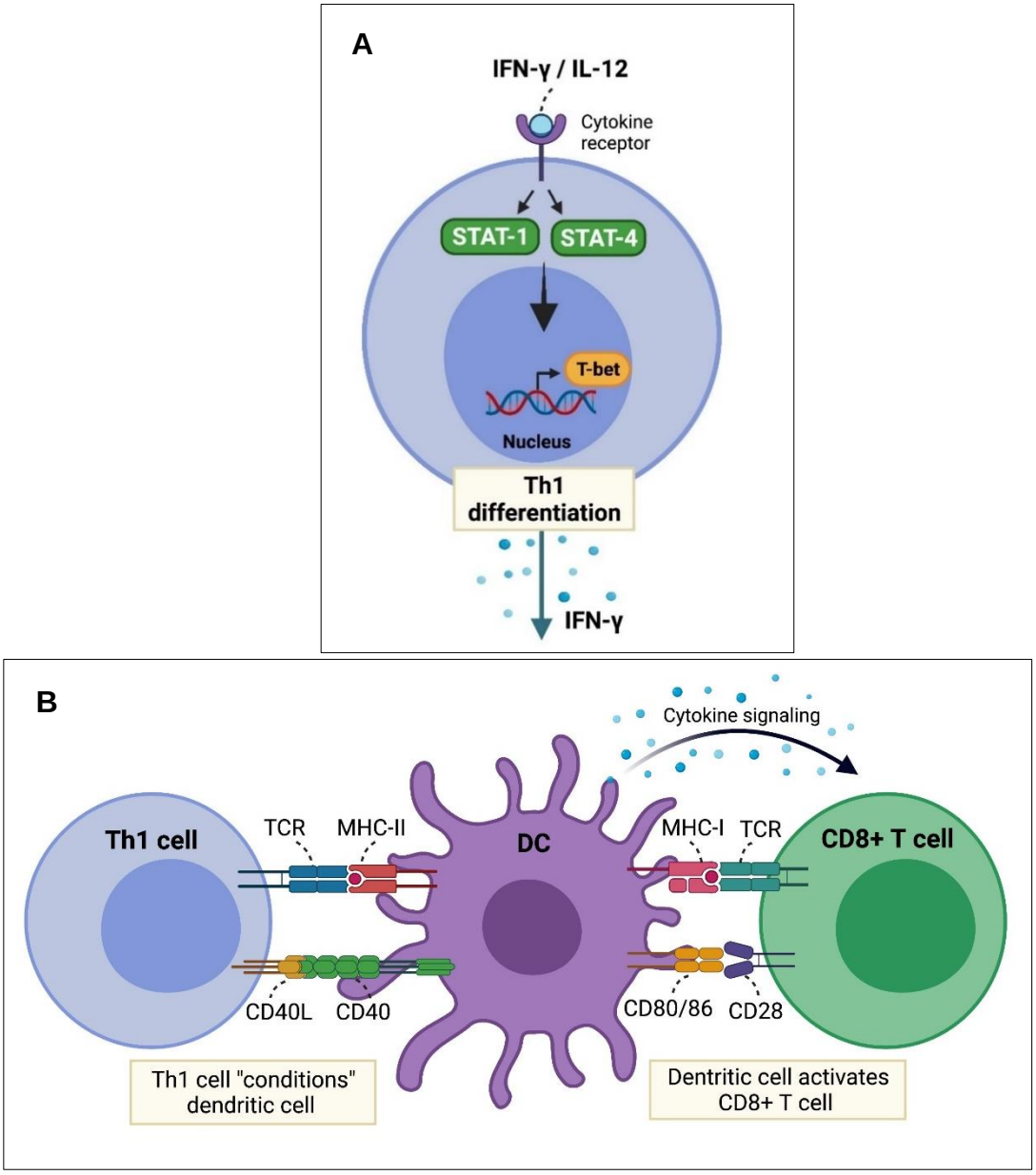

Figure 1.2: Th1 help to the activation of CD8+ T cell. A) IFNγ and IL-12 bind to their corresponding receptors on naïve CD4+ T cells during activation, which leads to Tbet expression and Th1 differentiation. This figure was adapted from previous reviews [59-61]. B) Once differentiated, Th1 effector cell conditions dendritic cell, which in turn activates CD8+ T cell. This figure was adapted from previous reviews [62-64], and was created in Bio Render software.

IFNγ is a critical cytokine performing multiple functions to assist Th1 response against intracellular pathogens in mice, humans and cattle [65]. Although many types of immune cells can produce IFNγ including NK cells, DCs, macrophages and B cells, it is the signature cytokine of Th1 subtype [30]. Th1 produced IFNγ plays a critical role in regulating

the Th1 response. IFNγ can recruit immune cells to the site of infection and promote anti-microbial activities of neutrophils and macrophages by inducing oxidative burst and production of reactive oxygen species (ROS) [65-68]. IFNγ is directly involved in blocking viral replication, as well as enhancing the cytotoxic activity of CD8+ T cells [69, 70]. Moreover, IFNγ can enhance the number, mobility, and cytotoxicity of CD8+ T cells [70, 71].

During infection caused by intracellular pathogens, Th1 produced IFNγ can induce IgG subtype switching in activated B cells. However, this subtype switching may differ among the species. For example, it induces production of IgG2a in mice and IgG2 in cattle but IgG1 and IgG3 in humans **(Table 1.1)** [21, 72, 73]. These IgG subtypes induced by IFNγ can facilitate multiple mechanisms such as ADCC to kill intracellular pathogens, such as *Coxiella burnetii*, *Listeria monocytogenes,* and *Toxoplasma gondii* in mice [22, 74].

1.3.2 Differentiated Th2 cells coordinate humoral response against extracellular pathogens

During infections caused by extracellular pathogens, innate immune cells such as basophils, eosinophils, and innate lymphoid cells (ILCs) produce and secrete IL-4 [75, 76]. Together with 1st and 2nd signals, IL-4 signaling on naïve CD4+ T cell upregulates GATA3 (GATA binding protein-3), a critical transcription factor for Th2 differentiation [77, 78]. GATA3 knockout mice mounted impaired Th2 responses [79, 80]. When IL-4 binds to its corresponding receptor on the surface of naive CD4+ T cells, it activates STAT-6, which turns on pathways leading to GATA3 expression (**Figure 1.3A**)[81, 82]. Consecutively, GATA3 promotes Th2 differentiation by inducing histone acetylation and enhancing transcription of the IL4 gene [75, 83]. In addition, GATA3 is capable of suppressing Th1

differentiation by downregulating transcription and expression of molecules such as the IL-12 receptor *β2,* IFNγ , STAT-4, and possibly Tbet [84].

Once differentiated, Th2 cells are capable of activating B cells to produce antibodies that defend the host against extracellular pathogens [85, 86]. During B cell activation, Th2 cells recognize peptide-MHC-II complexes expressed on B cells [87, 88] and provide co-stimulation via CD40L, which are both necessary for B cell activation [89] **(Figure 1.3B)**. Importantly, IL-4 signaling induces isotype and subtype switching of B cells towards IgE and IgG1 production, which are key antibodies for controlling extracellular pathogens in mice and cattle [90].

Although antibodies can assist CD8+ T cell responses during intracellular infections, they play a major role in controlling infections caused by extracellular pathogens [16, 91, 92]. Antibodies can prevent the attachment of extracellular bacteria to the host cell, facilitate phagocytic killing, and neutralize toxins [16, 93-96]. In addition, different antibody isotypes and subtypes can have different functions. For instance, IgE can bind to both low and high-affinity receptors (FcεRI and FcεRII) on mast cells and basophils, which results in the degranulation and release of chemicals (*e.g.,* histamine, leukotrienes) that either kill parasites directly, or induce hyper-contraction of intestinal smooth muscle to promote their expulsion [97-100]. In addition to IL4, other cytokines such as IL-5, IL-9 and IL-13 are also involved in the control of extracellular pathogens. For example, IL-9 promotes production of IgE and proliferation as well as maturation of mast cells, which rapidly infiltrate the site of infection [101, 102]. Similarly, IL-5 induces differentiation, maturation, and infiltration of eosinophils to the site of infection [102]. Infiltrated mast cells and eosinophils, when cross-linked by antigen-specific IgE,

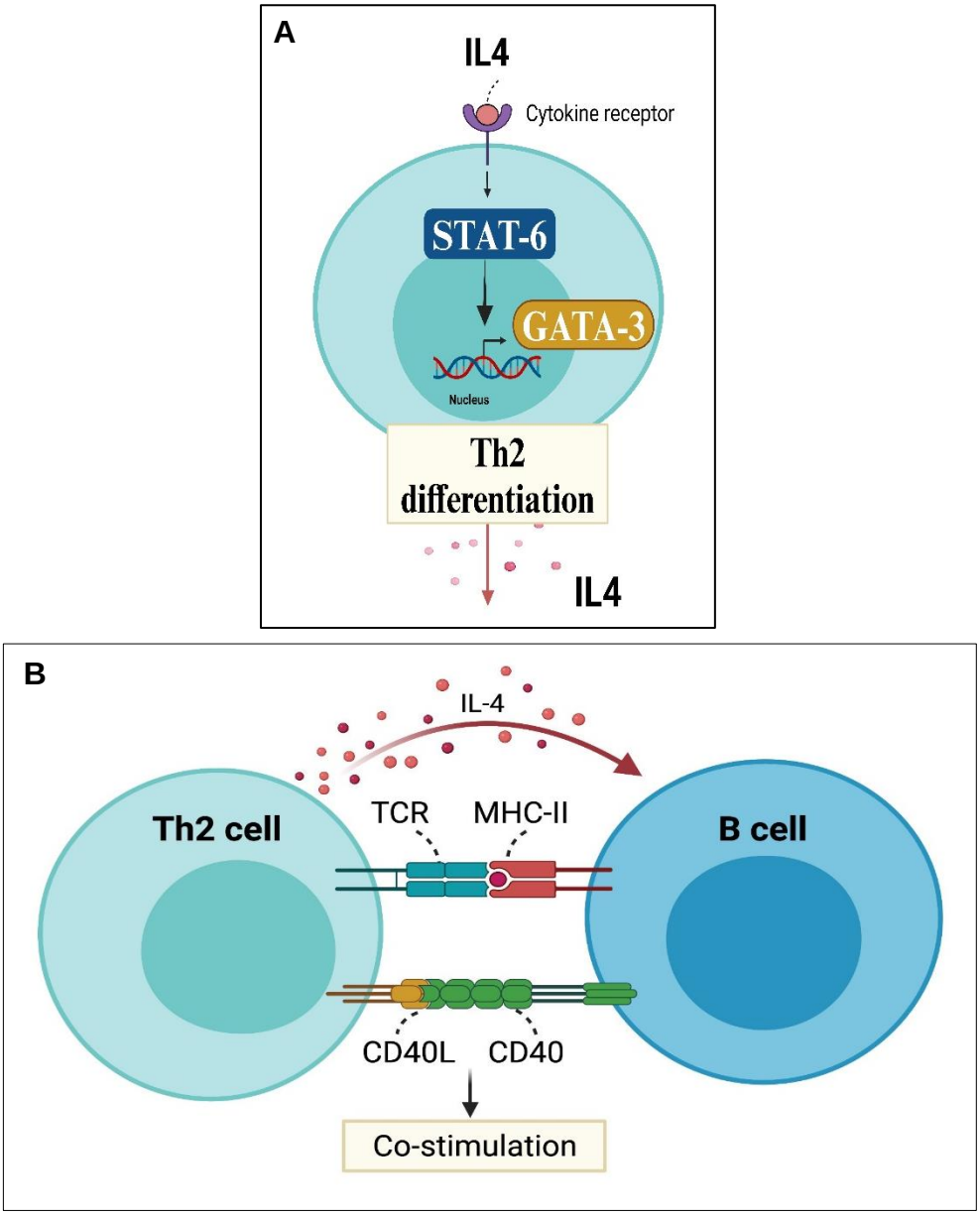

Figure 1.3: Th2 help to the activation of B cell. A) IL4 binds to its receptor on naïve CD4+ T cells during activation, which induces GATA3 activation and Th2 differentiation. This figure was adapted from previous reviews [60, 103, 104]. B) Once differentiated, Th2 cells secrete IL4 and provide antigen stimulation and co-stimulation to a B cell. This figure was adapted from previous reviews [105, 106].

degranulate (i.e., release histamine and leukotrienes) to kill or expel gastrointestinal parasites. IL-13 on the other hand, plays a significant role in the expulsion of parasites by inducing regeneration of the intestinal epithelium and contraction of smooth muscle cells in the intestine [86, 107]. Nevertheless, there are multiple cytokines involved in the differentiation of Th2 responses, but IL-4 is considered the most critical one.

1.4 Th1/Th2 cytokines induce Immunoglobulin class switching during infection

Antibodies produced by activated B cells during infections are classified into five different classes (*i.e.*, IgM, IgG, IgA, IgD and IgE) based on their structure [108]. Among them, IgG is the most abundant in serum, and it has four different subtypes, namely: IgG1, IgG2, IgG3 and IgG4 [108]. Each antibody has two structural segments (heavy and light chains) and two functional segments (F_{ab} and F_c portions). While association of heavy chain with the light chain at the F_{ab} portion forms antigen-binding sites, only the constant portion of the heavy chain constitutes the F_c segment that regulates the effector function of the antibody. During infection, activated B cells undergo isotype or subtype switching, a process that involves switching of F_c segment but not of the F_{ab} segment. Briefly, DNA in B cells contains multiple heavy chain constant genes (or C_H genes) that encode various types of Fc segments [109]. During infections, Th1 and Th2 cytokines provide signals to the activated B cells to select a specific C_H gene for the heavy chain, thus producing a specific isotype or subtype of immunoglobulins with the same antigen specificity [110]. For example, IFNγ can induce subtype switching to IgG2a to enhance the killing of infected cells in mice; similarly, IL-4 can induce switching to IgG1 to promote humoral immunity (Table 1) [111-114]. Historically, characterizing serum IgG subtypes was a common practice to define the immune response in clinically ill cattle; the greater concentration of serum IgG2 typically indicated a Th1 response, whereas greater IgG1 indicated a Th2 response. Interestingly, the Th1 induced IgG subtypes may vary among the mice, humans and cattle species as shown in **Table 1.1**.

Table 1.1: Th1- and Th2-associated IgG subtypes in mice, humans, and cattle

Species	Th1 immunity	Th2 immunity	References
Mice	IgG2a	IgG1	[72, 111-113]
Humans	IgG1 and IgG3	IgG4	[73, 115-120]
Cattle	IgG2	IgG1	[21, 121, 122]

1.5 Cytokines and transcription factors mediate Th1/Th2 cross-regulation

In humans and mice, multiple lines of evidence support that Th1 differentiation inhibits Th2 differentiation, and vice versa [123, 124]. For example, *in vitro* experiments reveal that IFNγ inhibits Th2 differentiation whereas IL-4 suppresses Th1 differentiation [125-127]. In addition, studies using knockout mice and retroviral-transduced CD4+ T cells demonstrate that Tbet blocks Th2 differentiation by inhibiting the transcription of genes associated with Th2 cytokine production [51, 128]. Similarly, GATA3 prevents Th1 differentiation by suppressing the transcription of genes associated with Th1 cytokines, and interfering with Th1-promoting transcription factors [129, 130]. Collectively, these findings confirm that Th1 and Th2 transcription factors and cytokines cross-regulate each other, ensuring that CD4+ T cells differentiate into either Th1 or Th2 cells. In cattle, however, most of the differentiated clones represent a "hybrid" that co-expresses both IFNγ and IL4 in the same cell (explained in detail in section 3) [25, 131]. While it is clear in mice and humans that Tbet and GATA3 are the transcription factors that regulate expression of IFNγ and IL-4 respectively, at this moment, it is unclear if this is equally true for cattle. In addition, we do not know if the co-production of both Th1 and Th2 cytokines in the hybrid bovine clones corresponds to the co-expression of both transcriptional factors. Therefore, further research is needed to understand the underpinning regulatory mechanism of hybrid clone differentiation in cattle.

1.6 Distinct Th1 and Th2 are the most dominant antigen-specific clones in mice and humans

In mice and humans, Mosmann et al. (1986) and Romagnani et al. (1992) stimulated single CD4+ T cells *in vitro* and established antigen-specific CD4+ T cell clones, which they classified mostly into Th1 and Th2 subtypes. Although, in both mice and humans, clear-cut Th1 or Th2 were the dominant clones, a small percentage of hybrid clones (named "Th0" clones), that co-produced Th1 and Th2 cytokines (IFNγ and IL4), were also observed [30, 31]. Subsequently, follow-up research verified the existence of these hybrid clones, which were only a small fraction of the total clones (*i.e.,* only 9.6 % clones were Th0) [127, 132-138]. While initially the consensus in the murine and human immunology field was that Th1 and Th2 are the major effector cells that orchestrate immune responses against intracellular and extracellular pathogens, respectively, and that Th0 are short-lived "intermediate" cells [134, 139, 140], more recently hybrid Th0 (also termed as Th_{1+2}) cells have been described in vitro and in vivo [141-143].

1.7 Th0 is the most dominant antigen-specific clone in cattle

Just a few years after the discovery of the Th1/Th2 subtypes in humans and mice, Brown et al. (1998) successfully investigated bovine Th1/Th2 response through the establishment and analysis of antigen-specific CD4+ T cell clones. Peripheral blood mononuclear cells (PBMC) were purified from cattle challenged by experimental pathogens: either intracellular pathogens (*Babesia bovis*, *Babesia bigemina*) or extracellular pathogens (*Fasciola hepatica*) [25]. These purified PBMCs (that contained pathogen-specific CD4+ T cells), were stimulated with antigens derived from the same pathogen used for the challenge, to generate pathogen-specific CD4+ T cell clones, which were then analyzed and classified based on the

detection of Th1/Th2 cytokine mRNA. The authors reported that, regardless of the type of pathogen used in the challenge, most bovine clones were Th0 that co-expressed transcripts of IFNγ and IL4 (*e.g.,* more than 60% *Babesia* species -specific and more than 90% *Fasciola hepatica*-specific clones were Th0) [25]. These observations indicated that bovine Th1/Th2 responses might be at least partially different from the typical murine and human Th1/Th2 responses, as the frequency of bovine Th0 clones was significantly higher than that of murine and humans. Later, when researchers used the Th0 clones specific to an antigen of *Babesia bigemina* to stimulate B cells *in vitro*, both, Th1-related IgG2 and Th2-related IgG1 were detected in the supernatant culture, suggesting that Th0 is capable of performing functions of both Th1 and Th2 cells [144].

1.8 Many critical bovine pathogens induce Th0 responses

In cattle, mixed Th1/Th2 cytokines (both IFNγ and IL4) have been detected in cultured PBMCs, or Draining Lymph Nodes (DLNs), or local tissues in large number of diseases. Most researchers commonly refer to this as the bovine Th0 response, which may include clones of all three types (Th1, Th2, and Th0) [131, 145-147]. It is important to note that while Th0 clones can produce both IFN γ and IL4, Th1 and Th2 clones can only produce a single cytokine, either IFN γ or IL4 **(Figure 1.4).** Therefore, a mixed population of Th1, Th2, and Th0 cells possibly contributes to the induction of Th0 responses in most of the bovine diseases as explained in section 4.

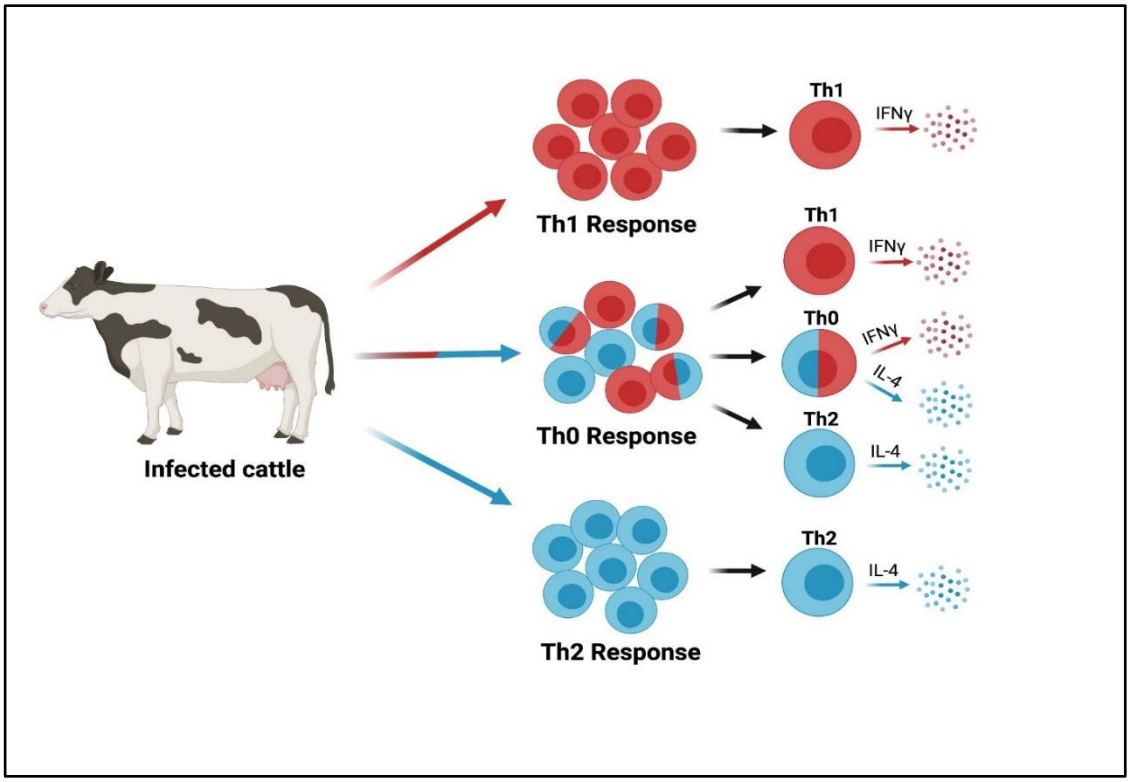

Figure 1.4: Helper T cell responses to infections in cattle. Pathogen infections in cattle may induce three types of CD4+ T cell responses: Th1, Th2 and Th0. Th1 responses are characterized by Th1 clones that produce IFNγ, Th2 responses include Th2 clones that produce IL4, and Th0 response could induce mixed populations of clones: Th1, Th2 and Th0. Th0 clones co-express both IFNγ and IL4.

1.8.1 Advancement of technology facilitates the progress in bovine immunology

Technology is a critical factor that drives the advancement of science, and bovine immunology is not an exception, particularly regarding bovine CD4+ T cell research. In the late 80s, the study of bovine Th1/Th2 responses depended heavily on the measurement of cytokines in the supernatant of cultured CD4+ T cells through simple biological assays such as ELISA, or detection of IgG subtypes in the serum of infected animals through ELISA or immunoblotting techniques. In this context, upregulation of supernatant IFNγ and serum

IgG2 would represent a Th1 response, upregulation of IL4 and detection of serum IgG1 would indicate a Th2 response [21, 121], and detection of both cytokines and both IgG subtypes (IgG1 and IgG2) would represent a Th0 response [148]. In the late 90s,

advancements in molecular biology enabled scientists to measure cytokines at the transcriptional level (mRNA).Thus, reverse transcription polymerase chain reaction (RT-PCR) was commonly used to detect the presence of mRNA of Th1/Th2 cytokines in PBMCs, DLNs, and tissues of infected cattle [149-151]. In the next decade, the advent of quantitative PCR (qPCR) improved the detection of Th1/Th2 transcripts from a qualitative to a quantitative level [152]. Later, with the invention and use of flow cytometry, scientists were able to measure protein production of Th1/Th2 cytokines on a population level [153]. More recently, some very exciting technological advancements have been developed, such as single-cell RNA sequencing, proteomics, metabolomics, confocal microscopy, which are considered excellent tools for a deeper understanding of immune mechanisms [154-158]. Therefore, the advancement of bovine immunology research is closely associated with the development of novel technology in science, especially in the context of understanding Th1/Th2 responses in cattle.

1.8.2 Most intracellular pathogens induce either a Th1 or Th0 response in cattle

During pathogen invasion, the host mounts a CD4+ T cell response that may or may not be effective enough to clear the infection. In humans, ineffective CD4+ T cell responses are associated with increased pathogenesis and progression towards chronic infections [159]. Cattle mostly launch either Th1 or Th0 responses against intracellular pathogens [160-163]. However, some bovine pathogens are able to establish chronic infections, which is possibly associated with ineffective CD4+ T cell responses [131, 164].

As observed in mice and humans, bovine Th1 responses are considered to be protective against diseases caused by intracellular pathogens such as *Theileria annulata* , and *Anaplasma marginale* [160, 161]. Indeed, researchers in the late 80s found that transferring

serum from an immune animal into animals infected with theileriosis was not effective at controlling infection [165]. Several groups later discovered that CD8+ T cell responses but not humoral responses were effective at controlling disease, since antigen-specific CD8+ T cells from recovered animals demonstrated effective cytotoxicity to the autologous infected cells *in vitro* [166-168]. Further research revealed that *in vitro* activation of T cells with *Theileria*-infected macrophages predominantly induced IFNγ expression [169]. Similar to theileriosis, Th1 responses were also protective against *Anaplasma marginale* [161, 170]. In both infected and vaccinated animals, circulatory IFNγ levels were higher relative to their healthy counterparts [161, 170]. Similarly, IgG2 was increased in cattle infected with *Anaplasma marginale* [171]. Collectively, in both theileriosis and anaplasmosis, hosts seem to induce effective Th1 responses.

Bovine pathogens such as *Mycobacterium tuberculosis* and *Mycobacterium paratuberculosis* can shift a Th1-dominant response towards a Th0- or a Th2-dominant response as the infection progressed [131, 164]. In bovine tuberculosis, high levels of circulatory IFNγ are detected at the early stage of disease that could inhibit Mycobacterial growth, suggesting that the host most likely mounts an early Th1 response [172-174]. However, in the chronic tuberculosis increased serum IgG1 (a Th2 associated antibody) is detected in the serum [131]. In line with these observations, in mice and humans, IFNγ expression was upregulated during the early phases of tuberculosis, however, at the chronic phase IL4 expression was enhanced [175-178]. Collectively, these results suggest that *Mycobacterium tuberculosis* can shift an IFNγ (Th1) dominant response towards an IL4 (Th0 or Th2) dominant response at the later stages of disease. Interestingly, the frequency of antigen-specific Th0 clones was higher in animals showing severe lung pathology than in

animals having less severe lesions [131]. Therefore, the authors speculated that Th0 clones may play an important role in skewing the immune response from Th1 (IFNγ) response towards Th0 or Th2 (IL4) response during the progression of infection **(Figure 1.4)** [131]. As in *Mycobacterium tuberculosis* infections, the immune responses to *Mycobacterium paratuberculosis* switches from Th1 response to Th2 response while the disease progresses from subclinical to clinical stage [164]. In *Mycobacterium paratuberculosis* infections, cattle show high levels of IFNγ in the supernatant of cultured PBMCs and high levels of IFNγ mRNA in the intestinal ileal tissues, suggesting an induction of Th1 response against this pathogen [179, 180]. Importantly, cattle clinically infected with *Mycobacterium paratuberculosis* had significantly lower expression of IFNγ in ileal and caecal lymph nodes compared to cattle at sub-clinical stage of infection [181]. This finding supports the notion that the suppression of the Th1 response at the sub-clinical stage of the disease might have contributed to the progression of disease into the clinical stage. Furthermore, increased antigen-specific IgG1 was detected in animals infected with *Mycobacterium paratuberculosis* at the clinical stage, suggesting a Th2 response [182, 183]. Together, these findings suggest that the shift of an early-induced Th1-dominant response towards a Th0 or Th2-dominant response is associated with disease progression in both bovine tuberculosis and bovine paratuberculosis.

During the early phases of Respiratory syncytial virus (RSV) infection in humans and mice, the host launches a Th1/Th2 mixed response (*i.e.*, both IFNγ and IL4), which then shifts towards a Th2 response (*i.e.*, increased circulatory IL4) during chronic infection [184-186]. Consistently, cattle infected with Bovine respiratory syncytial virus (BRSV) seem to mount a Th0 response, which turns into a Th2 response during chronic infection [149, 187].

In the past, reports suggested that both IFNγ and IL4 were detected in the peripheral blood, lymph sample and pulmonary tissues of BRSV infected animals at the early stage, indicating the induction of a Th0 response [150, 187, 188]. Similarly, both IgG1 and IgG2 were detected in the serum, although they peaked at different times during infection [188]. Conversely, IgE and IgG1 levels increased as the infection progressed towards the chronic stage, suggesting a gradual shift from a Th0 towards a Th2 response [149, 187-189]. Collectively, these studies indicate that these pathogens can switch the early-induced Th0 response towards a Th2 response during chronic infection.

The efficacy of Th0 responses in controlling infections caused by bovine intracellular pathogens is unclear. While Th0 responses seem ineffective against some bovine diseases such as tuberculosis, they can be protective against bovine babesiosis and non-cytopathic Bovine viral diarrhea virus (ncp- BVDV) infection [162, 163, 190]. In Babesiosis, both CD8+ T cell responses and humoral responses appear critical to clear infection. For instance, increased numbers of antigen-specific CD8+ T cells were detected in the peripheral blood of vaccinated animals [162]. Similarly, transferring serum from an immune animal containing both IgG1 and IgG2 can clear infection of sick animals [190]. In this regard, *in vitro* experiments have demonstrated that the majority of Babesia-specific clones are Th0, which are able to stimulate B cells to produce both IgG1 and IgG2 [25, 144, 190]. Furthermore, IgG1 and IgG2 antibodies were found effective to prevent invasion of bovine erythrocytes by *Babesia bovis* merozoite *in vitro* [191]. Collectively, these findings suggest that Th0 responses promote both the cytotoxic activity of CD8+ T cells, and neutralizing activities of IgG subtypes [162].

Cattle might launch different immune responses against different biotypes of the same intracellular pathogen [151, 192, 193]. For instance, while Th0 response was induced against the non-cytopathic (ncp) biotype of Bovine viral diarrhea virus (BVDV), Th1 response was induced during infection caused by the cytopathic biotype (cp) [194]. In experiments with T cells isolated from the ncp-BVDV infected cattle, IL4 protein in the supernatant of CD4+ T cell culture and IFNγ protein in CD8+ T cell culture were detected, suggesting possible induction of Th0 response [163]. More recently, Palomares et. al., (2014) analyzed cytokine expression in tracheo-bronchial lymph nodes and found that both IFNγ and IL4 were detected in ncp-BVDV-infected cattle, but IL-12 mRNA was only detected in cp-BVDV-infected cattle [151]. Additionally, while only IgG2 was detected in the serum of cp-BVDV-infected cattle, both IgG1 and IgG2 were detected in ncp-BVDV infected cattle after day 35 of infection [193]. These results collectively reveal that ncp-BVDV induces a Th0 response whereas cp-BVDV induces a Th1 response in infected cattle.

Thus, available literature supports the notion that cattle launch either Th1 or Th0 responses against most infectious diseases caused by intracellular pathogens (Table 2). Moreover, although further research is required to confirm these findings, the shift from an early Th1 or Th0 response towards a Th2 response is associated with progression of disease towards chronic condition.

Table 1.2: Characterization of helper T cell responses in diseases induced by bovine intracellular pathogens

Disease	Detected cytokines	Serum antibodies	References
Theileriosis	IFNγ (RT-PCR)	-	[160, 169]
Anaplasmosis	IFNγ (ELISA)	IgG2 /IgG1 +	[161, 195]
Babesiosis	IFNγ + IL4 (RT-PCR)	-	[25, 144, 162]

Respiratory syndrome	IFNγ+IL4(flow	IgE	[187, 188]
Bovine viral	IFN γ / IFNγ + IL4	IgG2/ IgG1 + IgG2	[151, 192, 193]
Tuberculosis	IFNγ to IL4 shift (PCR)	IgG2 to IgG1 shift	[196]
Paratuberculosis	IFNγ to IL4 shift	IgG2 to IgG1 shift	[164, 197, 198]

Table 1.2: Characterization of helper CD4+ T cell responses in diseases induced by bovine intracellular pathogens Th1/Th2 cytokines detected in the PBMCs and DLN tissues. While cytokines were measured either through ELISA or through PCR techniques and antigen-specific IgG1 and IgG2 antibodies were quantified from serum samples using ELISA.

1.8.3 Most extracellular pathogens induce either a Th2 or Th0 response in cattle

In mice and humans, Th2 responses are typically effective in controlling extracellular pathogens. In this regard, Th2 cytokines can induce processes such as IgG subtype switching and migration of mast and eosinophils to the site of infection that are critical for defending the host against extracellular bacteria and parasites [86]. In cattle, most of extracellular parasites induce either Th2 or Th0 responses [199-201]. However, some pathogens are capable of suppressing Th2 response , which is associated with the establishment of chronic infections [202].

Generally, Th2 responses are effective in controlling gastrointestinal nematodes such as *Cooperia oncophora* [203, 204]. Infected animals had increased level of antigen-specific IgG1 (Th2 associated antibody) in the serum [205]. Consistently, a high titer of pathogen specific IgG1 was associated with a better immune response [206]. Similarly, increased numbers of peripheral eosinophils (a Th2 response feature) was associated with increased expulsion of cooperial larvae [206]. Importantly, cytokine analysis of the intestinal tissue of disease resistant cattle demonstrated high expression level of IL4 and IL13 mRNA compared to those susceptible animals [207, 208]. These results offer compelling evidence that Th2

response is critical to control infection caused by some extracellular pathogens such as *Cooperia oncophora*.

Interestingly, some extracellular parasites such as *Dictyocaulus viviparus* (lung worm) are capable of shifting the initial Th2 or Th0 response into an ineffective Th1 response to establish chronic infections [209, 210]. At the early stage, both IL4 and IFNγ were detected in the lungs and DLNs after day 15 of lung worm infection, indicating an initial Th0 response [211]. However, subsequent research only detected increased IL4 mRNA for a short period of time in the Broncho-alveolar lavage fluid (BALF) of infected cattle, suggesting a possible Th2 response [212]. In line with this finding, high level of total IgE (antigen-specific plus non-specific) in the serum and BALF was associated with the clearance of lungworm [209]. Furthermore, in the chronically infected animals the detection of Th1 associated antibody (*i.e.*, IgG2) in the serum, was associated with increased lungworm larval excretion [210]. These data indicate that bovine lungworm might shift the early-induced Th0 or Th2 response towards a Th1 dominant response to establish chronic infection.

In cattle *Fasciola hepatica* (liver fluke) can modulate the early-induced Th1 or Th0 response into an ineffective Th2 response at the later phases of the disease [213, 214]. Of note, although an initial Th1 response was observed in the peripheral blood, a Th0 response was also observed inside the hepatic lymph node, as indicated by the detection of both IFNγ and IL4 [215-218]. Collectively, these experiments suggest that cattle might launch either a Th1 or a Th0 response at the early stages of liver fluke infection. However, at later stages, the response is shifted to a Th2 response as indicated by the significantly increased expression of IL4 mRNA (x6) and significantly reduced expression of IFNγ mRNA (x6) in the hepatic tissue of infected animals, which is consistent with several other reports [146,

219, 220]. In line with these observations, peripheral blood lymphocytes obtained from chronically infected animals failed to induce IFNγ secretion when co-cultured with adult fluke antigen *in vitro* [215]. Importantly, chronically infected cattle typically show high levels of antigen-specific IgG1 (Th2 antibody) in the serum [146]. Altogether, these findings suggest that *Fasciola hepatica* might switch a Th1 or a Th0 dominant response to a Th2 dominant response at the chronic stage of disease.

Ostertagia ostertagi (OO), an economically important abomasal nematode, typically induces Th0 response [221]. Bovine OO usually causes chronic infection and requires long-term repetitive exposure (at least 2 years) to develop effective immunity [222]. Both pathogen-specific IgG subtypes (IgG1 and IgG2) were detected in OO-infected cattle, with higher serum IgG1 titer than IgG2 [223]. Similarly, mRNAs of both IL4 and IFNγ were upregulated in the abomasal lymph nodes of experimentally infected cattle from day 11 to day 28 after infection, suggesting the induction of a Th0 [221]. In contrast to this observation, subsequent research demonstrated induction of Th2 response in the abomasal lymph nodes of OO infected cattle [224]. The differences observed between these two experiments might be explained, at least in part, by the differences in time points for cytokine detection and in the number of L3 larvae used for experimental infection. More specifically, while Canals et al (1997) measured cytokine expression from day 11 to day 28 post infection and used 200,000 L3 larvae for experimental infection, Claerebout (2005) measured cytokine expression after 8 weeks post primary infection and only used 25,000 L3 larvae [221, 225]. Recently, Mihi et al (2013) experimentally infected cattle with 200,000 L3 larvae and tested the gene expression of Th1/Th2 cytokines at different time points; interestingly, the authors observed a positive association between upregulation of both IFNγ and IL4 (in mucosa) with

migration of adult (L5) worms out of gastric gland toward abomasal mucosa [152]. These observations suggest that *Ostertagia ostertagi* may modulate the bovine immune response by inducing a Th0 response, which is ineffective in controlling OO and leads to the establishment of chronic infections.

Immune response against extracellular pathogens may vary at the systemic and local levels, such as in bovine trichomoniasis, where Th0 response is induced in the serum, and Th2 response in the mucosal secretion [226, 227]. More specifically, *Trichomonas foetus* upregulates both IgG1 and IgG2 in the serum but only IgG1 in local secretions from cervix, vagina, and uterus [226, 227]. Furthermore, animals immunized with specific antigen of *Trichomonas foetus* showed resistance to the experimental challenge, which was associated with the upregulation of both antigen-specific IgG1 and IgG2 in the serum [228, 229]. *Trichomonas foetus* seems to induce a Th0 response in the circulation, but a Th2 response in the mucosa. In addition, the systemic Th0 response may be protective against *Trichomonas foetus* re-challange.

Generally, Th2 response is effective in controlling extracellular bacteria [230]. For instance, Th2 response controls *Clostridium difficile* infection *in* humans *and Streptococcus suis* infection in pigs [230, 231]. In cattle, only few reports are available on CD4+ T cell response to extracellular bacteria such as *E. coli.* At this moment, the common understanding is both CD8+ T cell and antibodies seem to be critical to generate protective immunity (consistent with humans) in In E. coli 0157:H7 infection [199, 200, 232].

Collectively, the results obtained from multiple experiments indicates that extracellular pathogens typically trigger Th2 or Th0 responses in cattle as shown in Table (3), and some

extracellular pathogens modulate initial Th2 or Th0 responses to ineffective Th1 responses that are associated with the development of chronic infection.

Table 1.3: Characterization of helper T cell responses in diseases caused by bovine extracellular pathogens.

Disease	Detected cytokines	Serum antibodies	References
Cooperiosis	IL4 (q-PCR)	IgG1	[207, 208, 233]
Lung worm infection	IL4 /IL4+ IFNγ	IgG1, /IgG1+IgG2	[234, 235]
Trichomoniasis	-	IgG1 + IgG2	[228, 236, 237]
Fasciolosis	IL4	IgG1	[217, 238, 239]
Ostertagiasis	IL4 + IFNγ	IgG1 + IgG2	[152, 221, 224]

Table 1.3: Characterization of helper CD4+ T cell responses in diseases induced by bovine extracellular pathogens; Th1/Th2 cytokines were detected in the PBMCs and DLN tissues. While cytokines were measured either through ELISA or through PCR techniques, antigen-specific IgG1 and IgG2 antibodies were quantified from serum samples using ELISA.

1.9 Pathogens regulate the availability and the strength of three critical signals to suppress effective CD4+ T cell responses

Whenever a pathogen invades and starts multiplying, the host mounts a coordinated attack in order to clear the infection. To counteract the host attacks, some pathogens can interfere with helper T cell responses to establish chronic infections. This can be achieved through unique strategies that impair the availability or strength of the signals required for the activation and differentiation of CD4+ T cells (**Figure 1.1**). For example, pathogens such as *Salmonella*, and *Mycobacterium tuberculosis* can downregulate MHC-II expression in APC, which diminishes the strength of the 1st signal (antigen stimulation) [240, 241]. In addition, pathogens can reduce the expression of co-stimulatory molecules (2nd signal) and change the type of APCs (*e.g.* dendritic cell vs macrophage), which can collectively impair all of the three signals required for the activation and differentiation of T cells (**Figure 1.1**) [242, 243].

Bovine pathogens escape from effective CD4+ T cell responses in a very similar way to those of mice and humans. They can regulate the availability, type, and strength of three signals. Some pathogens such as Bovine herpes virus type-1 (BHV-1), *Bovine papilloma virus (BPV)*, and *Mycobacterium paratuberculosis* can undermine the strength of antigen stimulation (1st signal) by downregulating MHC-I expression, which is actively involved in antigen presentation to CD8+ T cells [244-246]. Similarly, some pathogens can disrupt the host T cell response through inhibiting the co-stimulatory signals [217, 247, 248]. Co-stimulatory molecules expressed on the surface of CD4+ T cells (as shown in **Figure 1.1**) are of two types: one provides activating signals, and the other provides inhibiting signals [249]. Pathogens such as, *Bovine leukemia virus*, *Anaplasma marginale*, and *Fasciola hepatica* can upregulate the expression of inhibitory molecules like program cell death protein-1 (PD-1), which severely impairs the T cell response when these inhibitory molecules bind to their ligands on the surface of APCs [217, 247, 248]. Additionally, pathogens such as *Ostertagia ostertagi* and *Myctobacterium paratuberculosis* can induce immune-regulatory cytokines that can inhibit the activation, differentiation, and expansion of effector CD4+ T cell subtypes [29, 250, 251]. More specifically, *Ostertagia ostertagi* may stimulate neutrophils to produce IL-10, which can suppress bovine CD4+ T cell activation [29]. Furthermore, pathogens like *Fasciola hepatica* can reduce the number of APCs by apoptosis, which curtails the availability of all activating signals [217]. Moreover, pathogens such as *Anaplasma marginale, Bovine herpes virus -1* and *Bovine viral diarrhea virus* can directly cause apoptosis of antigen-specific CD4+ T cell and starkly compromise the ability of the host to co-ordinate effective CD8+ T and antibody responses [248, 252-254]. In short, bovine pathogens regulate the CD4+ T cell responses by reducing the availability and strength of

the three activating signals by changing the type and number of APCs, or by interfering with co-stimulation and cytokine production.

1.10 Pathogens regulate the CD4+ T cell differentiation process to establish chronic infections in cattle

In addition to regulating activation signals, during the course of infection, pathogens can also regulate CD4+ T cell differentiation to evade the effective immune response mounted by the host. As already explained, intracellular pathogens can shift effective Th1 response to an ineffective Th2 response; similarly, extracellular pathogens can shift an effective Th2 response to an ineffective Th1 response, in order to promote the chronic infection in the host. For example, *S. japonicum* in mice can shift a Th2 response to an ineffective Th1 response by triggering apoptosis of Th2 cells via granzyme B signal pathway [255]. Similarly, some authors suggested that in chronic diseases such as bovine tuberculosis, immune complexes circulating in the blood might interfere specifically with Th1 response thus leading to a relatively increased Th2 response [256]. In cattle, intracellular pathogens including *Mycobacterium tuberculosis*, *Mycobacterium paratuberculosis and Bovine respiratory syncytial virus* (BRSV) shift the immune responses from a Th1 or a Th0 to an ineffective Th2 response, to establish chronic infections [131, 164]. In the same manner, extracellular pathogens such as *Dictyocaulus viviparus* modulate the immune response from a Th0 or a Th2 response to an ineffective Th1 response and establish the chronic infection [209, 210]. In summary, a fraction of bovine pathogens can skew the CD4+ T cell polarization to an ineffective subtype that cannot control their infection, which leads to the establishment of chronic infections.

1.11 Conclusion and future directions

After receiving three stimulation signals from APCs, naïve CD4+ T cells differentiate into effector subtypes such as Th1, Th2, and Th0 cells. While clear-cut Th1 and Th2 are the common subtypes detected in mice and humans, hybrid Th0 is common in cattle infected by both intracellular and extracellular pathogens. In fact, Th0 responses induced in many bovine diseases might consist of a mixed population of Th1, Th2, and Th0 subtypes. Thus, despite similarities in general, bovine CD4+ T cell responses seem to be partially different from the Th1/Th2 responses classically defined in mice and humans. Therefore, understanding the mechanisms of bovine CD4+ T cell differentiation and its regulation by pathogens may facilitate the development of more effective vaccines and designing immune intervention strategies against important chronic bovine infectious diseases.

Chapter:2 Differential expression of CD45RO and CD45RA in bovine T cells

Anmol Kandel[1], Lei Li[1], Akanksha Hada[1], and Zhengguo Xiao[1*]

2.1 Abstract:

Effective vaccination induces immune memory to protect animals upon pathogen re-encounter. Despite contradictory reports, bovine memory T cells are identified based on two isoforms of CD45, expression of CD45RO plus exclusion of CD45RA. In this report, we contrasted CD45RA/RO expression on circulatory T cells with IFNγ and IL4 expression induced by a conventional method. To our surprise, 20 % of cattle from an enclosed herd did not express CD45RO on T cells without any significant difference on CD45RA expression and IFNγ or IL4 induction. In CD45RO expressing cattle, CD45RA and CD45RO expressions excluded each other, with dominant CD45RO (>90%) expression on gamma delta (γδ) followed by CD4+ (60%) but significantly higher CD45RA expression on CD8+ T cells (about 80%). Importantly, more than 80% of CD45RO expressing CD4 + and CD8 + T cells failed to produce IFNγ and IL4, however, within the cytokine inducing cells, CD4+ T cells highly expressed CD45RO but those within CD8+ T cells mostly expressed CD45RA. Hence, CD45RO is not ubiquitously expressed in cattle, and rather than with memory phenotype, CD45RA/RO expression are more associated with distinct T cell subtypes.

2.2 Introduction

Memory T cells mount rapid and robust immune responses and are the hallmark of effective vaccination. The quality and quantity of induced immune memory are therefore critical for evaluating the efficacy of vaccines. Conventionally, memory T cells were detected as cells producing cytokines such as IFNγ and IL4 after brief stimulation *in vitro* [257-259]. However, more recently, memory T cells are often identified based on the inclusion of

CD45RO and exclusion of CD45RA on T cells, which is a relatively easy and practical approach for downstream treatments [259-261]. In humans and bovine T cells, CD45RA and CD45RO expressions were found mutually exclusive, and while naïve cells are defined as CD45RA positive (CD45RA+) and CD45RO negative (CD45RO-), memory T cells are CD45RO positive (CD45RO+) and CD45RA negative (CD45RA-) [262-264]. The small population of double positive (DP) cells that co-express CD45RA and CD45RO (*i.e.*, CD45RA+/CD45RO+) were believed to reflect the transition stage from naïve to memory induced by pathogen infection or vaccination [265-267]. Despite of some controversial reports, the conventional memory markers, CD45RO and CD45RA, are used extensively to detect memory T cells in humans and cattle [261, 268-281].

CD45 is a tyrosine phosphatase membrane protein expressed abundantly on the surface of a wide range of immune cells, including T lymphocytes [282, 283]. In humans, CD45 is expressed in a combination of multiple isoforms generated through alternative splicing of the CD45 pre-mRNA at exons 4, 5, and 6 [282, 284, 285]. Inclusion and exclusion of these three exons differentially generate CD45 isoforms, such as the high molecular weight CD45RA and the low molecular weight CD45RO [286, 287]. Specifically, the shortest CD45RO transcript is generated through the exclusion of exons (4, 5 and 6) while still retaining 3 and 7, which are shared by all the other isoforms [286, 287]. Therefore, selective detection of CD45RO transcript by quantitative PCR (qPCR) is not practical. Instead, due to the highly conserved nature of CD45 protein across the mammalian species [284, 288], bovine CD45RO expression is commonly examined at the protein level using a monoclonal antibody (clone # ILA116) in flow cytometry [261, 264, 280, 289-296]. Bembridge et al (1995) in their seminal research first used this monoclonal antibody (clone # ILA116) to precipitate

bovine CD45RO, which had a molecular weight very close to that of humans (180 kDa) and suggested that this isoform could be associated with memory [264]. Subsequently, Sopp et al (2005) reported that CD45RO+ CD4+ T cells were the dominant producer of IFNγ and IL4 [279]. These reports, along with several others supported that CD45RO expression is associated with memory phenotype in cattle [264, 279, 280, 297].

In the past few decades, a considerable number of reports in humans and cattle challenged the classical CD45RA/RO paradigm that defines the memory phenotype as CD45RO+ and CD45RA- cells. Specifically, a large number of reports in humans revealed the existence of terminally differentiated CD45RA+ memory T cells both *in vitro* and *in vivo*, indicating that memory T cells could be found within both CD45RO+ and CD45RA+ subpopulations [269, 272, 274, 275, 277, 278]. Interestingly, some data in bovine research also contrasted this classical paradigm. For instance, Hagberg et al. (2008) suggested that CD45RO+ CD4+ and CD8+ T cells isolated from immune cattle failed to proliferate in response to the homogenate derived from the *Dictyocaulus viviparous* parasite [298, 299]. Further, memory CD8+ T cells were detected in both CD45RO+ and CD45RA+ fractions, suggesting that CD45RO is not necessarily a memory marker for bovine CD8+ T cells [264, 265, 294, 300, 301]. In addition, Guerra Maupome et al (2019) suggested that CD45RO is not an appropriate marker to detect bovine memory γδ T cells [302]. These contradictory reports warranted re-assessment of the classical paradigm in cattle to verify if CD45RA/RO isoforms are genuinely associated with memory phenotype in bovine T cells.

This project aimed to contrast the CD45RA/RO expression on circulatory T cells with memory T cell subtypes from the same cattle. Our data suggested that the classical

CD45RA/RO paradigm may not be accurate in cattle. First, about 20% of cattle (six out of 28) did not express CD45RO. Furthermore, in those expressing CD45RO, the expression of this isoform was not correlated with IFNγ and IL4 induction. Instead, CD45RO and CD45RA clustered differentially in bovine T cell subtypes, suggesting that their expression is more associated with T cell subtypes than with memory phenotype.

2.3 Materials and Methods

2.3.1 Cattle

A total of 28 healthy cattle (Wye Angus), including 20 grass-fed and eight grain-fed, aged from one year to 24 months, were used. The closed herd was housed at the Wye Research and Education Center, University of Maryland Experimental Station (Queenstown, MD, USA) [29, 303-305]. All calves were weaned at six months of age, then randomly assigned to either grain-fed or grass-fed groups. The grain-fed group was kept on a feedlot with a mixture diet [304], and the grass-fed group was maintained on the pasture as reported previously [306]. All the animals were examined monthly to ensure health. Animal Care and Use Protocols were approved by UMD (R-FEB-18-06 and R-JAN-21-02), Institutional Animal Care and Use Committee (IACUC), with the relevant guidelines and regulations.

2.3.2 Peripheral blood mononuclear cells (PBMCs) isolation and stimulation

This procedure was similarly performed as in our previous reports [29, 305]. Briefly, fresh blood was collected from the jugular vein using EDTA coated vacutainers (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) and transferred to 15 ml conical tubes (Fisher Scientific, Pittsburgh, PA, USA), which were centrifuged for 30 min at 1200 × *g*. Following centrifugation, the buffy coat at the interface was gently collected into a new 15ml tube and

re-suspended with 1x phosphate-buffered saline (PBS) (Fisher Scientific, Fair Lawn, NJ, USA) to 8 ml. Then it was overlaid with a 5 ml lymphocyte separation medium (LSM) with the density of 1.077g/ml (Corning, Manassas, VA, USA), followed by centrifugation for 30 min at 900 x g with break off. The second buffy coat at the interface was collected and washed twice with PBS. After the last wash, the cell pellet was re-suspended in 5 ml Allos medium (RPMI1640 [Corning, Manassas, VA, USA] plus 10% FBS), and a small aliquot was used for cell counting.

Following the purification, one million cells per sample were aliquoted and re-suspended in 1 ml Allos medium supplied with Brefeldin A (BFA) (BioLegend, San Diego, CA USA) or cell activation cocktail (CT) (R&D Systems, Minneapolis, MN, USA) that contains monensin sodium salt (1.5 mM), Phorbol 12-myristate 13-acetate (0.0405 mM) and Ionomycin calcium salt (0.67 mM). The cell suspensions were incubated at 37°C with 5% CO_2 for 4 hours before intracellular staining.

2.3.3 Antibodies and reagents

All the antibodies used in this study are listed in the following tables: primary antibodies and secondary antibodies plus isotypes. Staining buffer (SB) is PBS with 2%FBS, and fix solution is 4% paraformaldehyde (W/V) in PBS with pH 7.4. Intracellular staining permeabilization wash buffer (P/W) (BioLegend, San Diego, CA USA) was purchased and used following the manufacturer instructions.

Primary antibodies

Specificity	Clone	Isotype	Source
bCD3	MM1A	IgG1	WSUMAC
bCD4	CC8	IgG2a	Bio-Rad
bCD25	LCTB2A	IgG3	WSUMAC
bTCRδ	GB21A	IgG2b	WSUMAC

bTCRδ	CACT61A	IgM	WSUMAC
bCD45RA	GC6A	IgM	WSUMAC
bCD45RO	ILA116	IgG3	WSUMAC
bCD62L	BAQ92A	IgG1	WSUMAC
bIFNγ	CC302	IgG1	Bio-Rad
bIL4	CC303	IgG2a	Bio-Rad

WSUMAC, Washington State University Monoclonal Antibody Center.

Bio-Rad, Bio-Rad Laboratories, Inc.

Specificity	**Clone**	**Conjugated**	**Source**
bCD4	CC8	PE	Bio-Rad
bCD8	CC63	FITC	Bio-Rad
bCD8	CC58	PE	Bio-Rad
bCD25	IL-A111	PE	Bio-Rad
bCD25	IL-A111	FITC	Bio-Rad
bCD62L	CC32	FITC	Bio-Rad
bIFNγ	CC302	PE	Bio-Rad

Secondary antibodies and isotype controls

Specificity	**Secondary antibodies**	**Source**
IgG1	Anti-mouse IgG1-APC	BioLegend
IgG1	Anti-mouse IgG1-Biotin	BioLegend
IgG2a	Anti-mouse IgG2a-APC	BioLegend
IgG2b	Anti-mouse IgG2b-PE	BioLegend
IgG2b	Anti-mouse IgG2b-Biotin	BioLegend
IgG2b	Anti-mouse IgG2b-FITC	BioLegend
IgG3	Anti-mouse IgG3-Biotin	BioLegend
IgM	Anti-mouse IgM-BV421	BioLegend
Biotin	Streptavidin Per-CP	BioLegend

Isotype controls	**Source**

Mouse IgG1	BioLegend
Mouse IgG2a	BioLegend
Mouse IgG2b	BioLegend
Mouse IgG3	BioLegend
Mouse IgM	BioLegend
Mouse IgG1-FITC	BioLegend
Mouse IgG1-PE	BioLegend
Mouse IgG2a-FITC	BioLegend
Mouse IgG2a-PE	BioLegend

2.3.4 Flow Cytometry analysis

For surface staining, cells were stained with primary antibodies, followed by secondary antibodies, or fluorescence conjugated antibodies at 4°C for 25 minutes, then washed with SB to remove the unbounded antibodies. Cells were further incubated with fix solution (1:1 dilution) for 15 minutes and washed with SB. Finally, cells were re-suspended in SB before being analyzed in a flow cytometer. For intracellular staining, the cells were stimulated for 4 hours as described in 2.2, and then fixed with 2% paraformaldehyde for 15 minutes. Following two washes, cells were incubated with permeabilization wash buffer (P/W) (BioLegend, San Diego, CA, USA) for additional 15 minutes at 4°C. Similar to the surface staining, cells were incubated with antibodies at 4°C for 25 minutes. Finally, cells were re-suspended in SB for reading. Staining of isotype and unstained controls was performed using the same method described above. Cells were examined by using FACSCalibur™ flow cytometer. The Flow cytometer data were analyzed with the FlowJo version 10 (Tree Star, Ashland, OR, USA).

2.3.5 Statistical analysis

Statistical analyses were performed using Prism 9 (GraphPad Software, Inc., La Jolla, CA, USA), and details are described in the figure legends. Overall, all data passed the Anderson–Darling normality test and were analyzed by one-way ANOVA with Tukey's Multiple Comparisons Test. Asterisks indicate statistical significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. NS indicates not significant.

2.4 Results

2.4.1 Bovine lymphocytes do not always express CD45RO

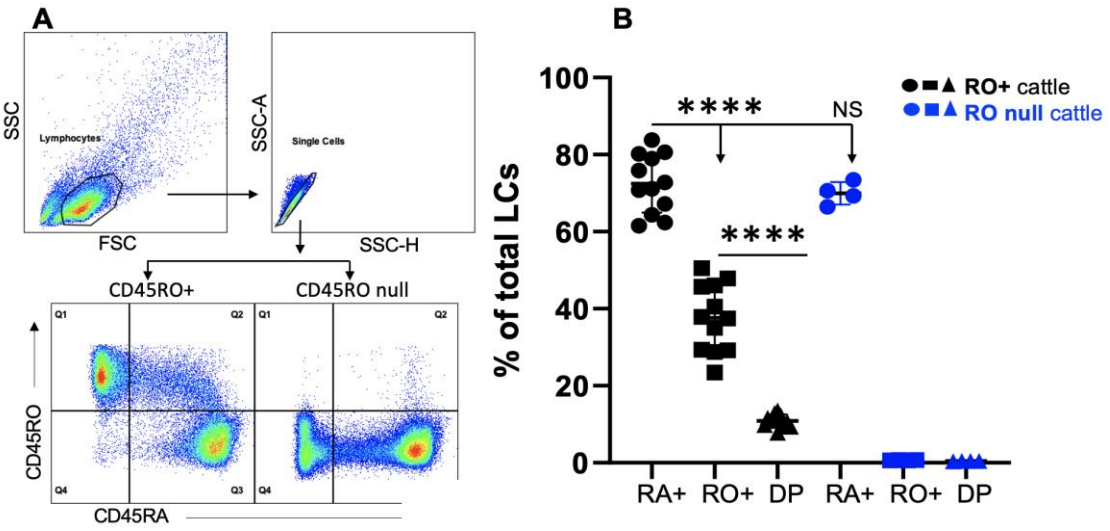

Figure 2.1: CD45RO is not expressed by lymphocytes in some cattle. Lymphocytes were gated in PBMCs and examined for expression of CD45RO and CD45RA. (A) Gating strategy for lymphocytes; (B) Comparison of CD45RO and CD45RA expression in lymphocytes. Data were pooled together from two experiments, and expressed as mean of 12 CD45RO positive (**RO+**) cattle samples, or four CD45RO negative (**RO null**) cattle with standard deviation (SD). Data in BLUE will indicate samples from **RO null** cattle in the rest of figures. Q1: lymphocytes only expressing high level of CD45RO, but not CD45RA, designated as RO+; Q2: lymphocytes both CD45RO and CD45RA, designated as double positive (DP). Q3: lymphocytes only expressing high level of CD45RA, but not CD45RO, designated as RA+. Background in isotype control was subtracted from all the data in B. Data were analyzed by one-way ANOVA with Tukey's Multiple Comparisons Test. Asterisks indicate statistical significance. *P < 0.05; **P < 0.01; ***P < 0.001. NS: not significant. The same analysis will be performed in the rest of figures unless mentioned otherwise.

Detection of an established memory T cell population is the gold standard for evaluating effectiveness of the vaccines [292, 307, 308]. In both humans and cattle, memory cells are

identified using CD45RO as a marker [279, 309-312]. We examined CD45RO and CD45RA expressions in bovine PBMCs. Almost all of the lymphocytes expressed high levels of either CD45RO(RO+) or CD45RA(RA+), and a small population expressed both at intermediate levels, which are referred to as double positive (DP) cells throughout this manuscript (**Fig. 2.1A-B**). To our surprise, four out of sixteen cattle did not express CD45RO (designated as **RO null**) but their CD45RA expression level was similar to those in CD45RO expressing cattle, labeled as CD45RO positive **(RO+)** (**Fig. 2.1A-B**). It seems lack of CD45RO isoform does not necessarily affect CD45RA expression. Among the examined cattle, while half of the cattle were raised on pasture (grass-fed), the other half were raised in feedlot (grain-fed) [29, 304]. Due to more exposure of grass-fed cattle to the environmental pathogens present in the pasture such as *Ostertagia Ostertagi* (OO) [304], the frequency of the memory cells in these animals could be more than in grain-fed counterparts, despite under the same vaccination procedure [304, 313]. The data were further analyzed by comparing the grass-fed cattle with the grain-fed but no significant difference was observed **(Appendix Figure A.1B)**.

2.4.2 CD45RO and CD45RA are expressed differentially in T cell subtypes

There are three major T cell subtypes in cattle, CD4+, CD8+ and gamma delta (γδ) T cells, all of which could differentiate into memory cells, with CD45RO as the marker [264, 279, 314]. Consistent with previous literature, CD4+ and γδ were the biggest T cell populations in PBMCs, while the CD8+ population was significantly lower than that of CD4+ T cells (Fig.2B) [279, 315]. Interestingly, CD45RO and CD45RA expression clustered differently in each T cell subtype with γδ T cells (90%) dominantly expressing CD45RO, followed by

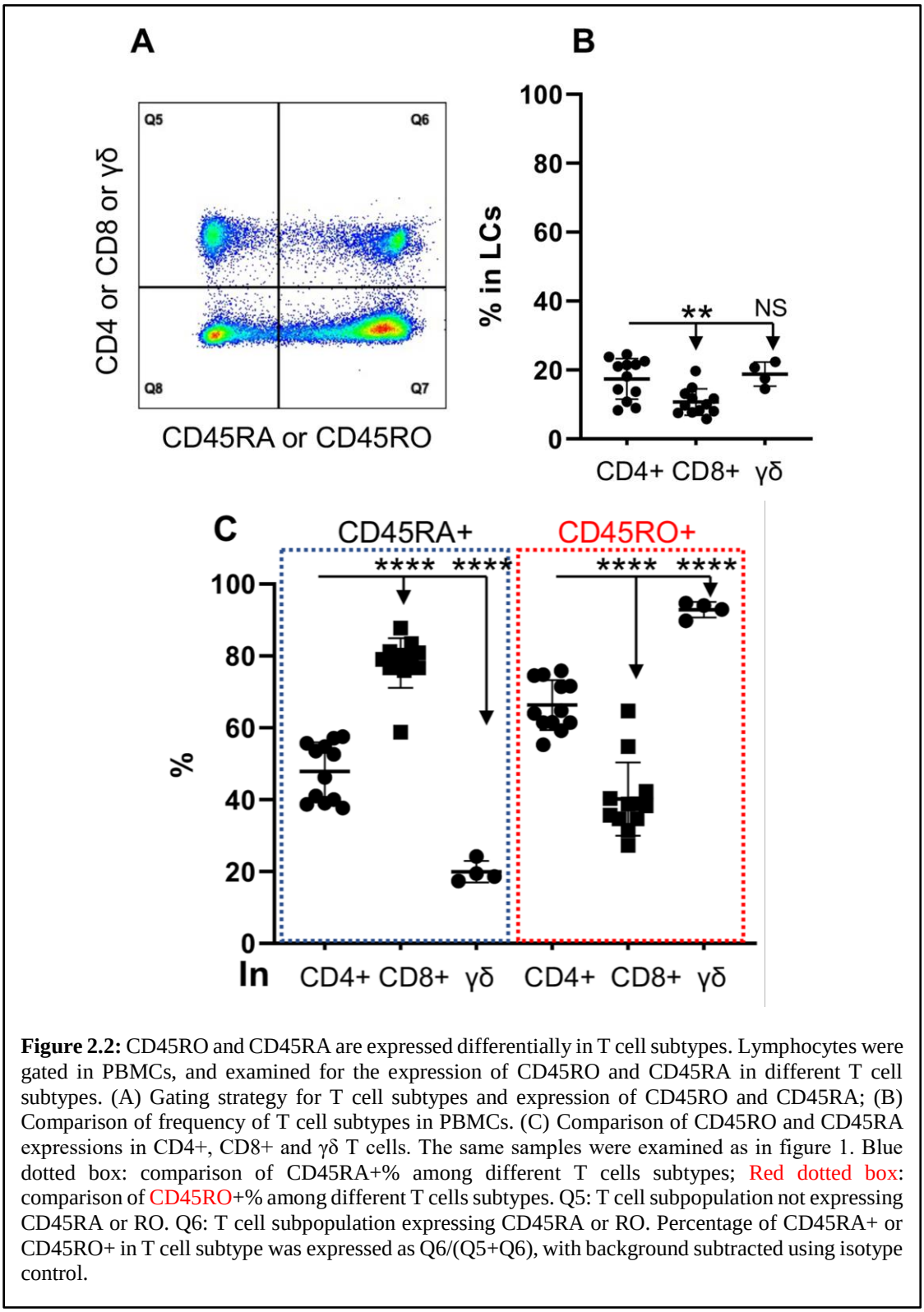

Figure 2.2: CD45RO and CD45RA are expressed differentially in T cell subtypes. Lymphocytes were gated in PBMCs, and examined for the expression of CD45RO and CD45RA in different T cell subtypes. (A) Gating strategy for T cell subtypes and expression of CD45RO and CD45RA; (B) Comparison of frequency of T cell subtypes in PBMCs. (C) Comparison of CD45RO and CD45RA expressions in CD4+, CD8+ and γδ T cells. The same samples were examined as in figure 1. Blue dotted box: comparison of CD45RA+% among different T cells subtypes; Red dotted box: comparison of CD45RO+% among different T cells subtypes. Q5: T cell subpopulation not expressing CD45RA or RO. Q6: T cell subpopulation expressing CD45RA or RO. Percentage of CD45RA+ or CD45RO+ in T cell subtype was expressed as Q6/(Q5+Q6), with background subtracted using isotype control.

CD4+ (around 60%), and CD8+ (around 40%) as shown in **Fig. 2.2C**. The pattern of CD45RA expression, as expected [264, 302], was opposite to CD45RO, lowest in γδ T cells but highest in CD8+ T cells **(Fig. 2.2C)**. It seems that the expression of CD45RA and CD45RO is associated with specific T cell subtypes. With the reason unknown, CD45RO expression was higher in CD4+ from the grass-fed cattle than those from the grain-fed **(Appendix Figure. A.2B)**.

2.4.3 Differential activation status in CD45RO+ and CD45RA+ fractions of CD4+, but not of CD8+ T cells

CD25, the α subunit of IL-2 receptor, has been a reliable marker for identifying activated T cells in humans and cattle [29, 44, 316, 317], which is also expressed by regulatory CD4+ T cells in cattle [304]. In the PBMCs from healthy cattle, only a small fraction of CD4+ T cells expressed CD25, thus potentially activated **(Fig. 2.3A-B)**. Majority of the cells in the CD25+ CD4+ T cell subpopulation were expressing CD45RO (RO+), with a smaller fraction expressing CD45RA (RA+) (Fig. 3B). The frequency of RA+ cells in CD25+ CD4+ T cells was similar between **(RO+)** and **RO null** cattle **(Fig. 2.3B)**. In contrast, the frequency of CD25+ cells were significantly lower in CD8+ than in CD4+ T cells ($p<0.0001$) **(Fig. 2 B-C and data not shown)**. CD62L, an adhesion molecule related to trafficking into the lymphoid tissues [318], was expressed at a higher level in CD45RA+ than in the CD45RO+ subpopulation from both CD4+ and CD8+ T cells **(Fig. 2.3 D-E)**. The expression of CD62L was not affected by the lack of CD45RO expression in the **RO null** cattle **(Fig. 2.3E)**.

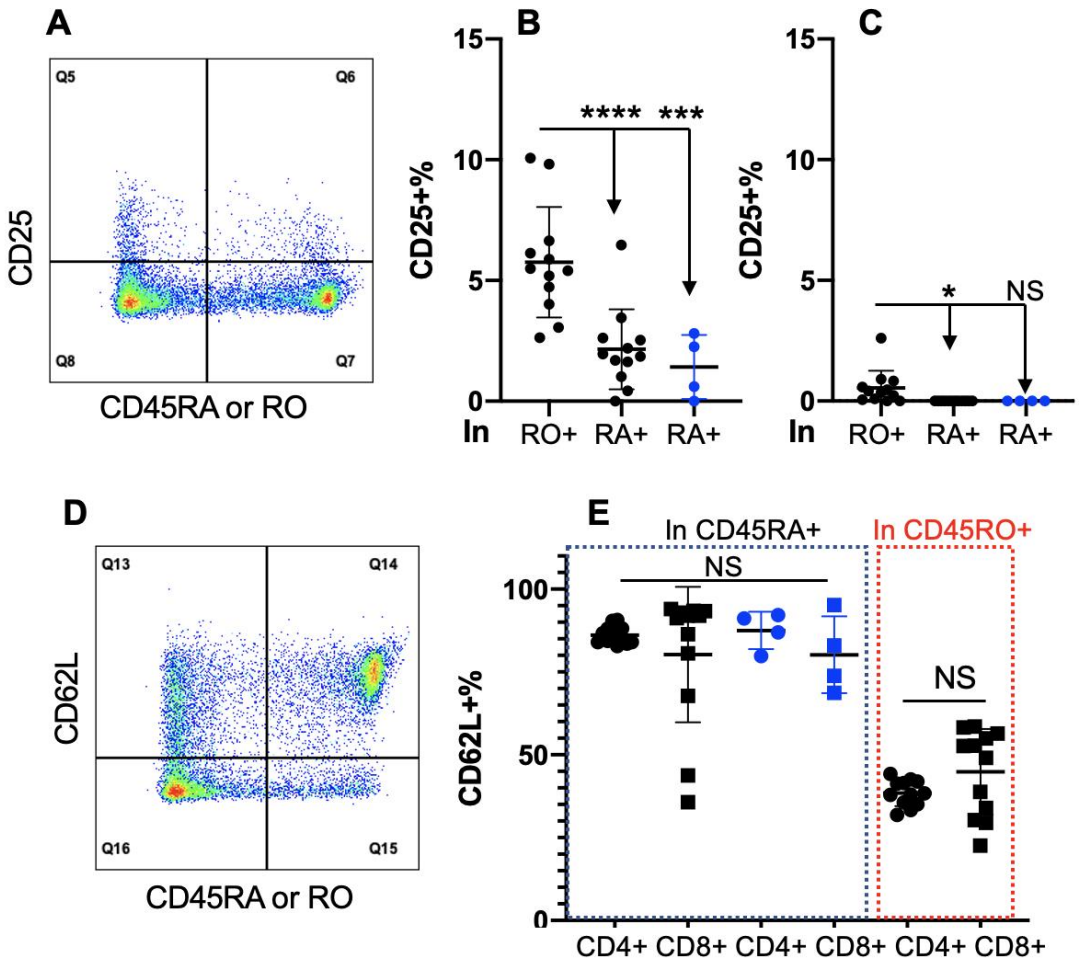

Figure 2.3: Activation status varies in CD45RO+ and CD45RA+ of CD4+ (B), but not of CD8+ T cells (C). CD45RO+ cells are designated as RO+ and CD45RA+ are labeled as RA+ in the figure (B and C). Lymphocytes were gated on CD4+ or CD8+ T cells in PBMCs, and examined for the expression of CD25 and CD62L in either CD45RO+ or CD45RA+ subpopulations. (A and D) Gating strategies for CD25 (A) or CD62L (D) and expression of CD45RO and CD45RA based on gated CD4+ or CD8+ T cells. (B-C) Comparison of CD25+% in RO+ and RA+ subpopulations in CD4+ (B) and CD8+ (C) T cells. The same samples were analyzed as in figure 1. Percentage of CD25+ expression in RO+ and RA+ subpopulations was expressed as Q6/(Q6+Q7). (E) Comparison of CD62L+% in RO+ and RA+ subpopulations in CD4+ and CD8+ T cells. Percentage of CD62L+ expressing in CD45RO or CD45RA subpopulations was expressed as Q14/(Q14+Q15). Background in isotype control was subtracted from all the data in B and D.

2.4.4 Expression of IFNγ and IL4 is not associated with CD45RO in CD4+ T cells

Induction of IFNγ and or IL4 after brief *in vitro* stimulation is a conventional method to detect memory T cells in humans and cattle [257-259, 319]. Using this method, about 15% of the CD4+ T cells expressed IFNγ, whereas a smaller population expressed IL4, plus an even smaller fraction produced both IFNγ and IL4 **(Fig. 2.4A-B).** Interestingly, the lack of CD45RO expression did not affect the frequencies of these IFNγ or IL4 producing cells (**Fig. 2.4B**), suggesting CD45RO may not be an exclusive marker for memory CD4+ T cells. To further examine the association of IFNγ and/or IL4 with CD45RO, data were analyzed in two different ways. First, we calculated the percentages of cytokine producing cells within CD45RA+ (RA+) and CD45RO (**RO+**) subpopulations; second, we analyzed the frequencies of CD45RA and CD45RO within the cytokine producing population. Close to 20% of the RO+ subpopulation produced IFNγ, which was much lower (about 5 %) in RA+ (**Figure 2.4D**) This trend was similarly reflected in IL4 producing cells but at a lower level, and, and lack of CD45RO expression did not affect the frequency of IFNγ/IL4 production in **RO null** cattle **(Fig. 2.4D)**.

In the second analysis, more than 50% of IFNγ or IL4 producing CD4+ T cells expressed CD45RO, with a significantly small fraction also expressing CD45RA (**Fig. 2.4E**). There was no significant difference in the frequencies of IFNγ or IL4 producing CD4+ T cells between grass-fed and grain-fed cattle (**Appendix Figure A.4A**). Additionally, the percentage of CD45RO+ or CD45RA+ cells within IFNγ or IL4 producing populations was also similar (**Appendix Figure A.4B**). These data suggested that most cytokine-producing CD4+ T cells express CD45RO, consistent with previous reports in cattle [264, 279, 280, 320], but a majority of the CD45RO expressing cells are not producing effector cytokines.

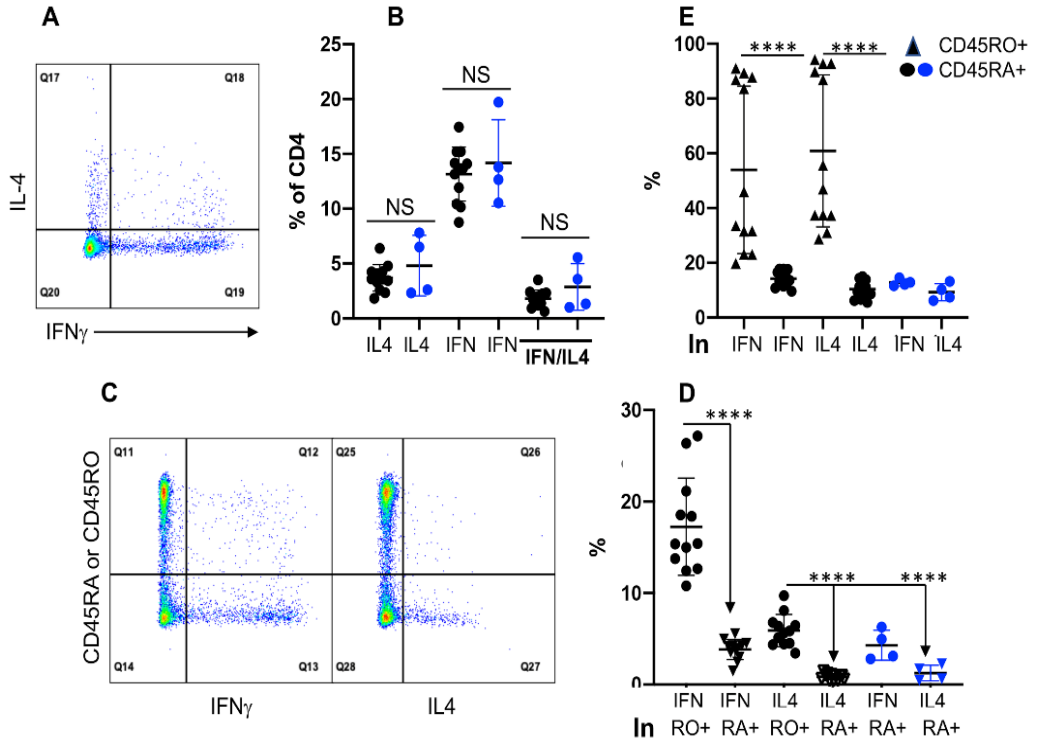

Figure 2.4: Expression of IFNγ and IL4 is not associated with CD45RO in CD4+ T cells. Purified PBMCs were stimulated with the activation cocktail for 4 hours before intracellular staining for IFNγ and IL4, plus surface staining with antibodies to identify CD4+ T cells, and their expression of CD45RO and CD45RA. (A) Gating strategy for IFNγ and IL4 gated on CD4+ T cells. Q17: IL4+ subpopulation not expressing IFNγ, designated as IL4**.** Q18: IFNγ and IL4 double positive subpopulation, designated as **IFN/IL4**. Q19: IFNγ+ subpopulation not expressing IL4, designated as IFN. (B) Comparison of IFNγ and or IL4 producing CD4+ T cells between **RO+** and **RO null** cattle samples. (C) Gating strategies for IFNγ or IL4 producing CD4+ in CD45RO or CD45RA subpopulations. (D) Comparison of IFNγ or IL4 expression in RO+ or RA+ CD4+ T cells. Percentage of IFNγ+ was calculated as Q12/(Q11+Q12), whereas IL4+ as Q26/(25+26). (E) Comparison of CD45RO+ or CD45RA+ in IFNγ+ or IL4+ CD4+ T cells. CD45RO+ or CD45RA+% in IFNγ+ was calculated as Q12/(Q12+Q13), whereas in IL4+% as Q26/(26+27), as indicated in (C). Background in isotype control was subtracted from all the data in B, D and E.

2.4.5 Expression of IFNγ is not associated with CD45RO in CD8+ T cells

Vaccination against intracellular pathogens such as viruses induces memory CD8+ T cells, which are critical for rapid control of infections [321, 322]. We detected memory CD8+ cells using a similar methodology as applied for identifying memory CD4+ T cells in **Figure 2.4**. Unlike in CD4+, CD8+ T cells only produced IFNγ, but essentially no IL4 (**Fig. 2.5A-B**).

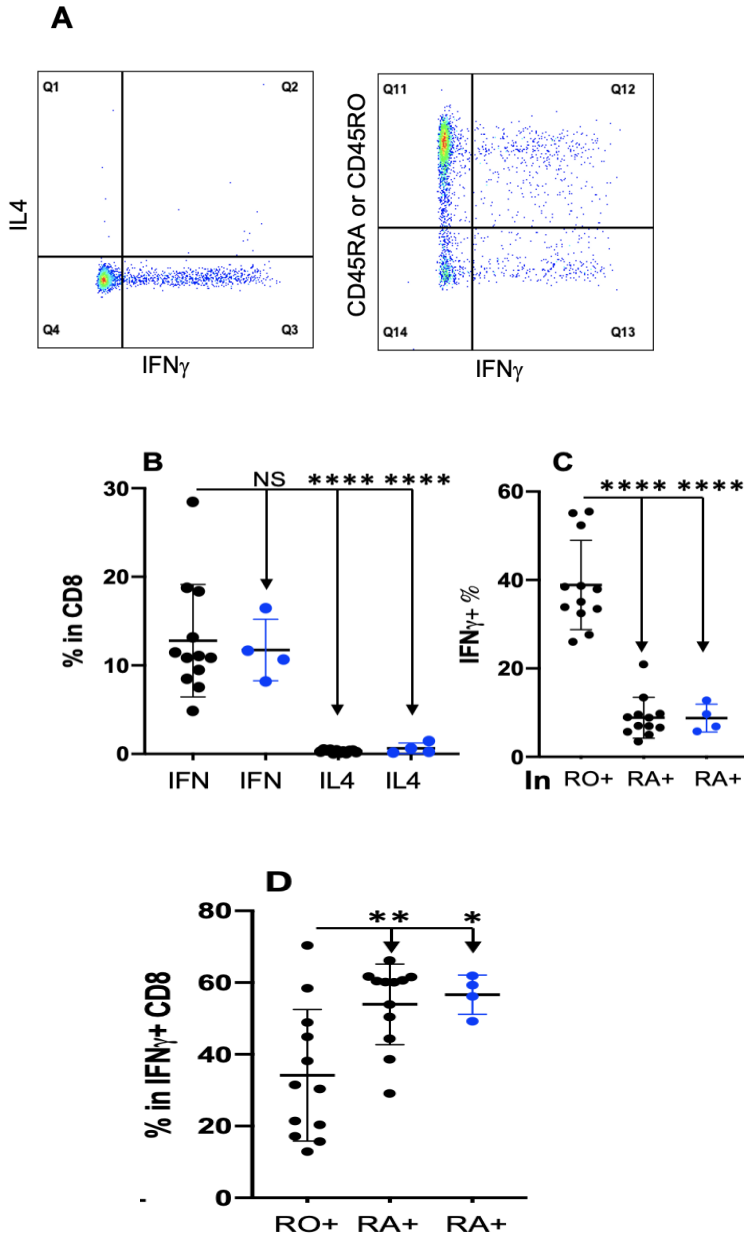

Figure 2.5: Expression of IFNγ is not associated with CD45RO in CD8+ T cells. The same samples and treatment were applied as in Figure 4, but the analysis was gated on CD8+ T cells. (A) Gating strategies for IFNγ+ and IL4+, plus IFNγ+ and CD45RO/CD45RA expression gated on CD8+ T cells. (B) Comparison between IFNγ (designated as IFN: Q2+Q3) and IL4 (Q1+ Q2) producing cells in CD8. CD45RO+ cells were designated as RO+ and CD45RA+ cells as RA+ in (C, D). (C) Percentage of IFNγ+ in RO+ or RA+ CD8+ T cells: Q12/(Q11+Q12), as indicated in (A). (D) Percentage of RO+ or RA+ in IFNγ producing CD8+ T cells: Q12/(Q12+Q13), as indicated in (A). Background in isotype control was subtracted from all the date in B-D.

This fact agrees with their cytotoxic function instead of the regulatory function defined for CD4+ T cells that expressed both cytokines [322, 323]. However, consistent with the results for CD4+ (**Fig. 2.4**), the lack of CD45RO expression did not affect IFNγ production in CD8+ T cells from **RO null** cattle (**Fig. 2.5B**). In CD8+ T cells, CD45RO expressing cells had a significantly higher frequency of IFNγ+ cells than those expressing CD45RA (**Fig. 2.5C**), which was similar to observations in CD4+ T cells (**Fig. 2.4D**). However, within IFNγ+ CD8+ T cells, the frequency of CD45RA expressing cells was significantly higher than those expressing CD45RO, opposite to the data in CD4+ T cells (**Fig. 2.4E**), suggesting that CD45RA/RO expression was different between cytokine-producing CD4+ and CD8+ T cell subtypes. These data further support the notion that CD45RA/RO expression is associated with distinct T cell subtypes.

2.4.6 Expression of IFNγ and IL4 is not associated with CD45RO in γδ T cells

γδ T cells have been suggested to be involved in both innate and adaptive immunity, thus their memory has also been identified with CD45RO in cattle [324, 325]. Our data revealed that 2 out of 12 cattle were **RO null**, and therefore, did not express CD45RO in the γδ T cells (**Fig. 2.6B** and data not shown). In the **RO+** cattle, consistent with **Fig. 2.2**, more than 90% of γδ T cells expressed CD45RO with majority of them also positive for CD62L (**Fig. 2.6A-B**). Despite almost all of the γδ T cells (>90%) expressed CD45RO, only a small fraction of them produced IFNγ, suggesting weak or no association between CD45RO and memory phenotype (**Fig. 2.6C-D)** Among the IFNγ expressing γδ T cells, a small frequency of cells was double positive for of IFNγ/IL4, which was not affected by the lack of CD45RO expression in the **RO null** cattle (**Fig. 2.6C** and dotted boxes in **2.6D**). Previously, γδ T cells have been reported to express low levels CD25, [326-328], being activated at least partially.

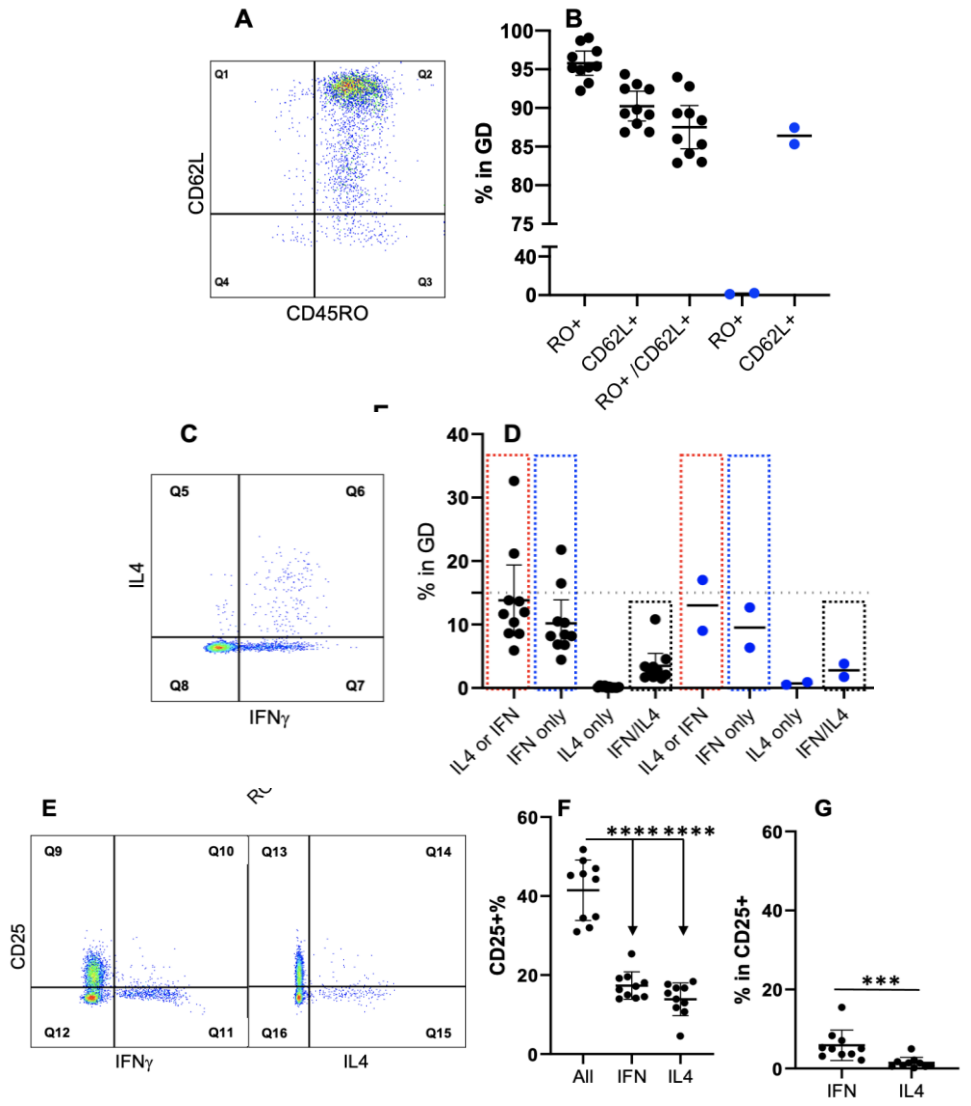

Figure 2.6: Expression of IFNγ and IL4 is not associated with CD45RO in γδ T cells. PBMCs were isolated from 12 cattle, which were tested for expression of CD45RO (RO+), CD62L (CD62L+), IFNγ (IFN) and IL4 in a way similar to that in figure 4. (A) Gating strategy for RO+ and CD62L+ based on γδ T cells. (B) Comparison of CD62L expression in **RO+** cattle and **RO null** cattle. (C) Gating strategy for IFNγ and IL4+ gated on γδ T cells. (D) Comparison of IFNγ and IL4+ between **RO+** and **RO null** cattle. IL4 or IFN: Q5+Q6+Q7. Q5: IL4 only; Q6: IFN/IL4; Q7: IFN only. Red dotted box: IL4 or IFN; blue box: IFN only; black box: IFN/IL4. (E-G): Gating strategy for CD25+ and IFNγ+ or IL4+ based on γδ T cells (E), and comparison of their expression in both **RO+** and **RO null** cattle (F-G). In F, CD25+% in all: Q9+Q10; CD25+% in IFN: Q10/(Q10+Q11); CD25+% in IL4+: Q14/(Q14+Q15). In G, IFN in CD25+: Q10/(Q9+Q10); IL4 in CD25+: Q14/(Q13+Q14). Data were expressed as mean plus 95% confidence interval (CI) in B, D, F and G. Background in isotype control was subtracted from all the date in B, D, F and G.

In our data, only a small fraction of IFNγ+ and IL4+ cells expressed CD25 (**Fig. 2.6E-G**), suggesting that these cytokine expressing cells were mostly memory but not activated cells.

2.5 Discussion

Vaccine induces pathogen-specific memory T cells to protect animals from re-infection. Memory cells in cattle are defined as CD45RO+ and CD45RA- T cells. However, our research revealed that a fraction of cattle does not express CD45RO on T cells but still differentiate into IFNγ and IL4 producing memory cells. Additionally, in those that expressed CD45RO, the clustering of CD45RO and CD45RA isoforms is uniquely related to bovine T cell subtypes rather than the memory phenotype.

The classical paradigm supported that naive cells are CD45RA+ CD45RO– and memory cells are CD45RO+ CD45RA-. However, several results from humans and cattle, at least partially, contrasted this characterization [264, 269, 272, 274, 275, 277, 278, 299, 302, 329]. In fact, multiple publications have suggested that the memory CD4+ and CD8+ T cells could express CD45RA. Therefore, the relevance of CD45RO as a unique memory marker for T cells remains controversial [268-278, 330-333]. In support to these reports, our data demonstrated that, in a fraction (about 20%) of healthy cattle, T cells failed to express CD45RO but expressed a normal level of CD45RA. Interestingly, the frequency of IFNγ and IL4 producing memory cells in **RO null** cattle was not different from those in **RO+** cattle. This pattern was also consistent within cytokines producing memory CD4 +, CD8 +, and γδ T cell subtypes, indicating that in the **RO null** group, the memory T cell differentiation was not affected by the absence of CD45RO expression. Furthermore, we noticed that the average frequency of CD45RA+ T cells in the **RO+** cattle was similar to those in the **RO null**, which suggests that CD45RA expression might not depend on CD45RO. In the **RO+** cattle, only less than 20% of CD45RO+ CD4+ T cells produced IFNγ, which means that more than 80% of CD45RO+ cells did not produce effector cytokine upon *in vitro* stimulation. These

experimental observations strongly suggested that using CD45RO as a marker for memory T cells in cattle might be overestimating and even misleading.

The classical paradigm assumed that switching of CD45 isoforms from CD45RA to CD45RO is an essential process associated with the memory T cell differentiation [263, 264, 311, 334-336]. However, the data from the **RO null** cattle suggested that, at least in a fraction of cattle, switching of CD45 isoforms is not required for the induction of memory T cells. We noticed that the average percentage of CD45RA+ lymphocytes in the **RO null** cattle was similar to that in **RO+** cattle. Further, the average frequencies of CD45RA+ cells within CD4+ and CD8+ T cell subtypes in the **RO+** cattle were also close to those in the **RO null**. The evidence suggested that the isoform switch might not be necessary to induce memory T cell population in all cattle. Therefore, the relevance of CD45RO as a signature marker for memory bovine T cells is questionable.

Our data demonstrated that CD45RA/RO expression pattern differs in distinct bovine T cell subtypes. While the proportion of CD45RA+ cells in the total lymphocytes was always higher than that of CD45RO+ cells, the pattern varied significantly within the distinct bovine T cell subtypes. In γδ (more than 90%) and CD4+ (60%), CD45RO expression was dominant, however, in CD8+ T cells, CD45RO expression was relatively low (about 30%) but CD45RA expression was significantly high. These data indicated a distinct pattern of CD45RA/RO clustering in bovine T cell subtypes. The tendency of our data wasin partial agreement with several previous reports, where γδ T cells were extensively CD45RO+, CD4+ T cells were predominantly CD45RO+, but CD8 + T cells were mostly CD45RO- [264, 279, 302]. Interestingly, this pattern has been similarly reported in human T cell subtypes [337-341].

Therefore, the distinct pattern of isomer clustering reflected that the CD45RA/RO expression was strongly associated with bovine T cell subtypes.

We noticed that even within the cytokine producing T cell population, the CD45RA/RO expression pattern was associated with distinct T cell subtypes, which agrees with several previous reports. It has been reported that the antigen-specific memory CD4+ population is within the CD45RO+ population [261, 264, 279, 280, 295, 297], but that of CD8+ T cells contains both CD45RA+ and CD45RO+ fractions [264, 291, 292, 294]. Previously, cytokine-producing ovalbumin (OVA)-specific memory bovine CD4+ T cells were found within the CD45RO+ sub-population, which is in line with another report, where the CD45RO+ fraction of the CD4+ T cells dominantly produced IFN-γ and IL4 in both blood and lymph nodes [264, 279]. In contrast, a different pattern has been reported in the context of bovine CD8+ T cells. While the *S uberis* and *BCG* specific memory CD8 + T cells were mostly found within CD45RO+ population, those specific to *Theileria parva* were under both CD45RA+ and CD45RO+ fractions [264, 291, 292, 294]. Our analysis suggested that the memory bovine T cells could be both CD45RO+ and CD45RA+ with the dominant expression of CD45RO in the CD4+ T cells but CD45RA in the CD8+ subtype. Therefore, the association between CD45RA/RO expression and bovine T cell subtypes was also reflected within IFNγ and IL4 inducing cells. Our analysis further suggests that CD45RA/RO expression is associated with distinct bovine T cell subtypes. Within the total lymphocytes, most (~90%) of the cells expressed either CD45RA or CD45RO, and the frequency of CD45RA+ cells was significantly higher than that CD45RO+ [342]. However, when T cells were analyzed, differential clustering of CD45RA/RO expression were noticed in each subtype. While CD45RA expression was high in CD8+ (around 80%), CD45RO was

dominantly expressed in CD4+ (about 60%) and γδ (about 90%) subtypes [342]. Furthermore, when CD45RA/RO expressions in IFNγ- and IL4-inducing memory T cell subtypes were examined, similar pattern of clustering were consistently detected. While the cytokine-producing cells within CD8+ T cells were mostly CD45RA+, those within the CD4+ and γδ subtypes were CD45RO+ [342]. Interestingly, our data is supported, at least partially, by some evidence reported in cattle [279, 302, 343], and humans [339-341, 344-346]. Therefore, we believe that CD45RA/RO expression is strongly related to T cell subtypes in cattle.

As CD45RA/RO expression on T cells was more related to distinct subtypes than the memory phenotype, we speculate that their expression pattern could be associated with T cell subtype-specific functions, which has been reported in humans [330, 347-349]. For instance, *Dengue virus (DENV)* specific CD45RA+ CD4+ T cells, *Epstein-Barr virus* (EBV) specific CD45RA+ CD8+ T cell, and *M tuberculosis* as well as *cytomegalovirus* (CMV) specific CD8+ γδ T cells expressing CD45RA demonstrated cytotoxic activity [330, 347-349]. Additionally, CD45RA+ but not CD45RA- CD8+ T cells isolated from *HIV-1* infected patients selectively demonstrated cytotoxicity under *in vitro* assay, indicating that killing ability could be related to CD45RA expression on these cells [333]. In contrast, CD45RO expression in the CD4+ T cells could be more related to immunoregulatory functions such as helping B cells to produce immunoglobulins. CD45RO+ CD4+ T cells showed an improved ability to adhere with immortalized B cell lines than the CD45RA+ cells [350]. Furthermore, CD45RO+ CD4+ T cell isolated from the blood of healthy humans stimulated B cells to produce high levels of IgM and IgG *in vitro*, which were drastically reduced when CD45RO+ CD4+ T cells were removed from the culture [267, 351]. These lines of evidence

provide a new perspective for interpreting the distinct pattern of CD45RA/RO clustering on T cells that their expression could be related to the distinct T cell subtypes and their specific effector functions. Therefore, more research is required to appropriately understand how distinct CD45RA/RO expression is related to the cell-specific functions in cattle. Nevertheless, more biomarkers are warranted to precisely identify the memory bovine T cell population in cattle.

2.6 Conclusions

Our data contrasted the classical CD45RA/RO paradigm and suggested that CD45RO expression on CD45RA- T cells is not strongly correlated with memory identification. Rather than with memory phenotype, the pattern of CD45RA/RO distribution on the T cells is associated more with distinct T cell subtypes. Therefore, future research should target to identify novel markers for memory T cell population, and also define the function of CD45RA/RO isoforms in cattle.

Chapter:3 Differentiation and Regulation of Bovine Th2 Cells in Vitro

Anmol Kandel[1]**, Lei Li[1]**, Yan Wang[2]*, Wenbin Tuo[3] and Zhengguo Xiao[1]*

1 Department of Animal and Avian Sciences, University of Maryland, College Park, 20742, MD, USA; akandel1@umd.edu (A.K.); lixxx242@umd.edu (L.L.)

2 Mass Spectrometry Facility, National Institute of Dental and Craniofacial Research, National Institutes of Health. Bethesda, MD 20892-4370, USA
Email: yan.wang2@nih.gov

3 Animal Parasitic Diseases Laboratory, USDA/ARS, Beltsville, MD 20705 USA
Email: wenbin.tuo@usda.gov

* Correspondence: xiao0028@umd.edu; Tel.: +1-301-405-6258.
yan.wang2@nih.gov; Tel.: +1-301-347-802

** Equal contribution

3.1 Abstract

Bovine Th2 cells have usually been characterized by IL4 mRNA expression, but it is unclear whether their IL4 protein expression corresponds to transcription. We found that grass-fed healthy beef cattle, which were regularly exposed to parasites on grass, had a low frequency of IL4+ Th2 cells using flowcytometry, similar to animals grown in feedlots. To assess the distribution of IL4+ CD4+ T cells across tissues, samples from the blood, spleen, abomasal draining, and inguinal lymph node were examined, which revealed limited IL4 protein detection in CD4+ T cells across the tissues. To determine if bovine CD4+ T cells may develop to Th2, naïve cells were stimulated with anti-bovine CD3 under a Th2 differentiation kit in vitro. Cells produced primarily IFNγ protein, with only a small fraction (<10%) co-expressing IL4 protein. Quantitative PCR confirmed elevated IFNγ transcription, but no significant change in IL4 transcription. Surprisingly, GATA3, the master regulator of IL4, was highest in naive CD4+ T cells, but was considerably reduced following differentiation.

To determine if differentiated cells were true Th2, an unbiased proteomic assay was done. The assay identified 4212 proteins, 422 of which were differently expressed compared to those in naïve cells. Based on these differential proteins, Th2-related upstream components were predicted, including CD3, CD28, IL4, and IL33, demonstrating a typical Th2 differentiation. To boost IL4 expression, T cell receptor (TCR) stimulation strength was reduced by lowering anti-CD3 concentrations: weak TCR stimulation essentially abolished Th2 expansion and survival. In addition, extra recombinant bovine IL4 (rbIL4) was added during Th2 differentiation, but despite of enhanced expansion, IL4 level remained unaltered. These findings suggest that, while bovine CD4+ T cells can respond to Th2 differentiation stimuli, the bovine IL4 pathway is not regulated in the same way as in mice and humans. Furthermore, presence of *Ostertagia ostertagi* (OO) extract, a gastrointestinal nematode in cattle, inhibited signaling via CD3, CD28, IL4, and TLRs/MYD88, indicating that external pathogens can influence bovine Th2 differentiation. In conclusion, though bovine CD4+ T cells can respond to IL4-driven differentiation, IL4 expression is not a defining feature of differentiated bovine Th2 cells.

Keywords: bovine, Th2 differentiation, IL4, IFNγ, proteomics

3.2 Introduction

Although vaccination is the most cost-effective strategy for mitigating infectious diseases in animals, existing vaccines against most extracellular pathogens like gastrointestinal nematodes offer only partial protection upon parasite re-challenge in cattle [352-355]. Th2 cells are critical in controlling extracellular pathogens [356-358], however, it is unclear if and how naïve bovine CD4+ T cells differentiate into Th2 cells [25, 323]. While IFNγ-producing Th1 cells that defend against the invading intracellular pathogens have been

commonly detected in infected or vaccinated cattle [261, 290, 359-364], whether IL4 protein producing Th2 cells differentiate in cattle remain mostly unclear. Increased IL4 mRNA expression is considered hallmark for bovine Th2 responses [122, 204-206, 365].

In mice and humans, IL4 decreases the production of IFNγ, a signature Th1 protein. Additionally, the master regulator of IL4, GATA3, cross-regulates transcription of Tbet, the master regulator of IFNγ [75, 81-83]. This promotes the development of IFNγ-negative Th2 cells that produce high levels of IL4. In contrast to data from mice and humans [366-369], antigen-specific bovine CD4+ T cell clones significantly (60-90%) co-express IFNγ and IL4 transcripts [25, 323], suggesting their predominant differentiation into double positive (IFNγ+ IL4+) Th0 cells capable of co-producing both Th1 and Th2- associated hallmark proteins. Nevertheless, despite of the controversy, an elevated ratio of IL4/IFNγ mRNA detection has been considered distinguishing feature of bovine Th2 cells [323, 370, 371].

Exposure to antigens derived from extracellular pathogens typically leads to increased frequency of IL4+ Th2 cells in the blood and lymphoid tissues of mice and humans [372-376]. However, in a recent study of healthy pasture-raised beef cattle [3], which are frequently exposed to extracellular parasites such as *Ostertagia ostertagi* [377], we discovered a small percentage of IL4 protein producing CD4+ cells in the blood, which was not significantly different from that of grain-fed cattle, which were less likely to be exposed to these environmental extracellular parasites [342]. These observations led us to question whether IL4+ IFNγ- CD4+ T cells are differentially distributed across other tissues such as spleen and draining lymph nodes in the pasture-raised cattle. However, our examination consistently revealed that bovine CD4+ T cells produce limited amount of IL4 protein irrespective of the examined tissues. Thus we investigated how naïve bovine CD4+ T cells

differentiate into typical Th2 condition in vitro, created by adopting the standard practice from mice and humans [5, 378, 379]. The differentiated cells were analyzed through unbiased proteomic assay, flow cytometry, and quantitative PCR. Also, the regulation of bovine Th2 cell differentiation was tested under distinct culture conditions such as reduced TCR stimulation strength, exposure to additional recombinant bovine IL4 (rbIL4), and the presence of *Ostertagia ostertagi* worm extract in vitro.

Our results suggest that bovine CD4+ T cells differentiates into dominantly IFNγ producing IL4 negative cells along with a small fraction (< 10%) of IFNγ and IL4 co-expressing hybrid Th0 cells, which reflects observations from the blood and lymphoid tissues of cattle. Based on differential regulation of proteins relative to the naïve cells, proteomic analysis verified bovine Th2 differentiation, however multiple pieces of evidence contrasted IL4 expression as the signature feature of bovine Th2 cells. Therefore, expression of IL4 may not be the only signature cytokine for Th2 in cattle.

3.3 Materials and Methods

3.3.1 Cattle

The Wye Angus herd has been a closed herd since 1958. It is maintained by the Wye Research and Education Center at the University of Maryland Experimental Station in Queenstown, Maryland [380]. The steers were grazed on a pasture composed of clover, orchard grass, and were supplemented with bailage and alfalfa during the winter season [381]. When the animals were 5 or 6 months old, they were weaned and split into grass-fed or feedlot (grain-fed) groups. While the grain-fed steers were processed at 14 months, the grass-fed steers were processed at 20 months [44, 377], at a commercial plant run by George G Ruppersberger & Sons in Baltimore, MD. Animal Care and Use Protocols were approved

by UMD (R-FEB-18-06 and R-JAN-21-02) Institutional Animal Care and Use Committee. All methods were performed in accordance with the relevant guidelines and regulation.

3.3.2 CD4+ T cell isolation

The blood was collected from the jugular veins of cattle at the Wye Angus Farm (UMD, Queenstown, Maryland). PBMCs were extracted from anticoagulated blood [44, 377]. The resultant single cell suspension was incubated with FITC-conjugated anti-bovine CD4 (Clone #CC8, BioRad, Hercules, CA) and PE-conjugated anti-bovine CD25 (Clone #CC63, BioRad, Hercules, CA) for 30 minutes at 4°C, followed by two medium washes. The final suspension included $2 * 10^7$ cells per mL. The purity of sorted CD4+ T cells was confirmed to be greater than 92% using a FACSAria II sorter (BD, San Jose, CA). These were subsequently stained with anti-bovine CD25 (Clone #LCTB2A, Washington State University (WSU), Seattle, WA) to confirm CD25 negative status using a FACSCanto I. (BD, San Jose, CA).

3.3.3 Differentiation of CD4+ T cells

The culture was similar as described previously [382, 383]. Briefly, anti-bovine CD3 (Clone #MM1A, WSU, Pullman, WA) was added to 24 - well plates at 10 µg/mL in 250 µL 1X PBS (Hyclone, Logan, UT), and the coated wells are covered with replacing RP-10 medium at 4^o C overnight, which was discarded before seeding the cells. Sorted out CD4+ T cells are resuspended either into freshly made Th1 or Th2 differentiation medium at a desired density, and 1.5 ml cell suspension containing $2 * 10^{\wedge 5}$ CD4+ T cells were seeded in the anti-bovine CD3 coated wells. Th1 differentiation medium was made by mixing RPMI 1640, 5% FBS, L-Glutamine (200mM), Penicillin/streptomycin (100X) and reagents in the Cell X Vivo

Human Th1 cell differentiation kit (R & D system, catalogue# CDK001). Th2 differentiation medium was prepared by mixing X-VIVO^{TM}15 serum-free Hematopoietic cell medium (Lonza, catalogue # 04-418Q), Penicillin/streptomycin (100X), and reagents present in the human Th2 kit (R & D system, catalogue# CDK002). The seeded plates were incubated in 37^{o} C, 5 % $C0_2$ for 13 days for Th1 and for 5 days for Th1 differentiation. Depending on the cellular growth, around 300 ul differentiation medium were replaced at every 3-4 days.

3.3.4 Flowcytometry analysis

Th1 and Th2 cells were collected for staining at days 5 and 13, respectively. Harvested cells were aliquoted for surface and intracellular staining. For surface labeling, cells were stained with primary antibodies for 30 minutes at 4 °C before being washed with 2% staining buffer (SB) to eliminate any unbound antibodies. Staining with secondary antibody was performed in the same way. After the final wash, cells were incubated with a fix solution for 15 min at 4 °C. Followed by one more round of SB wash, cells were resuspended in 100 µL SB and analyzed with the FACSCalibur™ flow cytometer.

Before performing intracellular staining, cells were stimulated with cell activation cocktail (Bio-techne, Minneapolis, MN, USA) at 37°C with 5 % CO_2 for 4 hours. The cocktail contains monensin sodium salt (1.5 mM), phorbol 12-myristate 13-acetate (0.0405 mM) and ionomycin calcium salt (0.67 mM) [384, 385]. The cells were then permeabilized with Perm/Wash buffer for 15 minutes at 4°C, and subsequently incubated with primary and followed by secondary antibodies for 30 minutes at 4 °C. Isotype controls were stained using isotype antibodies, and an unstained control was also included. Finally, cells were resuspended in 100µL SB, and then Flow cytometry was performed, acquiring a minimum

of 20,000 events. Data analysis was conducted using FlowJo version 10 (Tree Star, Ashland, OR, USA).

Primary antibodies

Specificity	Clone	Isotype	Colors	Source
bCD3	MM1A	IgG1	-	WSUMAC
bCD4	CC8	IgG2a	FITC	Bio-Rad
bCD25	LCTB2A	IgG3	PE	WSUMAC
bCD62L	BAQ92A	IgG1	FITC	WSUMAC
bIFNγ	CC302	IgG1	PE	Bio-Rad
bIL4	CC303	IgG2a	-	Bio-Rad

Secondary antibodies

Specificity	Secondary antibodies	Source
IgG1	Anti-mouse IgG1-APC	BioLegend
IgG2a	Anti-mouse IgG2a-APC	BioLegend

3.3.5 Proteomics

For proteomics analysis, 50 µg of each protein sample was digested with trypsin using the EasyPep mini digestion kit (Thermo Scientific) separately. Each sample was then labeled with a different TMTPro (Thermo Scientific) reagent following manufacturer's protocol. Labeled peptides were then mixed and the mixture dried under vacuum. The mixed peptide sample was then dissolved in 300 µL 0.1% TFA and fractionated into 10 fractions using the

high pH fractionation kit (Thermo Scientific). Each fraction was dried under vacuum and reconstituted in 0.1% TFA.

A Dionex U3000 nanoHPLC system interfaced to a Thermo Scientific orbitrap Fusion Lumos mass spectrometer was used for the LC-MS/MS analysis. ~1 µg of each fraction was injected into an Acclaim^{TM} PepMap^{TM} 100 trap column (75 µm × 2 cm, Thermo Scientific)) and desalted at 5 µL/min with 100% Solvent A for 5 minutes. The peptides were then separated using an Acclaim PepMap^{TM} 100 nano column (3 µm,, 75 µm×250 mm) over 160 minutes with a linear gradient of 5-50% solvent B (80% ACN, 0.1% formic acid). The Orbitrap detected precursor (R=120,000) and fragment (R=50,000) masses at. Data dependent MSMS was performed at a cycle time of 2 seconds. Dynamic exclusion was set to 20 seconds within 10 ppm error. Collision energy was 37%, which is higher than normal to assure efficient fragmentation of TMTPro labeled peptides.

Proteome Discoverer software (v. 2.5, Thermo Scientific) was used for protein identification and quantification, peptide matching and protein inference was carried out using Sequest HT search engine against bovine proteome downloaded from Uniprot (uniprot.org, 2020-03-06) using search engine. M oxidation (+15.995) and protein N-terminal acetylation were designated as variable modifications, carbamidomethylating of C (+57.021), TMTPro labeling (+304.207) of K and peptide N-Terminal were designated as fixed modifications. Mass tolerance was 10 ppm for precursor and 0.02 Da for fragment masses. Percolator was used to control false discovery rate to <1%. TMT reporter ions were quantified based on signal to noise when available, when data doesn't have signal to noise, reporter ion intensity is used. Protein abundances were normalized to the abundance of GAPDH. A nested study design was applied for relative peptide and protein quantification.

Peptide and protein ratios were first calculated within cells derived from the same cattle, background-based T-test was then applied to evaluate significance. Peptides with >35% consolation or < 2 reporter ions were not included in quantification. Principle Component Analysis (PCA) and Heatmap of proteins related to immune response (Gene Ontology) were generated from Proteome Discoverer.

Proteomics data is available as MassIVE dataset MSV000091527.

3.3.6 Quantitative PCR

Cells were collected, washed with 1X PBS, and the pellets lysed with RLT lysis buffer (Qiagen, Germantown, Maryland). The RNeasy Micro kit (Qiagen) was used to extract total RNA, with DNase treatment included in the procedure. A NanoDrop 1000 spectrophotometer was used to test RNA quality, and 400 ng of total RNA was used to synthesize first-strand cDNA using the Thermo Maxima First Strand cDNA synthesis kit (Fisher, Waltham, MA) in an Eppendorf Master Gradient Cycler. 400 ng of synthesized cDNA was divided into 5 ng/µL concentrations. Quantitative PCR (qPCR) amplification was done in the C1000 Touch Thermal Cycler (BioRad, Hercules, CA) using 2 µL of diluted cDNA and the IQ SYBR Green Supermix. In each reaction, 2 µL of cDNA template and two primers (final concentration 400 nM) were used, followed by a two-step technique of denaturation at 95°C for 3 min, 40 cycles of 10 sec at 95°C, and extension at 55°C for 30 seconds. Melting curves were obtained after 40 cycles, and all amplified products were validated via gel electrophoresis and sequencing.

3.3.7 Statistical Analysis

Statistical analyses were performed using Prism 8 (GraphPad Software, Inc., La Jolla, CA); details can be found in the figure legends. All data were analyzed using a one-way ANOVA

with Tukey's Multiple Comparisons Test. Asterisks denote statistical significance. *P < 0.05; **P < 0.01; ***P < 0.001.

3.4 Results

3.4.1 Bovine memory CD4+ T cells are a mixture of IFNγ dominated Th0

The detection of an established memory T cell population is the gold standard for assessing efficacy of vaccinations [292, 307, 308]. Previously we reported that beef cattle raised on pasture demonstrated a small fraction of IL4+ CD4+ T cells in the blood [3], which was not significantly different from those in the grain-fed cattle. To assess if IL4+ CD4+ T cells in the grass-fed cattle are distributed more in the lymph node draining abomasum, bovine CD4+ T cells from blood (BL), spleen (SP), draining lymph nodes (DLN) and inguinal lymph nodes (ILN) samples were examined for the expression of surface (CD3 & CD4) and intracellular (IFNγ & IL4) molecules. The frequency of total CD4+ T cells was highest in the BL and lowest in the DLN **(Fig 3.1A-B)**.

The highest percentage of IFNγ-producing CD4+ T cells was detected in the blood, which was lowest in the lymph nodes draining the abomasum **(Fig 3.1C-D)**, consistent with the notion that memory T cells predominantly exist in circulation or somatic tissues, whereas most CD4+ T cells are naïve in the lymphatic tissues like lymph nodes, thus not producing much of IFNγ [386-388]. Of all the investigated tissues, CD4+ T cells in PBMCs had the highest proportion (~ 40%) of IFNγ and/or IL4 producing cells, with IFNγ (designated as **IFN**) being significantly dominant (**Fig.3.1D**). A small fraction of IL4+ CD4+ T cells were similarly distributed among blood and different lymphoid tissues of pasture-raised cattle with no significant statistical difference, consolidating the observation from grain-fed cattle reported previously [342]. Notably, the majority of IL4 expression was restricted to the

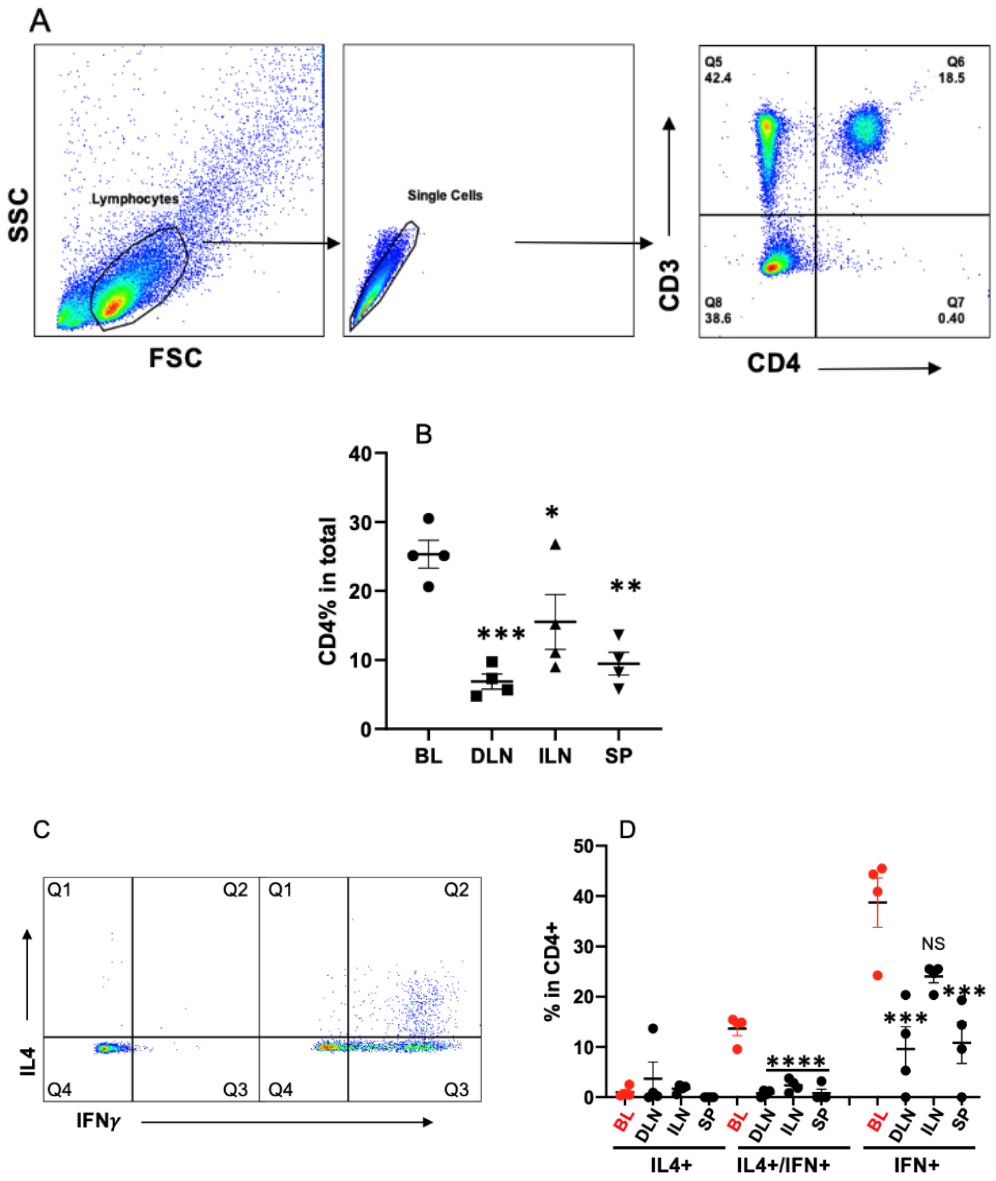

Figure 3.1. Bovine memory CD4+ T cells in healthy beef cattle are a mixture of Th1 and Th0. Lymphocytes were gated in peripheral blood mononuclear cells (PBMCs) or lymphoid tissues, and examined for expression of surface markers and cytokines. (A-B) Gating (A) and comparison of CD4+ T cells % in different tissues of grass-fed cattle; (C-D) Purified PBMCs or single cell suspension of lymphoid tissue were stimulated with the activation cocktail for 4 hours before intracellular staining for IFNγ and IL4, plus surface staining with antibodies to identify CD4+ T cells [3]. (C) Gating strategies for IFNγ or IL4 producing cell in total CD4+ T cells. (D) Comparison of IFNγ or IL4 expression in CD4+ T cells. Percentage of IL4+ only: Q1; IL4+/IFNγ+ (double positive): Q2; IFNγ+ only: Q3. Background in isotype control was subtracted from all the data in D. BL: blood; DLN: abomasal draining lymph nodes. ILN: inguinal lymph node, SP: spleen. Data were analyzed by one-way ANOVA with Tukey's Multiple Comparisons Test. Asterisks indicate statistical significance compared to BL in B and D. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. NS: not significant. The same analysis will be performed in the rest of figures unless mentioned otherwise

double positive Th0 cells (**Fig. 3.2D**) [25]. These results suggested differentiation of CD4+ T cells into Th1 dominant Th0 profile in cattle.

3.4.2 In vitro programming under Th2 leads to IFNγ dominated Th0

Polarized Th1 and Th2 cells are well-documented in mice and humans [367, 389, 390]. Human Th1 differentiation kit that mainly contains human IL12 (hIL12), and Th2 differentiation kits, primarily composed of human IL4 (hIL4), are commonly used for the vitro studies [391, 392]. Notably, both of these human cytokines have demonstrated effective biological activities in the bovine cells [393-395]. With this in mind, we explored the use of human kits to differentiate bovine CD4+ T cells under typical Th2 culture conditions, keeping Th1 as a control. Previously, we discovered that, unlike in mice and humans [262, 263], CD45RA expression was not associated with naïve CD4+ T cells in cattle [342, 396]. Therefore, we sorted out resting CD25 negative CD4+ T cells, as shown in **Fig. 3.2A**, and stimulated them with anti-bovine CD3 under

Th2 conditions using human differentiation kit (**Fig. 3.2B**). Indeed, CD4+ T cells differentiated under human Th1 condition, hereafter referred to as Th1 cells for the convenience, resulted in typical IFNγ+ effector cells, a small fraction of which also co-expressed IL4, consistent with the observations from the pasture-raised cattle **(Fig. 3.2C-D)** [397]. Interestingly, majority of the cells differentiated under Th2 culture, referred to as Th2 cells, were also not producing IL4 protein, rather were dominantly secreting IFNγ. Though a small fraction of IL4+ cells were induced, most of them were also positive for IFNγ, suggesting that double positive (IFNγ+ IL4+) Th0 cells were the major source of IL4 (**Fig. 3.2D**). Collectively, irrespective

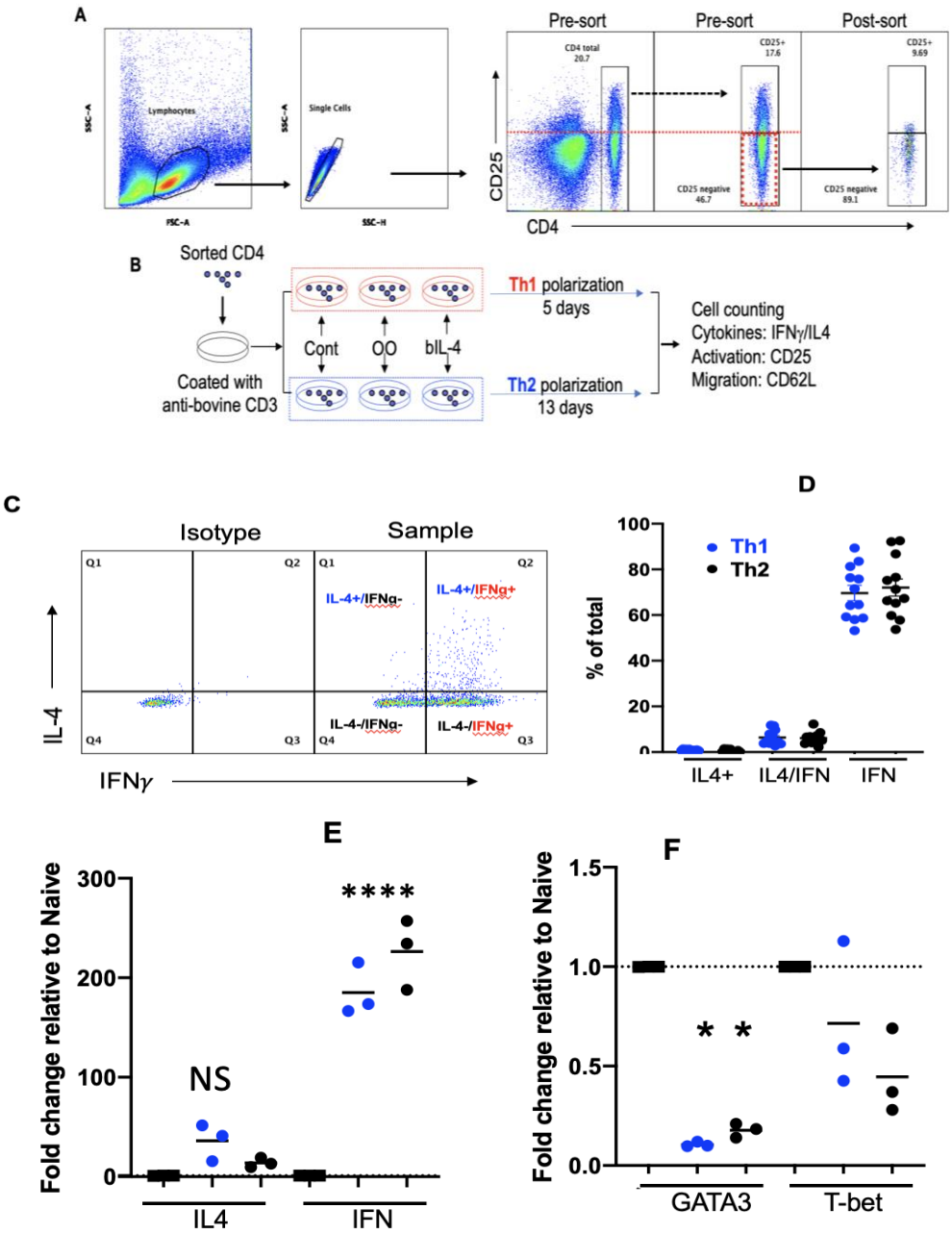

Figure 3.2: In vitro programming under Th1 or Th2 leads to Th0. (A) Purified PBMCs were stained with anti-bovine CD4 and CD25 antibodies (direct conjugates). CD4+ T cell sorting was based on CD4+/CD25-, with pre- and post-sorting purity check. Sorted cells were stimulated with anti-bovine CD3 antibody in the presence or absence of OO protein extract (3 µg/mL), and incubated in human Th1 polarization medium for 5 days, or Th2 polarization medium for 13 days [1, 2]. (A) Naïve CD4 T cells were gated on lymphocytes (small and large size), singlets, and CD25-/CD4+. (B) Experimental settings for differentiation of Th1 and Th2. Cont: control. (C-D) After 5-day (Th1) or 13-day (Th2) differentiation, cells were washed and stimulated with a cell activation cocktail for 4 h [3]. (C) Gating for cytokine producing CD4+ T cells after differentiation. (D) Comparison of cytokine producing cells after Th1 and Th2 differentiation. (E-F) Sorted naïve CD4+ T cells or differentiated CD4+ T cells were examined for transcription of IFNγ and IL4 (E), and their corresponding master transcription factors Tbet and GATA3 (F), all relative to naïve CD4+ T cell. Each dot represented individual cattle. Each dot represented individual cattle.

of the culture conditions, bovine CD4+ T cell differentiated mostly into IFNγ+ IL4- cells along with a fraction (<10%) of hybrid Th0 cells.

To examine the transcriptional regulation of IFNγ, IL4, and their corresponding master transcription factors Tbet and GATA3, quantitative PCR was performed using housekeeping gene GAPDH as the reference [398]. In line with protein expression data, we observed significant (>100 fold) enhancement of IFNγ mRNA expression in both Th1 and Th2 cells compared to naïve CD4+ T cells. In contrast, IL4 mRNA was moderately enhanced (>10 fold) but with no significant statistical difference (**Fig. 3.2E**). Of note, the master transcription factors, Tbet and GATA3 were highly co-expressed in the naïve CD4+ T cells, but the level of GATA3 in Th2 and that of Tbet in the Th1 was significantly lower than those in the naïve CD4+ T cells (**Fig. 3.2F**), suggesting that hallmark master regulators observed in mice and humans shows an opposite trend in cattle. In summary, in vitro CD4+ T cell differentiation under Th2 culture generates Th1 dominant Th0 phenotype, consistent with memory CD4+ T cell profile in the blood and lymphoid tissues of grass-fed beef cattle (**Fig. 3.1D**), and in line with data published in our recently published papers [342, 396].

3.4.3 Validation of Th2 differentiation

To validate if the differentiated cells under Th2 condition were actually Th2 cells, rather than focusing only on IFNγ and IL4 expressions, we conducted an unbiased proteomic assay and analyzed the proteins using ingenuity pathway analysis (IPA) software. Multiplexing with Tandem Mass Tag (TMT) followed by high pH fractionation and LC-MS/MS led to identification of 4212 proteins from three biological replicates in each population. Indeed, the Th1 and Th2 polarization regulated different groups of proteins (**Fig. 3.3A**), most of which were overlapping structural proteins. Notably, Th1, Th2 and the naïve cells fell into

different clusters upon principal component analysis (**Fig. 3.3B**), suggesting that cells induced under Th2 condition demonstrated a distinct phenotype compared to naïve and Th1 cells. Considering 1.5-fold as the cutoff value ($P< 0.05$), a comparison with naïve CD4+ T cells revealed 422 differentially expressed proteins in the bovine Th2 cells, and 397 in Th1 (**Supple. Protein List**). Furthermore, based on the Th2 differential proteins, the ingenuity pathway analysis (IPA) predicted typical upstream regulators associated with Th2, including IL4, IL7, IL1β, and IL33 **(Fig. 3.3C, Appendix B.1)**, suggesting a typical Th2 differentiation.Th1/Th2 differentiation is tightly regulated by specific master transcription factors, correspondingly such as Tbet for IFNγ, and GATA3 for IL4 [5, 51, 79, 399]. Indeed, based on the differentially expressed Th1 and Th2 proteins, TBX21, the gene encoding Tbet, was predicted to be activated in both Th1 and Th2 **(Table 3.1)**, consistent with the enhanced IFNγ expression in **Fig. 3.2D-E**. Nonetheless, based on differentially expressed Th2 proteins, GATA3, which play major role in maintaining IL4 expression in mice and humans, was not predicted as upstream molecules (**Table 3.1**), consistent with reduced GATA3 transcript and unchanged IL4 mRNA/protein detection after differentiation under typical Th2 culture **(Fig. 3.2 E-F)**. Of note, consistent with those in the mice and humans, transcription factors like AP-1, E2F2, Maf, MYB and NF-ĸB (**Table 3.1**) [400-411], as well as, cytokines such as IL4, IL1, and IL33 (**Fig. 3.3C, Appendix B.2**) [411-417], were predicted as upstream factors in bovine Th2 differentiation. Overall, proteomic assay validated differentiation of bovine Th2 cells that bear some differences from those in murine and human models.

Table 3.1: Predicted activation (Z score≥ 2) of upstream transcription factors based on differentially expressed proteins in the Th1 vs naïve and Th2 vs naive groups

Upstream regulator		**Th1/Naive**		**Th2/Naive**				
	Z-score	Predicted activation state	P-value	Target molecules in database	Z-score	Predicted activation state	P-value	Target molecules in database
CEBPB	3.466	Activated	2.54E-12	36	2.859	Activated	3.16E-15	41
ETV5	2	Activated	0.0167	5	2.449	Activated	0.000158	8
MYC	4.449	Activated	4.51E-06	40	3.667	Activated	7.34E-11	52
TBX21	2.449	Activated	9.78E-04	6	2	Activated	0.0308	4
FOXM1	2.47	Activated	2.3E-06	12	N/A			
AP1	N/A				2.372	Activated	2.05E-02	7
E2F2	N/A				2	Activated	0.00593	5
MAF	N/A				2.219	Activated	8.91E-05	7
MYB	N/A		2.157	Activated	0.00012		11	
NFKB1	N/A		2.578	Activated	6.56E-5		15	

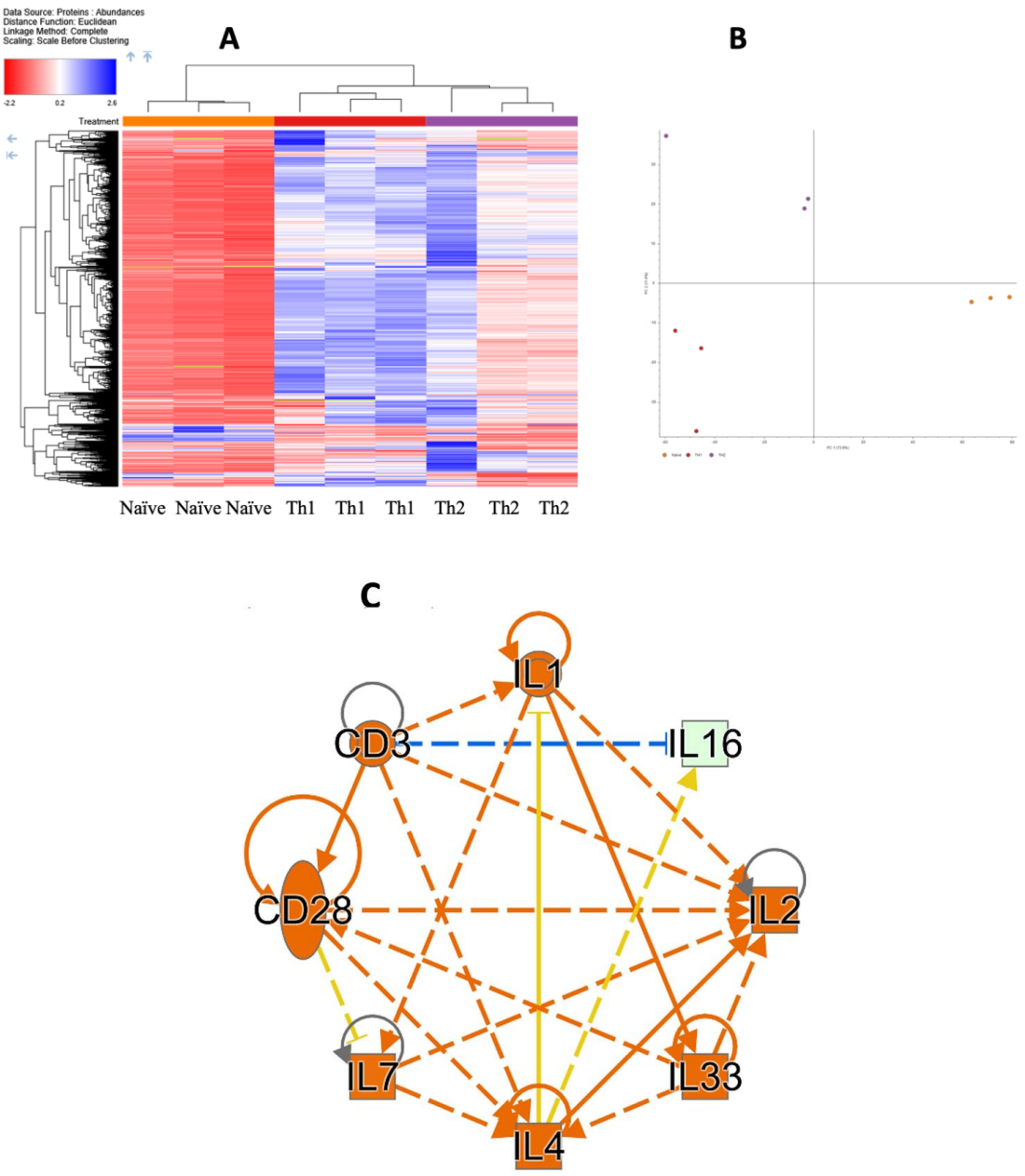

Figure 3.3: Th1/Th2 differentiations drive different effector functions. Quantitative proteomics analysis of naïve and differentiated CD4+ T cells was carried out using LC-MS/MS following multiplexing with TMT and high pH fractionation. Heat map of differentially expressed proteins in naïve, Th1, and Th2 cells with red indicating upregulation and blue indicating downregulation (A). Principle component analysis (B) demonstrate cells differentiated under naïve Th1- and Th2- cultures are distinctly clustered (C) IPA pathway analysis was performed to predict upstream cytokines, and network analysis based on the predicted activation and inhibition states of the upstream regulators identified in the Th2 vs naïve group. The data were based on the 1.5-fold up or down based on the proteins of Th2/Th1 based on the average of three biological replicated.

3.4.4 Th2 differentiation is sensitive to TCR stimulation strength

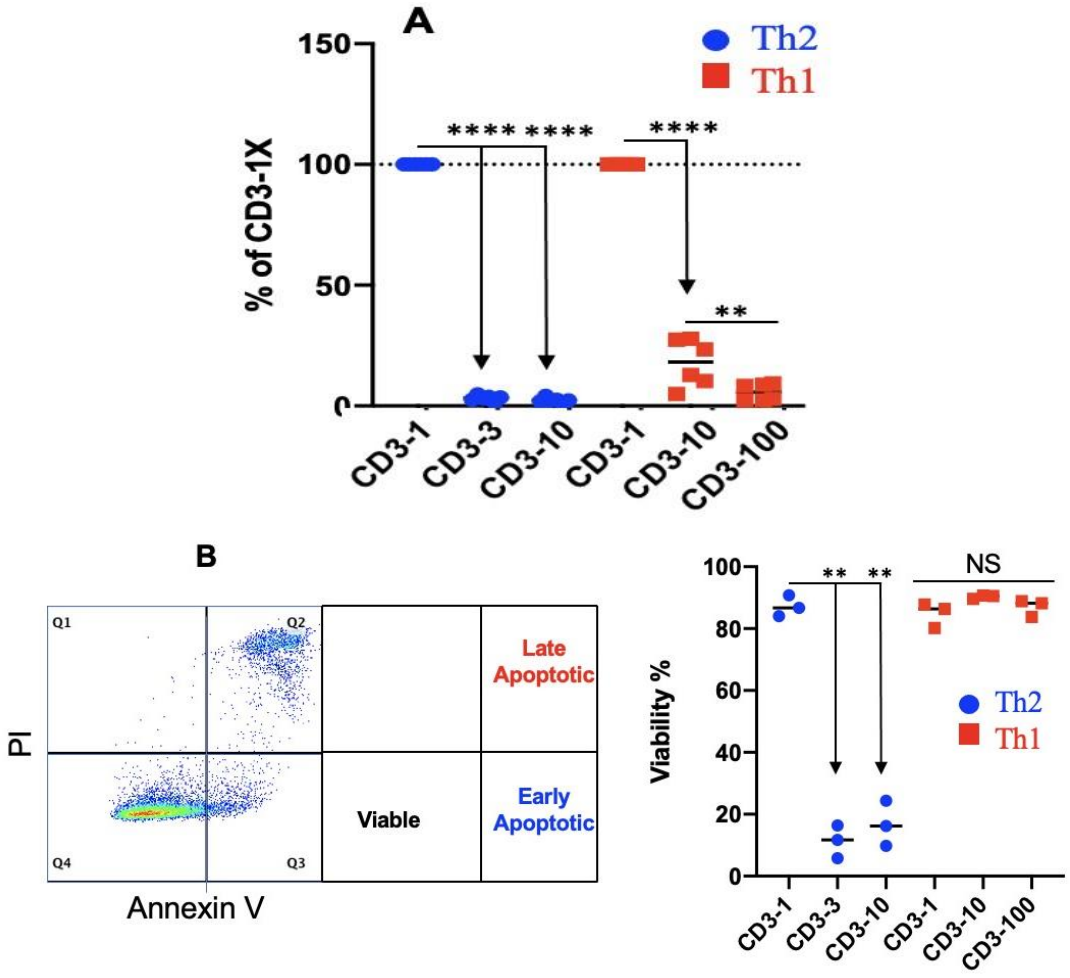

Figure 3.4: Th2 differentiations is sensitive to strength of TCR stimulation. Naïve CD4+ T cells were differentiated under Th2 and Th1 conditions as described in Figure 2B, with reduced CD3 concentration for coating. 1X: original concentration, 5 ug/ml, 10X: 0.5 ug/ml, 100X: 0.05 ug/ml. Differentiated T cells were harvested for examination. (A) Comparison of expansion relative to 1X anti-CD3. (B) Comparison of viability.

In mice, reducing TCR stimulation strength was able to shift CD4+ differentiation toward the IL4+ phenotype [418-423]. To investigate if IL4 expression could be boosted in a similar way, isolated naïve CD4+ T cells were stimulated with plate-bound anti-bovine CD3 antibodies coated at varied concentrations under both Th1 and Th2 conditions, as illustrated

in **Fig. 3.2B**. Even three-fold dilution (3X) of anti-bovine CD3 reduced Th2 proliferation by more than 95%, but cellular expansion under Th1 conditions was reduced by 80% at 10X dilution **(Fig. 3.4A)**. Furthermore, the viability of the tiny number of surviving cells in Th2 cells was reduced to roughly 20%, demonstrating that even a 3X dilution of CD3 essentially eliminated Th2 survival (>99%) compared to the 1X CD3 control **(Fig. 3.4B)**. In Th1 cells, the viability of remaining cells at 10X diluted CD3 was similar to that induced in the original anti-CD3 concentration (1X), about 90% **(Fig. 3.4B)**, indicating that the survival of differentiating bovine Th2 cells is more dependent on intense TCR stimulation than Th1 cells. Therefore, in contrast to mice and humans, lower TCR stimulation strength is detrimental to survival of bovine Th2 cells.

3.4.5 Extra recombinant bovine IL4 leads to enhanced Th2 differentiation.

To test if exposure to extra exogenous rbIL4 could enhance IL4 expression, purified naïve CD4+ T cells were cultured under Th2 condition for 13 days as mentioned in **Fig. 3.2B,** in the presence of rbIL4 introduced at various concentrations. The cells grew robustly when rbIL4 was added at 0.05 ug/ml-5 ug/ml concentrations, ended up with 2-fold increase compared with control (**Fig. 3.5A**). However, no changes were observed in CD25 (**Fig. 3.5B**) or IFNγ/IL4 expression (**Fig. 3.5 C-D**). To confirm if this enhanced proliferation was induced through IL4, 0.5 ug/ml recombinant bovine IL4 was applied to Th2 condition, and protein profiles of Th2 cells were contrasted with those derived from the Th2+rbIL4 culture. There were 175 differential proteins, of which 20 molecules were related to the IL4 related effector pathways.

In mice and humans, the presence of IL4 into the Th2 culture can enhance the expression of Th2 transcription factors, STAT6 and GATA3 [81, 82], which are indispensable for

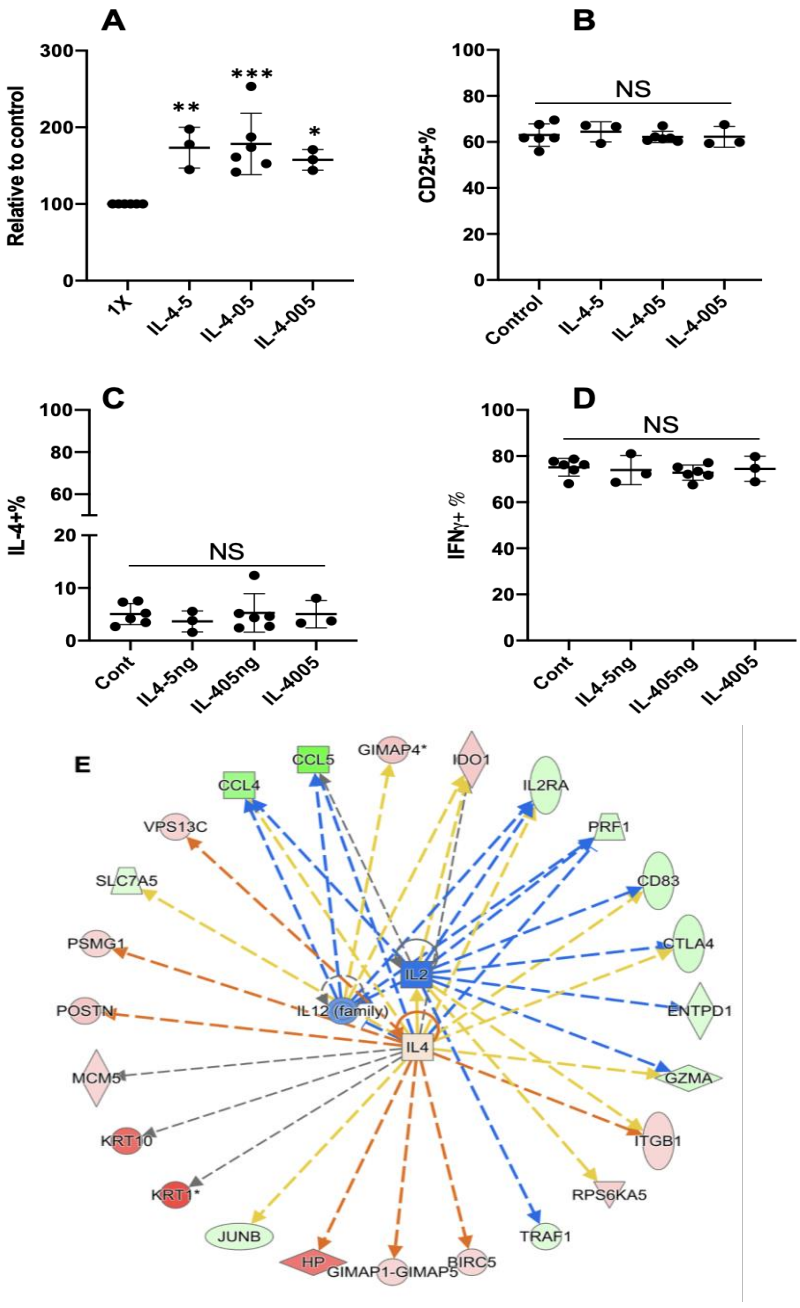

Figure 3.5: Extra recombinant bovine IL4 leads to enhanced Th2 differentiation. Naïve CD4+ T cells were differentiated under Th2 condition in the presence of additional recombinant bovine IL4. IL4 was tested at three different concentrations: 0.05 ug/ml, 0.5 ug/ml and 5 ug/ml. (A): Comparison of expansion (A), CD5 expression (B), IFNγ and IL4 expression (C). (D) Th2 cells differentiated in the presence of 0.5 ug/ml recombinant bovine IL4 were analyzed for protein profiles, in contrast to Th2 control cells. IPA was used to predict the effect function of the additional rbIL4. Network analysis was based on the predicted activation and inhibition states of the upstream regulators identified in the rbIL4+Th2 vs Th2.

establishment of IL4 secreting positive feedback loop. Nevertheless, analysis based on 175 differentially expressed proteins did not predict STAT6 and GATA3 as upstream factors in rbIL4 treated bovine Th2 cells **(Appendix Table b.4)**, which were observations in line with the lack of IL4 upregulation in qPCR and flow cytometry data **(Fig. 3.2 D-E)**.

While predicted effector pathways were related to IL4, supported by detection of 20 targeted proteins **(Fig. 3.5E)**, the activation score of IL4 was predicted to be low **(0.219, Appendix Table B.5)**, which was because the software detected few discrepancies between functional features of bIL4 with its murine and human counterparts **(Appendix Table B.3)**. IPA analysis suggested that rbIL4 plays a partially different role in the bovine Th2 differentiation process compared to its counterparts in mice and humans.

3.4.6 *Ostertagia ostertagi* extract (OO) leads to inhibition of Th2 differentiation

Extracellular parasites regulate Th2 differentiation in mice and humans in vitro [424]. OO, which is proteins extracted from the *Ostertagia ostertagi* worm, has effectively demonstrated ability to modulate neutrophil functions in cattle [425], eventually inhibiting the activation of bovine CD4+ T cells [44, 398]. To test if the differentiated bovine Th2 cells are sensitive to OO extract, sorted naïve bovine CD4+ T cells were cultured under anti-CD3 stimulated Th2 condition in the presence or absence of OO protein extract as indicated in **Fig. 3.2B**, and harvested cells were analyzed by flow cytometry and proteomic analysis.

To our surprise, treatment with OO led to cellular proliferation by 2-3-fold under Th2 conditions **(Fig. 3.6A)**, without noticeable changes in IFNγ and IL4 production (**data not shown**). Based on differential regulation of 83 proteins (**Appendix Table B.6**), OO presence led to inhibition of several key nodes including CD3, CD28, CD40, IL2, IL7, IL15 and

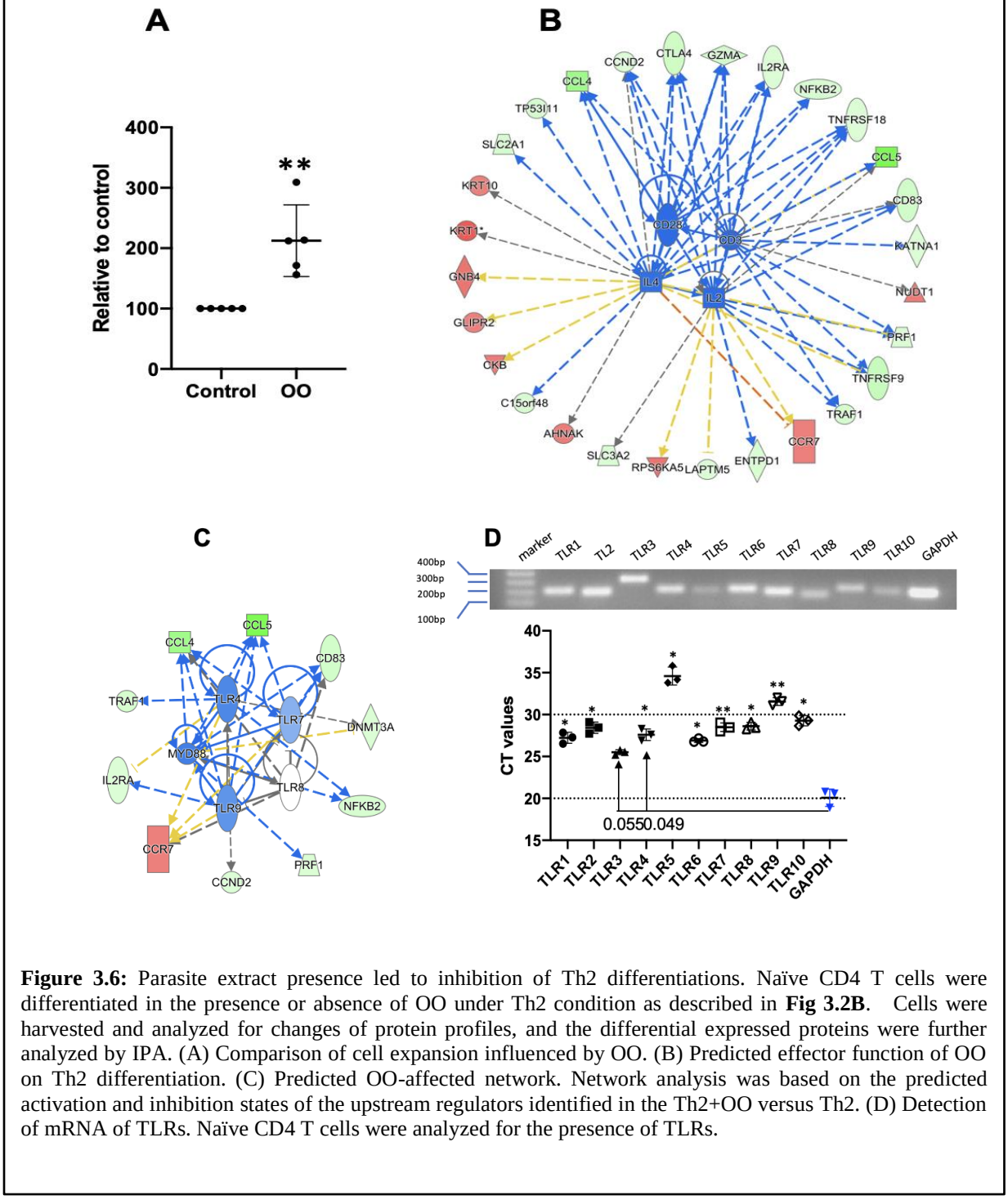

Figure 3.6: Parasite extract presence led to inhibition of Th2 differentiations. Naïve CD4 T cells were differentiated in the presence or absence of OO under Th2 condition as described in **Fig 3.2B**. Cells were harvested and analyzed for changes of protein profiles, and the differential expressed proteins were further analyzed by IPA. (A) Comparison of cell expansion influenced by OO. (B) Predicted effector function of OO on Th2 differentiation. (C) Predicted OO-affected network. Network analysis was based on the predicted activation and inhibition states of the upstream regulators identified in the Th2+OO versus Th2. (D) Detection of mRNA of TLRs. Naïve CD4 T cells were analyzed for the presence of TLRs.

NF-ĸB, which are critical regulators of Th2 cell differentiation (**Fig. 3.6B, Appendix Table B.6**) [323, 424]. Of note, TLR/MYD88 pathways were also found inhibited, especially

through TLR4, 7 and 9 (**Fig. 3.6C**). Despite the fact that bovine innate immune cells express a broad range of TLRs [426], it was unknown if the naïve bovine CD4+ T cells express TLRs as in mice and humans [427, 428]. For the first time, we confirmed that naïve bovine CD4+ T cells express all the 10 TLRs in PCR, and their expression levels, except that of TLR3, was abundant within 10 cycles compared with housekeeping gene GAPDH (**Fig. 3.6D**). It seems that bovine Th2 differentiation can be inhibited by extracellular pathogens through different pathways such as suppression via TLR/MYD88, at least in vitro.

3.5 Discussion:

In the blood and lymphoid tissues of pasture-raised cattle, majority of identified CD4+ T cells were IFNγ+ IL4 - and a small fraction of double positive Th0 cells were mostly inducing IL4 protein, which was similarly reflected by in vitro differentiated bovine Th2 cells. Analysis of differentially expressed proteins suggested that Th2 differentiation was not associated with IL4 expression in cattle, which remained unenhanced despite manipulating culture conditions in manners published in mice and humans.

Grass-fed cattle, which are fed with pasture are routinely exposed to infective larvae of extracellular parasites such as *Ostertagia ostertagi*, but grain-fed cattle raised on the feedlot-based diets are not exposed to these environmental pathogens. Therefore, we speculated that memory CD4+ T cells in the blood of grass-fed cattle would produce more IL4 protein than those in grain-fed group. Contradicting our hypothesis, peripheral CD4+ T cells from grass-fed cattle were producing significantly low amount of IL4 compared to that of IFNγ, demonstrating profile similar to those in the grain-fed group [323]. While mice exposed to nematodes such as *Nippostrongylus brasiliensis* and *heligmosomoides polygyrus* induce high frequency of IL4+ Th2 cells in the spleen and mesenteric lymph nodes compared

to those in the peripheral lymph nodes [372-375], we speculated whether IL4+ CD4+ T cells are distributed unevenly among different lymphoid tissues, such as the spleen (SP), and lymph nodes draining abomasum (DLN) compared to those in the inguinal lymph nodes (ILN) and the blood (BL). Once again, all these tissues reflected similar profile detected in the blood (**Fig. 3.1 D**), contrasting observations in the mice [429, 430]. As Th1 dominant Th0 profile was consistently detected irrespective of exposure to different dietary and environmental conditions, we speculated that perhaps bovine CD4+ T cells spontaneously differentiate into dominantly IFNγ producing mixed profile, which could be examined under the in vitro Th2 conditions, paralleling with the standard protocol in mice and humans [5, 378, 379].

In line with observations derived from the blood and lymphoid tissues of healthy pasture-raised cattle, naïve bovine CD4+ T cells did not exclusively differentiate into IL4 protein producing cells. Only a small fraction of differentiated cells was IL4+, which mostly consisted of double positive Th0 cells. To further investigate if the protein expression tendency was reflected at the mRNA level, we evaluated transcriptional regulation of hallmark cytokines and their master regulators, which were in line with the earlier observations. Different from mice and humans, where IL4 transcript level keeps on increasing after two days of stimulation [431], IL4 mRNA expression in differentiated bovine CD4+ T cells was not significantly enhanced compared to that in the naïve counterpart [370]. Furthermore, unlike gradual upregulation of GATA3 mRNA following a few hours of stimulation under the murine and human Th2 culture [141, 378, 432-435], GATA3 transcript was at the highest level in the naïve bovine CD4+ T cells, which downregulated significantly after differentiation **(Fig. 3.2F)**. While IL4 expression and IFNγ

exclusion is associated with GATA3 mediated repression of Tbet transcription in mice and humans [5, 51, 84, 128-130, 378, 379, 433, 436], absence of strict cross-regulations between these master regulators were somehow related to IFNγ dominant Th0 induction in cattle.

Since the cells differentiated under the Th2 culture were not highly producing IL4 protein, naturally, we then questioned if in vitro differentiated cells were real Th2 cells, so we analyzed them through unbiased proteomic assay combined with IPA analysis. Based on 422 differentially expressed proteins in reference to the naïve cells, our analysis revealed two key observations. While predicted upstream factors were mostly consistent with Th2 differentiation in mice and humans, some cattle-specific features were noticed that partly explained why IL4 expression in these cells were low. Consistent with mice and humans, IL4, IL33, IL1, IL2 and IL7, which are key cytokines known for differentiation, proliferation and survival of Th2 cells [411-417, 437-448], and transcription factors including AP-1, E2F2, Maf, MYB, Jun-B and NF-ĸB, [400-410, 449-451], that are essential for driving the transcriptional program, were similarly predicted in cattle. However, neither STAT6 nor GATA3 were predicted as upstream regulators in bovine Th2 differentiation, which are the molecules involved in chromatin remodeling and increased IL4 transcription in mice and humans [81, 82], partly explaining why IL4 protein detection in the differentiated bovine Th2 were consistently low. Of note, STAT5, which has been suggested in GATA3 independent Th2 differentiation in mice [452-454], was also predicted as upstream factor in cattle with its z-score suggesting activation, supporting the argument that bovine Th2 differentiation was not strongly associated with IL4 mRNA/protein detection in cattle.

Although IPA validated Th2 differentiation in cattle, IL4 expression in the differentiated cells was fairly low, which impelled us to explore strategies for optimizing IL4

expression in differentiated bovine Th2 cells. Therefore, we adopted standard practice from murine and human experiments. As weakened TCR stimulation has been correlated with IL4 enhancement under in vitro culture [418-422], we reduced the concentration of anti-bovine CD3 used for stimulating bovine CD4+ T cells under typical Th2 condition. Despite the fact that weakened TCR stimulation strength did not lead to a significant alteration in IL4 protein expression, it was linked to decreased viability and diminished expansion of cells under the Th2 conditions, a phenomenon not observed to the same extent in Th1 controls. While the survival of bovine Th2 cells relied on robust TCR stimulation, their IL4 expression remained unaffected by TCR stimulation strength.

Next, we tested if exogenously supplied bIL4 could somehow overcome limited IL4 secretion by the differentiated bovine Th2 cells. Despite sharing similar N-glycosylation sites [455, 456], a high concentration of hIL4 was required for mimicking biological effect of bIL4 on the bovine cells [457], which could be due to relatively low sensitivity and/or affinity of human analogue to the bovine receptors. To rule out this possibility, we added appropriately glycosylated rbIL4 into the Th2 culture, however its effect on IL4 production was almost null at any added concentration, which were partially in line with previously published reports in cattle [458]. Unlike in mice and humans, where addition of IL4 in the culture inhibits IFNγ secretion while promoting that of IL4 [431, 459-461], neither IL4 upregulation nor IFNγ downregulation was noted in cattle, highlighting the fact that IFNγ and IL4 cross-regulation is not common in bovine species. Distinctly, bIL4 failed to induce arginase activity in macrophages in vitro [462], and its porcine analogous mediated inhibition of the B cell responses in pigs [463], so heterogeneity in the functions of this cytokine across species could be speculated.

To find out why exposure to rbIL4 did not increase IL4 expression in the differentiated cells, we conducted further analysis in the IPA. Evidently, we found that bovine IL4 seems to have partially different role in bovine Th2 differentiation compared to those in mice and humans. Specifically, rbIL4 addition into the Th2 culture led to regulation of several proteins distinctly from those in mice and humans (**Appendix Table B.3**). Specifically, opposite from those in mice and humans, IL4 treatment was associated with downregulation of IL2 receptor (IL2R) [464, 465], downmodulation of Jun-B induction [466, 467], and suppression of CD83 (co-stimulatory molecule) expression in cattle (**Appendix Table B.3**) [468-471]. IPA analysis highlighted that lack of IL4 expression in the bovine Th2 cells might be related to unique biological functions of bovine IL4.

Finally, we asked the most critical question, which was whether the bovine CD4+ T cell differentiation under the in vitro Th2 conditions was sensitive to regulation from extracellular pathogens, which has been commonly observed in murine and human counterparts [424, 472-475]. Remarkably, OO extract presence elicited enhanced proliferation of Th2 cells by several folds without significantly changing IFNγ and IL4 expression in vitro, which were in contrast to its inhibitory effect on the expansion of PBMCs reported before [476-478], the discrepancy might be related to difference in methodologies, and culture duration. While we co-cultured CD3 stimulated sorted naïve bovine CD4+ T cells with the parasite protein extract, whole PBMCs were used in the previous experiments. Our results are partially in line with proliferation of CD4+ T cells from healthy cattle in response to *Dictyocaulus viviparous* homogenate and *Theileria annulata* infected cells [479, 480]. Based on differential expression of proteins, IPA strongly predicted activation of T cell proliferation pathways in Th2+OO group when contrasted with Th2 cells.

The presence of OO extract in the culture inhibited three critical signals involved in bovine Th2 cell differentiation in addition to those via TLR/MYD88 pathways, suggesting potential mechanism to avoid effective immunity in cattle [473, 481-484]. Noticeably, CD3 that mimics the antigen stimulation (1st signal), CD28, which provides co-stimulation (2nd signal), and cytokines like IL4 and IL33, which provides polarizing 3rd signals were found inhibited in the Th2+OO culture [437, 485, 486]. More importantly, NF-kB signaling, which can be inhibited by extracellular pathogens such as *Schistosoma* species in humans [424], were also found inhibited (**Appendix Table B.6**). Additionally, ligands present on the OO extract seems to have further suppressed TLRs/MYD88 pathways, which are partially consistent with published literature such as downregulation of TLR2 expression in *Filarial* and *Schistosoma Japonicum* infection [487, 488], and inhibition of MYD88 signaling by the products derived from helminths [489, 490]. While murine CD4+ T cells can express transcripts of TLRs from 1 to 9, which have been mostly similarly found in humans excluding that of TLR 6 and 8 [491-496], we report that the naïve bovine CD4+ T cells express 10 TLRs. We believe that at the resting stage naïve CD4+ T cells might also perform innate immune functions. Collectively, data indicated that OO might regulate Th2 differentiation through multiple pathways including those via TLR/MYD88 to avoid effective Th2 responses mounted in the cattle.

One key observation consistently noticed in cattle was majority of IL4+ cells were hybrid Th0. In mice and humans, IFNγ and IL4 expression in the CD4+ T cells is mostly mutually exclusive [366, 368, 369], and only a small fraction of hybrid cells co-express IFNγ and IL4, which were traditionally characterized as Th0 cells, and recently have been renamed as Th_{1+2} cells [135, 137, 138, 141-143]. Partially different from those in mice and humans,

co-expression of IFNγ and IL4 transcripts was found predominant (60 - 90 %) in cattle [25, 122, 497-499], which the authors designated as bovine Th0 feature. Our data partially supports the previously published data as at the transcript level co-expression was found relatively common in cattle, however we further demonstrate that at the protein level only a small fraction (<10%) of cells represent actual Th0. Of note, highest level of Tbet and GATA3 mRNA are found co-expressed in the naïve bovine CD4+ T cells, therefore characterization of Th0 cells based on transcript co-expression of master regulator has limitations in cattle.

While previously we published that hIL12 can activate bovine CD4+ T cells and enhance their IFNγ expression with limited IL4 protein [44, 458], lack of detection of IL12 in the Th1 proteome, and that of transcription factor STAT4 as an upstream factor suggest that bovine Th1 differentiation might be very distinct in cattle [48-51]. On the other hand, while IL4 driven Th2 kit was able to drive bovine Th2 differentiation, the process was not found associated with IL4 expression.

3.6 Conclusion & Future directions

Our findings from evaluation of Wye Angus cattle challenge the conventional belief that bovine Th2 cells are marked by a high IL4/ IFNγ mRNA ratio. Although naïve bovine CD4+ T cells responded to Th2 related cues, differentiated cells dominantly produced high amount of IFNγ protein with limited detection of IL4, which was supported by data from the blood and lymphoid tissues of pasture-raised cattle frequently exposed to the extracellular parasites. Results warrants that though IL4 may serve as a differentiation specific cytokine, it may not be the primary cytokine produced by the bovine Th2 cells, which underscores the need to revisit Th1/Th2 paradigm in cattle in the light of advanced technology. While this study

focused on polyclonal populations, our ongoing research aims to investigate antigen (OO)-specific CD4+ single cell population, which will generate more definitive answers in the future.

Chapter:4 Establishment of *Ostertagia ostertagi*-specific CD4+ T cell clones from naturally exposed cattle

Anmol Kandel[1], Lei Li[1], Wenbin Tuo[1]* and Zhengguo Xiao[1]*

1 Department of Animal and Avian Sciences, University of Maryland, College Park, 20742, MD, USA; akandel1@umd.edu (A.K.); lixxx242@umd.edu (L.L.)

2 Animal Parasitic Diseases Laboratory, USDA/ARS, Beltsville, MD 20705 USA

Email: wenbin.tuo@usda.gov

*Correspondence: xiao0028@umd.edu; Tel.: +1-301-405-6258.
*Correspondence: wenbin.tuo@usda.gov

3.1Abstract:

While interleukin 4 (IL4)-producing Th2 cells are traditionally associated with effective immune defense against extracellular parasites, our in vitro differentiated bovine Th2 cells revealed a notable discrepancy. Contrary to expectations, majority of these cells exhibited high IFNγ production, with less than 10% of them co-expressing IL4 (submitted under review). This finding diverges from previous research on anti-parasitic CD4+ T cell clones derived from experimentally infected cattle, which reported a dominant induction of a mixed phenotype characterized by mRNA of both IFNγ and IL4, termed as bovine Th0 clones. To investigate whether *Ostertagia ostertagi* (OO)-specific CD4+ T cell clones derived from naturally infected cattle actually display a similar Th0-dominated phenotype, CD4+ T cells isolated from the blood of pasture-raised cattle, confirmed to be infected with OO, were co-cultured with autologous irradiated PBMCs as antigen-presenting cells (APCs) in the presence of OO antigens. After several rounds of expansion, a single OO-responding CD4+ T cell was sorted into each well, and then was repeatedly stimulated with autologous irradiated PBMCs plus OO antigens for several weeks, before being characterized. All 21 CD4+ single T cell clones from 5 cattle produced high levels of IFNγ, and only 5 of them

co-expressed IL4 with remaining 16 detected negative for IL4 expression, supporting, a dominant IFNγ-producing Th0 phenotype. These findings suggest that even in the typical Th2 response observed in natural infection against extracellular parasites, pathogen specific Th2 clones exhibit a profile consistent with the phenotype detected in our previous experiments. Results support the notion that bovine Th2 responses are inherently predisposed towards an IFNγ-dominant Th0 phenotype, and IL4 expression may not be a hallmark feature of bovine Th2 cells.

4.2 Introduction:

It remains unresolved whether IL4 expression is a typical feature of bovine Th2 cells. In mice and humans, detection of IFNγ and IL4 in antigen-specific CD4+ T cell clones is mostly exclusive [4, 367-369, 500, 501], regulated by master regulators Tbet and GATA3 respectively [5, 51, 128, 130, 142, 399, 451, 502]. Nevertheless, our recent investigation in beef cattle revealed a distinct pattern of bovine CD4+ T cell differentiation intrinsically biased toward IFNγ dominated Th0 mixture. Challenging the traditional association between bovine Th2 differentiation and IL4 expression, multiple lines of experiments highlighted that IL4 expression in the differentiated bovine Th2 cells remain unchanged despite manipulation of TCR stimulation strength and addition of extra recombinant bovine IL4. Similarly, CD4+ T cells were found in the blood and lymphoid tissues of naturally OO infected grass-fed cattle were dominantly producing IFNγ with limited IL4 expression. This project aimed to validate whether OO-specific Th2 clones derived from natural OO-infected cattle exhibit IFNγ-dominated Th0 profile, as observed in our previous experimental settings.

Single CD4+ T cell clone, a population of antigen-specific cells that are derived from a common precursor [145, 503-508], have been extensively utilized for assessing Th1/Th2

responses [25, 366, 369]. Around three decades ago, single cell cloning was introduced into the bovine field in evaluating functional T cell responses against pathogens such as *Babesia bovis* and *Fasciola hepatica* [145, 171, 498, 507, 509-511]. Brown et al. (1998) in the published review summarized the functional responses shown by these clones and argued that most of the pathogen-specific bovine CD4+ T cell clones dominantly co-express IFNγ and IL4 transcripts, suggesting co-activation of both Th1- and Th2 associated differentiation. However, the research was limited by inconsistencies between expression of IL4 mRNA and level of its protein detected in the supernatant, as well as, lack of assessment of transcriptional activities of master regulators Tbet and GATA3. To confirm whether anti-parasitic CD4+ T cell clones specific to antigens from extracellular pathogen actually differentiates into Th0 dominant response as suggested by the review, or alternatively into IFNγ dominated Th0 mixture as indicated by our recent in vitro studies, a panel of CD4+ single T cell clones were established from the naturally OO infected grass-fed beef cattle, and their expression of hallmark cytokines and transcription factors were carefully analyzed.

Different from the previous bovine research, which used PBMCs from experimentally pathogen challenged cattle to generate antigen-specific T cell polyclones, we adopted cattle naturally exposed to OO on pasture, and used purified CD4+ T cells for developing T cell polyclones responding to the OO antigens. Moreover, overcoming the limitation of highly laborious limiting dilution technique used by the previous research [145, 503-508], we conducted flowcytometry based plate sorting to directly isolate single CD4+ T cell clone into the multi-wells plate for further expansion [512]. CD4+ single T cell clones that were responding to several rounds of weekly re-stimulations were examined through flow cytometry and qPCR assays. We were able to establish 21 CD4+ single T cell clones. All

clones produced high amount of IFNγ, but only 5 of them co-expressed IL4, the rest of 16 did not produce IL4 at all. Data support that OO-specific CD4+ T cell clones derived from cattle in a natural farm setting do not express IL4 as their signature feature. Seems OO specific CD4+ T cell clones are dominantly IFN+ IL4- cells, however a small number of clones can exhibit IFNγ dominated Th0 mixture, which reflects the CD4+ T cell profile identified in grass-fed bovine tissues and in vitro differentiated bovine Th2 cells. These established clones might offer a promising platform for identifying effective immunogens and mapping T cell epitopes, contributing to rapid development of protective vaccines against OO infection in the future [145, 171, 506, 511].

4.3 Material and Methods

4.3.1 Cattle

Since 1958, the Wye Angus herd has maintained a large number of genetically or closely related cattle under the care of the Wye Research and Education Center, University of Maryland Experimental Station (Queenstown, MD) [380]. At 5-6 months of age, the animals were weaned and subsequently divided into grass-fed (pasture-raised) or grain-fed groups. Grass-fed cattle were fed with pasture of orchard alfalfa and clover except during winter, when alfalfa and bailage were exclusively included in the diet [381].

4.3.2 OO-specific IgG ELISA

Blood samples were collected from twelve (12) grass-fed cattle and ten (10) grain-fed cattle, and OO-specific serum IgG antibody were assessed through direct ELISA as reported before [513]. Bovine immunoglobulin isotype-specific antibodies conjugated with horseradish peroxidase (HRP) from Bio-Rad Laboratories, Hercules, CA, was used as primary and goat

anti-mouse IgG-HRP (Thermo Fisher, Waltham, MA) was used as a secondary antibody. Briefly, 10 µg/mL crude adult OO antigens were coated on 96-well Nunc ELISA plates (Thermo Fisher Scientific, altham, MA) in coating buffer (0.2 M sodium carbonate/bicarbonate, pH 9.6) at 4 ◦C overnight. Phosphate-buffered saline, pH 7.2, 0.05 % Tween 20 (PBST), was used to wash the plate using a plate washer (BioTek Instruments, Winooski, VT), which was then blocked for 1 hour at 37°C using horse serum in PBST buffer. The serum samples were diluted from 1:8,000 to 1:32,000 ratio in blocking buffer, and one hundred microliters of the diluted serum samples were transferred to each duplicated well, which were then incubated at 37 ◦C for 1 h. Following one more round of wash, 100 µl of anti-bovine IgG HRP were added. Unbounded antibodies were washed again and then goat anti-mouse IgG-HRP (1:3,000) diluted in blocking buffer was added to each well before incubation at 37 ◦C for 1 h. After one more round of washing, 100 µl of 1- Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific, Waltham, MA) was added to the plates, which were then incubated at room temperature for 20 min before the substrate reaction was stopped by adding 50 µl of stop solution (2 M sulfuric acid in water). The optical densities were measured at 450 nm by a microplate reader (Spectra 90, Molecular Devices, San Jose, CA). The OD values of grass-fed serum was compared with that of grain-fed animals.

4.3.3 Preparation of OO adult worm extract

Helminth free calves were subjected to experimental infection with III-staged L3 *Ostertagia ostertagi* larvae, and antigens were prepared following previously described methods [514]. On days 9 and 21, the animals were euthanized, and adult worms were obtained from the abomasum using gel migration technique. After washing with cold PBS thrice, the samples

were subjected to centrifugation and then homogenization for five 15 second pulses on ice using a Polytron homogenizer (Kinematica AG, Luzern Switzerland). The resulting homogenates were spun down at 20,000 × g for 30 min at 4 °C and the soluble extract was collected and was stored at −20 °C prior to use. The concentration of OO adult worm extract were calculated using the bicinchoninic acid assay (BCA) kit (Thermo Fisher Scientific, Waltham, MA).

4.3.4 PBMC isolation and irradiation

Grass-fed blood samples were collected in 10 ml EDTA blood tubes, which were spun down at 1200g for 30 minutes at standard (5-7) break. Puffy coat layer was sucked and was mixed with 1X PBS at room temperature, and about 8 ml of cell suspension was gradually laid over 5 ml lymphocyte separation medium into the 15 ml tube. Followed by density gradient centrifugation at 900g for 30 minutes (break off), the second puffy coat layer was collected, resuspended in 1X PBS (RT), and was spun down again at 500g for 15 minutes on standard break. The pellet obtained would again be resuspended in 1X PBS and was centrifuged at 250 g for 10 minutes for two more rounds before being resuspended into 10% FBS Allos (RT). Cellular density was calculated with trypan blue exclusion method, and cells were subjected to x-ray irradiation at 30 Gy for 5-6 minutes, conducted at facility inside the department of Laboratory Animal Resources Imaging Core (DLARIC), University of Maryland, College Park.

4.3.5 OO specific T cell cloning assay

The clones were generated from 5 grass-fed cattle raised on two different batches; while the first batch included cattle #1, #2 and #3, the second batch included cattle #4 and #5. Different from studies that used magnetic beads [515, 516], or performed antibody mediated depletion

of specific subtype [517, 518], we used flow cytometry based sorting to obtain enriched CD4+ T cell (purity > 95 %) population from the *Ostertagia ostertagi* exposed bovine PBMC and cultured them either with autologous or heterologous irradiated PBMCs along with adult OO worm extract in the rhIL2 supplied medium. After four rounds of stimulation, growing T cell polyclones were harvested and frozen until used on liquid nitrogen tank. To perform single cell cloning, the polyclones were recovered, and were subjected to single cell sorting on 96 well plates. Individually sorted out cell was then stimulated with 5 * 10^4 autologous irradiated PBMCs and 5 ug/ml OO worm protein extract in the presence of rhIL2. Approximately 20% media volume was replaced once every weak, and autologous irradiated PBMC was replenished biweekly. On fourth week, the wells were screened and identified single cell colonies were gradually transferred to 48 and then to 24 well plates through weekly stimulation, before they were analyzed.

4.3.6 FACS

Cells containing successfully growing antigen specific single CD4+ T cell clones were harvested, divided into two tubes, and were subjected to surface and intracellular staining. In case of surface staining, cells were incubated for 25 minutes at 4 °C with primary antibodies, and then were washed with 2 % SB followed by incubation with fix solution (1:1 ratio) for 15 min at 4 °C. Fixed cells were washed again and were resuspended in 100 µL SB before being read in FACSCalibur™ flow cytometer.

Before intracellular staining, cells were treated with cell activation cocktail (Biotechne, Minneapolis, MN, USA) at 37°C with 5 % CO_2 for 4 hours. The cocktail comprises monensin sodium salt (1.5 mM), phorbol 12-myristate 13-acetate (0.0405 mM) and ionomycin calcium salt (0.67 mM) and triggers the activation of cells while ensuring retention of the cytokines produced within the cells. Permeabilization was performed with P/W buffer for 15 minutes at 4°C, and the cell suspension was treated with primary antibodies

for 25 minutes (4 °C) followed by staining with the secondary antibody. Gradual, P/W to SB transition was made before relabeling the surface molecules with directly conjugated surface antibodies. Isotype control samples were stained with isotype antibodies, and unstained control were treated similarly on each step with no exposure to antibodies at all. Cells were finally resuspended in 100µL SB, and then Flow cytometry was performed acquiring a minimum of 20,000 events. Data analysis was conducted using Flow Jo™ 10 (Tree Star, Ashland, OR, USA).

Primary and secondary antibodies

Specificity	Clone	Isotype	Colors	Source
bCD4	CC8	IgG2a	FITC	Bio-Rad
bCD25	LCTB2A	IgG3	PE	WSUMAC
bCD62L	BAQ92A	IgG1	FITC	WSUMAC
bIFNγ	CC302	IgG1	PE	Bio-Rad
bIL4	CC303	IgG2a	-	Bio-Rad
Anti-mouse IgG1	-	IgG1	APC	Bio-legend
Anti-mouse IgG2a	-	IgG2a	APC	Bio-legend

Isotype control antibodies

Isotype controls	Source
Mouse IgG1	BioLegend
Mouse IgG2a	BioLegend
Mouse IgG3	BioLegend
Mouse IgM	BioLegend
Mouse IgG1-PE	BioLegend

4.3.7 Quantitative polymerase chain reaction (qPCR)

Highly proliferating antigen-specific CD4+ T cell clones were collected, stained for CD4 expression, and around a million cells were sorted out using flow cytometry. Enriched CD4+

T cells were washed with 1X PBS before their pellets were lysed with RLT lysis bufer (Qiagen, Germantown, MD). RNA was isolated using the RNeasy Micro kit (Qiagen) driven protocol that incorporated DNase treatment steps to eliminate genomic DNA contamination. NanoDrop 1000 spectrophotometer was used to evaluate quality and concentration of extracted RNA, of which equivalent to 400 ng of RNA was aliquoted for cDNA synthesis through Termo Maxima First Strand cDNA synthesis kit (Fisher, Waltham, MA). Reverse transcription (RT) was performed in an Eppendorf Master Gradient Cycler (Eppendorf, Hamburg, Germany) with three cycles as follows: initialization by one cycle at 25 °C for 10 min, second cycle at 50 °C for 15 min, and third inactivation cycle at 85 °C for 5 min, after which the machine was temporarily maintained at 4 °C. Synthesized cDNA in 20 µL volume was diluted to 80 µL with RNase free water and 40 µL cDNA that contained 2.5 ng/ µL equivalent RNA was aliquoted for use in -20° C. 2 µl of the synthesized cDNA was used for Quantitative PCR (qPCR) amplification, which was carried out using the IQ SYBR Green Supermix (BioRad, Hercules, CA) kit in a C1000 Touch Termal Cycler (BioRad, Hercules, CA). 20 µL reaction volume that contained 2 µL of diluted cDNA template and two primers (final concertation 400nM) were subjected to amplification through following steps: initial denaturation at 95 °C for 3 min, 40 repeated steps at 95 °C for ten second, and extension at 55°C for 30 sec. After 40 rounds of cycles the melting curves were obtained, and the resulting products were further analyzed by gel electrophoresis.

4.3.8 Statistical Analysis

Statistical analyses were performed using Prism 8 (GraphPad Software, Inc., La Jolla, CA); details can be found in the figure legends. All data were analyzed using a one-way

ANOVA with Tukey's Multiple Comparisons Test. Asterisks denote statistical significance. *P < 0.05; **P < 0.01; ***P < 0.001.

4.4 Results

4.4.1 Grass-fed serum exhibit significantly high OO-specific IgG titers than those of grain-fed cattle

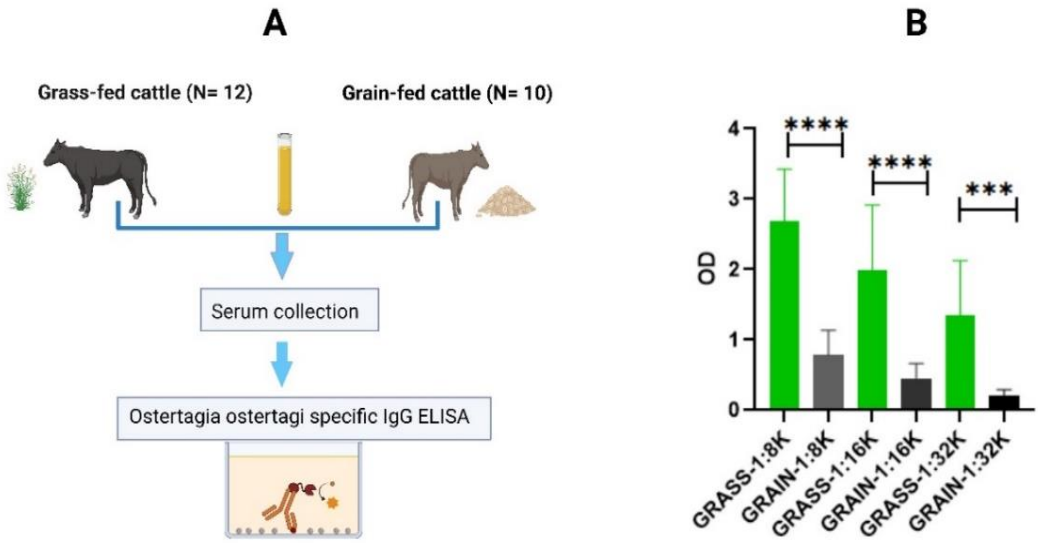

Figure 4.1: Comparison of OO-specific IgG titer between grass-fed versus grain-fed cattle serum. **Panel A**: Experiment approach; serum samples collected from twelve grass-fed and ten grain-fed cattle were used to perform direct ELISA in the OO crude antigen coated plate. OD values were measured and statistical analysis was done using non-parametric unpaired T test. **Panel B:** Data were plotted in prism and significance was tested with cutoff p value **** (P < 0.001) and ***(P < 0.002). Statistical analysis was done in Graph pad prism, and the figure was animated in Bio Render software.

Unlike the grain-fed cattle, which are fed with feedlot-based diet, grass-fed cattle are raised on pasture harboring infective staged III larvae of OO parasite, so we hypothesized that these chronically OO exposed cattle would demonstrate significantly high titer of OO-specific IgG in their serum relative to the grain-fed animals. Analysis of serum samples collected from twelve grass-fed and ten grain-fed cattle confirmed that grass-fed cattle were infected with

OO, which was observed regardless of the concentration of diluted serum **(Figure 4.1B)**. These findings are

mostly consistent with our previously published report, which suggest that grass-fed cattle are highly associated with OO exposure as demonstrated by lesions in abomasal mucosa [377]. We selected five grass-fed cattle as an experimental animal to perform antigen-specific T cell cloning assay.

4.4.2 OO specific CD4+ T cell polyclones were induced in MHC matched but not in the MHC mis-matched culture

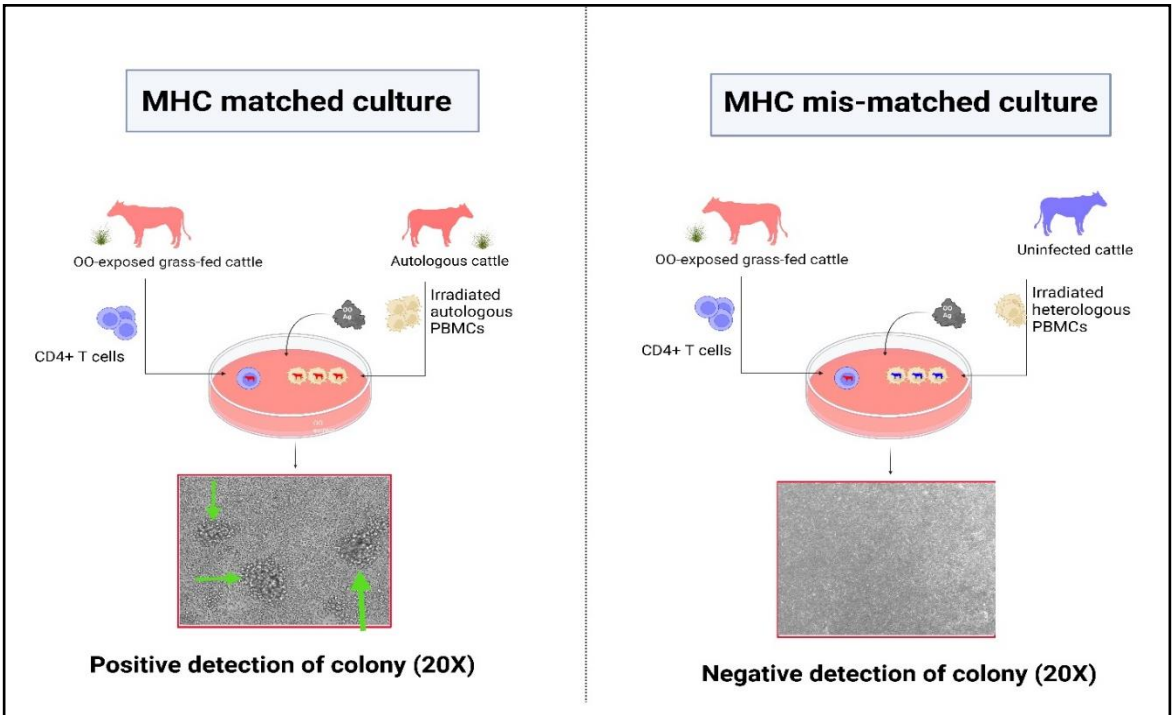

Figure 4.2: Generation of OO-specific T cell polyclones from OO infected grass-fed cattle. CD4+ T cells, sorted out from peripheral blood mononuclear cells (PBMCs) (purity ~ 95%), were cultured either with autologous (MHC matched) or heterologous (MHC mis-matched, uninfected) irradiated PBMCs in the presence of 25 µg/ml adult OO crude antigen in the presence of recombinant human interleukin-2 (rhIL2). Positive colonies were detected in the MHC matched condition but not in the MHC mis-matched condition until fourth round of re-stimulation. CD4+ Photographs were taken under 20X resolution. The figure was created in Bio Render software.

A small frequency (10-20%) of effector or memory T cells, having significantly longer life span than the naïve T cells [519], are circulating in the peripheral blood [519]. Considering that repeated exposure to antigens of OO worm, which is heavily found in the pasture grown in temperate climate, would induce relatively higher frequency of OO-specific memory T cells [520], we planned to generate OO-responding T cell polyclones by applying strategies used in previous research in humans and cattle [145, 507, 521]. Briefly, CD4+ T cells purified from the blood of naturally pathogen-exposed cattle was cultured either with autologous (MHC matched)

or heterologous (MHC mismatched, uninfected) irradiated PBMCs in the presence of 25 µg/ml crude adult OO antigen in the rhIL2 supplied medium [25, 144, 145, 509]. Consistent with the notion that induction of antigen-specific T cell response requires recognition of specific antigens by the T cell receptor (TCR) in the context of MHC matched antigen presenting cells, proliferating clones were detected only in the autologous condition, which were maintained in culture for 3- 4.5 months [145, 506, 507], however presence of allogenic irradiated PBMCs from the naïve or uninfected cattle did not support induction of polyclones even until fourth round of re-stimulations. These results support the observations from previous studies, which highlights abrogation of MHC-restricted response when bovine CD4+ T cells were stimulated with allogeneic irradiated PBMCs as the source of antigen-presenting cells [145, 498].

4.4.3 OO-specific responding cells were typical CD4+ T cells

Individual antigen-specific CD4+ T cell was isolated in each wells of three 96 well plates from a cellular suspension of T cell polyclones using the flow cytometry based plate sorting method (**Figure 4.3A**) [503]. After a month of co-culture with autologous irradiated PBMCs

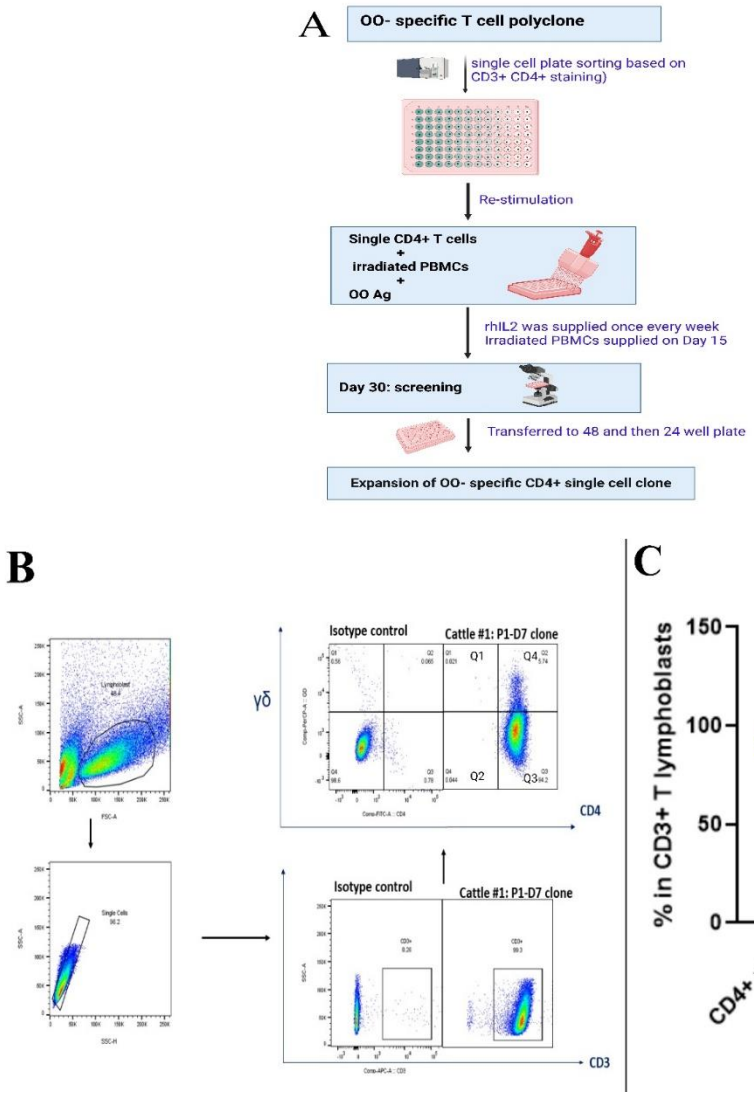

Figure 4.3: Establishment & phenotypic characterization of OO-specific CD4+ single T cell clones ***Panel A:** Workflow for generating OO- specific CD4+ T cell clones from the polyclone mixture. Individually sorted out CD3+CD4+ T cell from the polyclone was cultured with 50,000 autologous irradiated PBMCs, 5 µg/ml adult antigen and rhIL2. Antigen-specific colonies screened on Day 30 were progressively transferred to 48 and then 24 well plates and were analyzed for surface expression of CD3, CD4, and γδ expression. ***Panel B:** Analysis of surface markers on OO- specific CD4+ single T cell clones. Blasting Lymphocytes (70-250K) were gated, followed by single cell gating. Gated CD3 cells were evaluated for CD4 versus GD (γδ) expression and CD4 versus CD8 expression (data unpublished). ***Panel C:** Comparison between four fractions of cells based on CD4 and γδ expression on CD3+ gated T lymphoblasts **(Table 4.1)**; Q1: γδ+ CD4-, Q2: γδ- CD4-, Q3: CD4+ γδ-, Q4: CD4+ γδ+. Asterisks indicate statistical significance compared to BL in B and D. ******** P < 0.0001., and ******* P < 0.0002. NS: not significant. The same analysis will be performed in the rest of figures unless mentioned otherwise.

and antigens in the presence of rhIL2 supplied medium, few wells were detected positive for proliferating clones. Out of these positively screened clones, a small number (1-7) of CD4+

single T cell clones per cattle were successfully transferred to the 24 well plates through weekly re-stimulation carried out with autologous irradiated APCs and 5 ug/ml OO antigens (**Table 4.1**).

Consistent with the fact that CD4+ single T cell clones can be maintained under culture conditions for several months [25, 503], most of the OO antigen-responding CD4+ single T cell clones could be clonally passaged until 7^{th} to 12^{th} rounds of re-stimulations, before they were examined. Nonetheless, few vigorously expanding clones such as D7 and H7 from #1 were expanding until 14^{th} round of stimulation (> 3 months) while E4 from cattle #4 responded even up to the 18^{th} rounds (> 4 months). These findings are congruent with the semi-stem cell like nature of the memory cells. OO responding CD4+ single T cell clones were propagated through weekly antigen-specific stimulation with MHC matched irradiated PBMCs and 5 μg/ul OO extract before being examined for phenotyping and cytokine producing responses [25, 503]. Antigen-responsive CD4+ T cells, which were observed as morphologically blasting cells under microscopy. In the flow cytometry data, these blasting lymphocytes were characterized by high FSC-A value (70-250 K), and could be gated distinctly from the irradiated PBMC, which consistently had relatively small size, confirmed through a supplementary experiment from 6 irradiated PBMC controls (Data available on request). Based on gated blasting lymphocytes, except the 3 clones from cattle #5 that showed a relatively small (60-80%) CD3+ fraction, on average more than (>) 90 % of the cells in the expanding clones demonstrated CD3+ phenotype, meaning that responding cells were mostly T cells **(Table 4.1)**. When these CD3+ fraction was further analyzed, on average (> 90%) of the CD3+ T cells were found positive for CD4 expression **(Figure 4.3C)**, and negative for CD8α and γδ expression. For some unknown reason, a small fraction (on average

5 %) of CD4+ T cells co-expressed γδ. Of note, the small non-CD4 fraction detected in 2 CD4+ single T cell clones from cattle #2 could be residual T cells from irradiated PBMCs, which will eventually die due to effects of irradiation [522]. Overall, OO antigen responsive lymphoblasts were dominantly CD3+CD4+ T cells.

Table 4.1: Phenotypic analysis of single cell colony responding to OO antigens

Cattle ID		CD3+ % in	CD4+ γδ	CD4+ γδ +%	γδ+ CD4- %	Γδ- CD4- %
#1	P1-D7(12th	99.3	94.2	5.74	0	0.03
	P1-H7(12th	99.4	97.1	2.81	0	0.09
#2	P1-D3 (8th round)	95.8	91.4	7.67	0	0.93
	P2-C5(10th	92.55	58.6	6.22	0	35.18
	P2-E11(8th	94.85	89.5	9.33	0	1.17
	P2-F3(10th	92.15	91.8	7.43	0	0.77
	P2-C8(10th	94.65	94.2	5.29	0	0.51
	P3-C2(10th	94.35	92.9	6.17	0	0.93
	P3-B4	91.9	89.9	8.58	0	1.52
	P3-C5 (8th round)	95.05	80.7	5.84	0	13.46
#3	P3-C9 (8th round)	98.2	97	2.94	0	0.06
	P2-E3 (8th round)	94.2	95.1	4.25	0.30	0.35
#4	E4 (12th round)	98.5	97.3	2.22	0.26	0.22
#5	P3-C11(7th	79.6	99	0.74	0	0.26
	P1-E10(7th	79.5	95	1.54	3.08	0.38
	P2-B11(7th	60	98.3	1.15	0	0.55

4.4.4 OO- specific CD4+ T cells demonstrate IFNγ dominant profile with or without Th0

Previously, anti-pathogenic CD4+ single T cell clones were developed from PBMCs that responded to unfractionated antigens from *Fasciola hepatica* homogenate or soluble antigens from *Babesia species* [498, 506]. In line with these results, antigen-specific CD4+ single T cell clones were induced by propagating an individual antigen-specific CD4+ T cell clone in the presence of antigen presenting cells (APCs) and OO antigens, derived by homogenate prepared from adult worm picked up from the abomasum of infected cattle. To evaluate intracellular IFNγ and IL4 protein detection in these CD4+ single T cell clones, harvested cells were washed with complete allos medium, and were stimulated with activation cocktail for 4 hours before being examined for their IFNγ and IL4 expression profile [3]. Also, more than a million cells were sorted out from the clones expanded vigorously, and qPCR was performed to evaluate the transcriptional regulation of hallmark cytokines and their corresponding master regulators, Tbet and GATA3. Pathogen-specific CD4+ T cell clones demonstrated variable IFNγ and IL4 expressing profile in the past [506]. However, all the 21 OO specific CD4+ single T cell clones exhibited significantly high frequency of IFNγ producing cells, irrespective of their source (infected cattle), and rounds of clonal expansion (Figure 3.4B, Table 4.2). Notably, only 5 out of 21 (28%) CD4+ single T cell clones were co-expressing IL4 and IFNγ, with almost non-existing typically IL4+ IFNγ- cells in any of them. Of note, the data from these 5 clones reflected our previous findings that IL4 protein expression is mostly limited to the hybrid Th0 cells in beef cattle [523]. Just like what we observed in the pathogen infected lymphoid tissues and in vitro differentiated Th2 cells, Th0 cells in these 5 CD4+ single T cell clones occupied on average a small (~ 10%) subset of

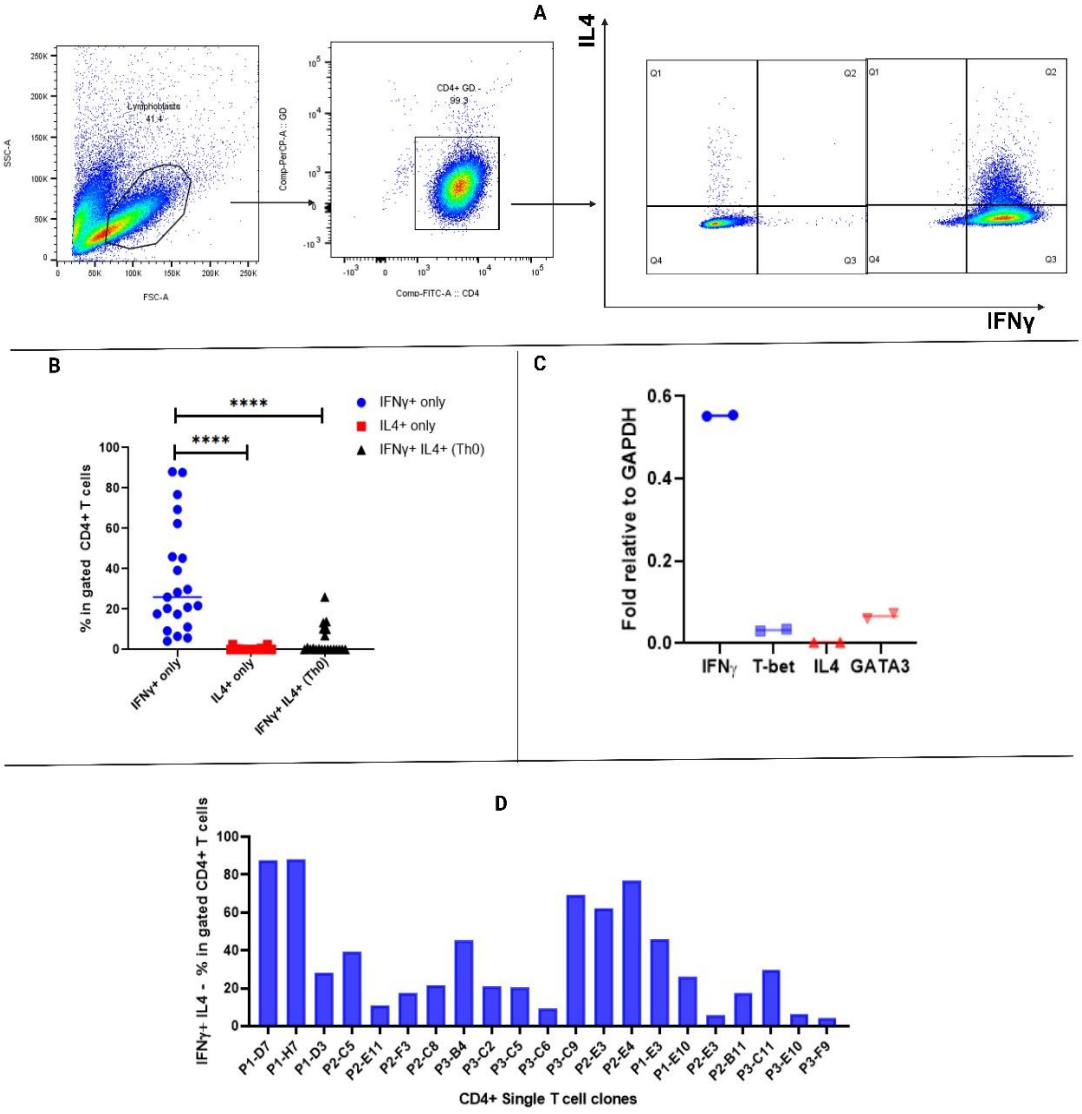

Figure 4.4: OO-specific bovine CD4+ single T cell clones demonstrated IFNγ dominant response with or without Th0 mixture. ***Panel A:** Gating strategies for IFNγ and/or IL4 producing CD4+ T cells in the OO responding clones. Single cell suspension of lymphoid tissue were stimulated with the activation cocktail for 4 hours before intracellular staining for IFNγ and IL4, plus surface staining with antibodies to identify CD4+ T cells [3]. Gating was performed based on isotype staining. **Panel B:** Comparison between IFNγ and/or IL4 producing cells in the CD4+ single T cell clones; Q1: IL4+ IFNγ -, Q2: IL4+ IFNγ+ (Th0), Q3: IFNγ+ IL4-, **Panel C**: Regulation of Th1/Th2 associated master transcription factors (Tbet, GATA3) and hallmark cytokines (IFNγ, IL4) defined in mice and humans. Quantitative polymerase chain reaction was carried out. **Panel D:** Comparison between IFNγ+ % among CD4+single T cell clones. Data were analyzed by one-way ANOVA with Tukey's Multiple Comparisons Test. Asterisks indicate statistical significance **** $P < 0.0001$., and *** $P < 0.0002$. NS: not significant.

IFNγ producing cells. Overall, 16 clones demonstrated dominant IFNγ production, and 5 CD4+ single T cell clones exhibited IFNγ dominated Th0 profile, which confirmed the central argument that bovine CD4+ T cells are intrinsically biased towards IFNγ dominated Th0 profile (Manuscript under review).

Interestingly, we noticed that the frequency of IFNγ+ cells was not consistent among the clones as shown in Figure 3.4D. In line with results from *Babesia bovis* specific CD4+ single T cell clones [25, 506], we saw that the clones which were highly responsive to weekly re-stimulations had higher % of IFNγ than those which were relatively less responsive. For instance, more than more than 80% the cells in the vigorously expanding CD4+ single T cell clones (Eg: D7, H7 & E4) were highly producing IFNγ, which was detected in considerably less frequency in the rest of the clones that were responding slowly to the weekly stimulation (Table 3.2). Results indicated that the clones demonstrated variable proportion of IFNγ inducing cells, revealing differential responses shown by the CD4+ single T cell clones.

Extracellular parasite derived antigen or allergen would typically induce IL4 producing CD4+ T cells in mice [369]. For instance, in the pathogen-specific CD4+ T cell clones, a small fraction of clones that were IL4 mRNA positive were strictly negative for IFNγ transcript/protein expression [25]. Nevertheless, none of the OO responding CD4+ single T cell clones displayed typically IL4 dominant phenotype, when intracellular protein expression was evaluated. In fact, 5 clones where IL4 was detected, its expression was highly associated with IFNγ producing cells. Consistent with lack of high IL4 mRNA/expression detection in our in vitro differentiated bovine Th2 cells, typical IL4 expressing Th2 cells was not seen in OO-specific CD4+ single T cell clones, which validates the results from OO

infected bovine tissues as well. While the previous research suggested inconsistency between IL4 mRNA expression and protein detection in the supernatant, transcriptional regulation of IFNγ and IL4 in the rapidly growing OO specific CD4+ single T cell clones were highly consistent with their intracellular protein detection data. The investigation on the transcriptional regulation of key cytokines, including IFNγ and IL4, and their putative master regulators, Tbet and GATA3, were carried out in two vigorously growing CD4+ single T cell clones. Relative to the housekeeping gene GAPDH, the regulation of IFNγ was found significantly upregulated, while that of IL4 remained minimal, which was further supported by highly reduced transcriptional activity of IL4 master regulator: GATA3. These qPCR results validated the flow cytometry-based data and provided transcriptional basis for induction of IFNγ dominated Th0 response in OO specific bovine CD4+ single T cell clones.

Table 4.2: Intracellular IFNγ and/or IL4 detection in OO antigens specific bovine CD4+ T cell clones

Cattle #	Clone ID	IFNγ+	IL4+ only	IFNγ+ IL4+ (Th0)	IFNγ- IL4-
#1	P1-D7 (12th round)	85.2	0	7.40	14.8
	P1-H7 (12th round)	84.2	0	12.6	15.8
#2	P1-D3 (8th round)	29.6	0	0.095	70.4
	P2-C5 (10th round)	40	0.1	0.05	59.9
	P2-E11 (8th round)	12.4	0.13	0.032	87.4
	P2-F3 (10th round)	18.9	0.14	1.7	79.4
	P2-C8 (10th round)	21.7	0	0	78.3
	P3-B4	16.5	0.22	0.26	83.02
	P3-C2 (10th round)	22.2	0.18	0.054	77.566
	P3-C5 (8th round)	21.6	0.075	0.056	78.269
	P3-C6	10.5	0.31	0	89.19

#4	E4 (12th round)	76.13	0	13.8	10.07
	B5 (12th round)	40.4	2.88	13.8	42.92
	E8 (12th round)	31.8	9.25	18.0	40.95
#5	P1-E3 (12th round)	56	2.46	10	41.54
	P1-E10 (7th round)	27.6	0	0	73
	P2 – E3 (7th round)	7.40	0	0	92.6
	P2-B11 (7th round)	18.7	0	0	81.3
	P3-C11(7th round)	31.5	0	0.51	68.5
	P3-E10 (7th round)	8.19	0.55	0	91.81
	P3-F9 (7th round)	4.20	0	0	95.8

4.5 Discussion:

Grass-fed cattle, confirmed with OO infection, were used as a source for generating OO-specific CD4+ single T cell clones. From 5 pathogen infected cattle, 21 CD4+ single T cell clones were established, which were analyzed for mRNA/protein expression of IFNγ and IL4, in addition to transcriptional regulation of their master regulators Tbet and GATA3. Contrasting the classical notion that extracellular parasite derived antigens highly induce typical IL4 driven responses, none of the OO-specific CD4+ single T cell clones demonstrated IL4+ IFNγ- profile; while 16 clones dominantly produced IFNγ with no IL4 expression at all, 5 CD4+ single T cell clones exhibited IL4 co-expression in a small fraction (average ~ 10.86 %) of double positive cells, traditionally designated as bovine Th0 cells. In line with data from blood and lymphoid tissues of pathogen-infected cattle, and in vitro differentiated bovine Th2 cells, analysis of OO-specific CD4+ single T cell clones supported that bovine CD4+ T cell differentiation is intrinsically skewed towards IFNγ dominant Th0 profile, at least in the Wye Angus beef cattle.

Phenotyping confirmed that significant number of blasting lymphocytes were CD3+ CD4+ CD8- γδ- T cells. Almost the whole panel of OO-specific CD4+ single T cell clones did not express CD8α **(Appendix Figure C.1)**, however a small fraction of γδ stained population that was not statistically significant, was also detected, which was in line with results from *Babesis bovis* specific CD4+ single T cell clones, where γδ expressing cells were noticed at the later stages of re-stimulation similarly [506]. Of note, in 2 clones, CD4- cells were detected, which we think is coming from the resistant cells present in the irradiated PBMCs. In line with published data [524], although our supplementary experiments strongly suggest that PBMCs irradiated at 30 Gy x-ray radiation do not demonstrate ability to proliferate but still retain the ability to function as the antigen presenting cells [506, 508], nonetheless, it is highly possible that some of these irradiated cells might had resistant phenotype [522]. Despite that noise signals were detected under few cases, blasting clones were significantly identified as CD3+ CD4+ CD8- γδ- T cells.

Our experiment suggest that the classical Th1/Th2 paradigm defined in mice and humans may not be applicable in cattle [144, 145, 506, 508]. Unlike in murine and human systems, where exposure to proteins from extracellular parasites like *Nippostrongylus brasiliensis* typically induces the development of IL4-expressing Th2 cells that excludes IFNγ expression [375, 424], antigens from OO worm extract favored dominant production of IFNγ, and their ability to induce IL4 protein was consistently low and limited to the hybrid phenotype, further corroborated by the lack of transcriptional regulation of *IL4* along with significantly upregulated transcription of *IFNγ* gene. Results confirm our in vitro generated data that bovine CD4+ T cell differentiation seems naturally skewed towards IFNγ dominant Th0 profile [342]. These results are further supported by the controversial findings in the

field which revealed lack of association between IL4 mRNA detection and protective immunity against OO infection in cattle [221, 225, 342]. Multiple lines of evidence suggest that whether classical Th1/Th2 paradigm popular in mice and humans is translatable in cattle is highly controversial.

While *Babesia bovis*- and *Fasciola hepatica*-specific bovine CD4+ single T cell clones that suggested a high (60 -90 %) prevalence of hybrid Th0 clones [25, 498, 506], only 5 out of the 21 CD4+ single T cell clones demonstrated IFNγ and IL4 co-producing hybrid Th0 phenotype in our experiments. The analysis on the previous experiments were largely based on mRNA data, which has been contradicted by protein level evaluation of IFNγ and IL4 in the OO-specific CD4+ single T cell clones. We consistently saw that Th0 cells only occupy a small subset (7-18%) of dominantly IFNγ producing cells. While our results agree with Brown et al (1998) that bovine CD4+ T cell differentiation partially differs from those in mice and humans, results from our different experiments convergently support that the frequency of bovine Th0 cells is comparatively lower than what has been previously assumed.

Previously, in a fraction of anti-parasitic CD4+ single T cell clones, IL4 mRNA expression was not found consistent with supernatant protein detection [25, 498, 506]. A few *Fasciola hepatica*-specific CD4+ single T cell clones generated in the past experiments induced high levels of IL4 mRNA but exhibited low IL4 protein (0-2 U) detection in the supernatant. Also, few other clones demonstrated low IL4 mRNA profile despite secreting relatively high IL4 protein in the supernatant (20 U). In the OO-specific CD4+ single T cell clones, intracellular protein detection data was consistent with IL4 transcript expression, and was further supported by reduced mRNA induction of its master regulator GATA3. We

maintain that transcriptional regulation of IL4 was consistent with intracellular detection of IL4 protein, which is supported by data from our clean in vitro system and blood and lymphoid tissue profile derived from naturally OO infected cattle.

The proportion of Th0 clones in this CD4+ single T cell cloning experiment parallels with that seen in the analysis of memory CD4+ T cell responses in the grass-fed cattle (**Chapter 3, Fig 3.1**). Although individual variations among the cattle were observed in both experiments, in account of statistical average, proportion of Th0 in total IFNγ producing cells calculated from OO-specific CD4+ single T cell cloning experiment (23 %) agrees with that detected in the memory bovine CD4+ T cell profile detected in the blood of grass-fed cattle (20%).

Technically, antigen-specific T cell cloning seems to be dependent on many factors. Lack of OO protein extract or MHC-matched irradiated PBMCs in the medium would not support development of proliferating colony. Also, the CD4+ single T cell clones were highly sensitive to the number of cultured irradiated PBMCs and presence of growth factors such as rhIL2, which was in line with observations from the past research [504]. Additionally, we noticed that the T cell growth factors present in the supernatant of the highly growing CD4+ single T cell clones such as D7 and H7 could temporarily mitigate delayed responsiveness of slowly expanding clones, indicating important role of the soluble factors in boosting bystander immune response. In short, density and source of irradiated PBMCs and concentration of growth factors majorly dictated successful propagation of the CD4+ single T cell clones.

The single cell cloning efficiency was less than 3%. Out of the 288 wells (~ 3 * 96 well plates) seeded with an individual antigen specific CD4+ T cell, only a small fraction of wells

was screened positive for detection of proliferating CD4+ single T cell clones, of which most of the clones could not be successfully transferred into the 24 well plates. In depth analysis suggested that the cloning efficiency (< 3%) of our experiment was significantly lower than those detected in the prior research (at least < 16 %,) [505, 508], which is most likely due to differences in the experimental approaches. While the previous research experimentally challenged cattle with high dose of antigens and then controlled the infection using anthelmintic drugs before performing ex vivo T cell cloning [505, 508], we selected naturally and chronically OO exposed cattle as our experimental models. We think that the discrepancy in the cloning efficiency is most likely related to the difference in the experimental animals.

4.6 Conclusion:

In contrast to the traditional understanding, our investigation revealed that the clonally expanding OO-specific CD4+ single T cell clones were not typically producing IL4, rather were dominant IFNγ-producing, with a small fraction (~ 10%) of bovine Th0 cells. In line with results from the in vitro Th2 differentiation studies and pathogen-infected tissues, examination of these clones suggested that bovine CD4+ T cells are intrinsically skewed towards IFNγ dominant Th0 response, and bovine Th2 induction may not be associated with IL4 mRNA/protein expression, at least in the Wye Angus beef herd. Of note, IL4 expression was limited to the hybrid Th0 cells in cattle.

Chapter 5: Objectives, conclusion and future studies

5.1: Objectives

Helper CD4+ T cells play crucial role in mounting cell-mediated as well as humoral immune responses against the pathogen infection [4, 5]. Among the different subtypes of helper CD4+ T cells, Th2 phenotype is crucial for defending infection caused by large parasites including gastrointestinal nematodes [10-12, 17]. Therefore, examining how helper CD4+ T cells differentiate into bovine Th2 phenotype is critical for understanding how to induce effective immunity against extracellular pathogens that causes huge economic losses in the cattle industry worldwide [523]. Nevertheless, a huge knowledge gap was detected in the phenotype and differentiation process of bovine Th2 cells [523], which we think was partly limiting the ability to design better vaccines against bovine extracellular parasites. Therefore, we studied differentiation and regulation of bovine Th2 cells in this dissertation project.

5.2: Conclusion

5.2.1: IL4 expression was not signature feature of bovine Th2 cells

The Wye Angus pasture harbors infective III staged larvae of *Ostertagia ostertagi* (OO), so grass-fed cattle are frequently exposed to the antigens from these parasites [520], but grain-fed cattle that are reared in the feedlot-based diets lack routine exposure to these environmental pathogens. Therefore, cattle naturally infected with OO under the farm setting were used as a model, whose memory T cell profile in the blood and lymphoid tissues were carefully analyzed **(Chapter 2, 3)**. In the naturally OO infected bovine tissues, including blood, draining lymph nodes and spleen, we noticed that IL4 expression was limited, and the majority of CD4+ T cells were dominantly producing IFNγ, which contradicted the

traditional notion, which suggest that increased exposure to extracellular pathogen would increase typical IL4 expressing memory Th2 cells. Therefore, we decided to examine these results under simple in vitro system, which had been a proven system in murine and human models. The resting CD4+ T cells isolated from the blood of the grass-fed cattle were polarized under either Th1- or Th2- differentiation conditions **(Chapter 3)**, whose results supported the observations from pathogen-exposed tissues. Results highlighted that bovine CD4+ T cells differentiate into IFNγ dominant profile along with a small frequency of hybrid (Th0) phenotype irrespective of the differentiation conditions. We then questioned whether these low IL4- producing cells were actually Th2 by performing unbiased proteomic assay. Evaluation validated bovine Th2 differentiation, which was largely consistent with murine and human species, nevertheless, the process was found to be weakly associated with IL4 induction. We adopted a couple of strategies from murine and human research and attempted to enhance IL4 expression in these differentiated cells. Nonetheless, none of the attempts were successful, and IFNγ dominant Th0 profile was consistently noticed. For the final evaluation, we generated and then examined pathogen-specific CD4+ single T cell clones that specifically responded to OO antigens for several rounds of stimulation **(Chapter 4)**: the resulting single cell clones (21) dominantly induced IFNγ, and a small fraction of them (5 out of 21) demonstrated IFNγ dominant Th0 profile. Overall, the project revealed that bovine Th2 differentiation is partially different from those in mice and humans, and even the anti-pathogenic Th2 phenotype that specifically responded to OO antigens heavily produced IFNγ. In contrast to the traditionally assumed notion, we found that bovine CD4+ T cells were consistently programmed into IFNγ dominant Th0 profile. Plus, although IL4 was induced as one of the cytokines produced by Th2 cells, and it could provide upstream

signaling for bovine Th2 differentiation, it was not expressed as hallmark bovine Th2 cytokine.

5.2.2: Differentiated bovine CD4+ T cells induced a small fraction of hybrid Th0 cells

Different from the previously published reports in cattle, we support that the frequency of hybrid Th0 cells that co-express IFNγ and IL4 protein is relatively low. While our data aligns with the findings from Brown et al. (1998), which speculated that bovine CD4+ T cell differentiation could be partially distinct from those in mice and humans, the frequency of hybrid Th0 cells induced in our experiments were consistently low (~ 10 %) compared to those in their experiments (60 - 90 %) [25]. Of note, we choose naturally infected farm animals as the model to perform experiments whereas the previous studies used experimentally challenged cattle, whose infection was later suppressed using pharmacological drugs [25]. Also, we quantified intracellular detection of IFNγ and IL4 proteins in addition to the mRNA expression, and tested the hypothesis under clean in vitro condition in addition to performing OO-specific CD4+ single T cell cloning. Experimental infection with OO parasite have resulted inconsistent results and therefore difficulty in interpreting data has been noticed [221, 225], so the profile reflected by the naturally infected cattle might provide precise information in this context. In line with controversial data that suggested lack of association between IL4 expression and reduced parasitic worm burden in OO vaccinated cattle [26, 224, 525], our results points out that IL4 may not be the signature molecule of differentiated bovine Th2 cells. Importantly, a small subset of differentiated bovine CD4+ T cells co-produced IFNγ and IL4, which has been designated as hybrid bovine Th0 cells.

5.2.3: Bovine immune system offers distinct species-specific differences

The bovine immunology field has observed several differences compared to murine and human counterparts [352, 523]. The high percentage of γδ T cells in cattle [27], simultaneous enhancement of Th1- and Th2- associated factors in tissues and body fluids collected from infected cattle [523], and slightly different composition of bovine intra-epithelial T lymphocytes are few evidences in this context [526]. Adding unto these observations, the results from the **Chapter 2** reveals that expression of CD45RO and exclusion of CD45RA is not strongly associated with memory T cell detection in cattle, which is different from the trend suggested in murine and human model [342, 396]. **Chapter 3** and **Chapter 4** further reveals that bovine CD4+ T cell differentiation slightly differs from those in the mice and humans, and IL4, which is considered as signature Th2 protein in mice and humans, was limitedly expressed by bovine Th2 cells. Given the anatomical and physiological differences of ruminants, and their constant exposure to a wide variety of microorganisms, evolutionary pressure might have favored divergent development of their adaptive immune system. In domestic animals such as pigs, immunophenotyping have revealed distinct phenotypes, and IL4 expression was not found associated with typical Th2 responses [463]. So, increasing numbers of evidence have pointed that during the course of evolution the immune system of domestic animals could have been shaped distinctly from the murine and human counterparts.

5.3: Limitations and future directions

Our results provide several insights on bovine Th2 differentiation, guiding future studies in the field. For instance, we found that the co-expression of Tbet and GATA3 master regulators were relatively common in bovine species, which was somewhat associated with IFNγ

dominant Th0 profile. Recent pieces of evidence from murine and human data suggest that when these molecules are co-expressed, Tbet activity dominates over GATA3. Specifically, Tbet DNA binding protein can bind to that of GATA3, thus facilitating re-distribution of GATA3 away from the *IL4* binding sites [433, 502]. Therefore, future research is warranted to pinpoint if these mechanisms are similarly involved in uniquely regulated bovine CD4+ T cell differentiation process. Additionally, while we noticed that GATA3 was not predicted to be activated in the bovine Th2 proteome, GATA4 and STAT5, which has been associated with transcriptional programming of Th2 differentiation were activated. Due to lack of knock out models, we could not test these questions in this project. Nevertheless, we believe that advancing technology might allow studying these mechanisms using alternative ways in the near future.

As an important aspect of the study, we also examined whether OO antigens could impact bovine Th2 differentiation. We noticed that bovine Th2 differentiation was sensitive to the presence of OO antigens as evidenced by increased proliferation of differentiated cells, and potential downregulation of key immunological signaling pathways. Specifically, we found that presence of OO antigens was contributing to the downregulation of signaling via CD3, CD28, IL4, NF-KB and those via TLRs/MYD88 pathways, based on differential expression of 83 proteins between Th2 control and Th2+OO group. Expanding on these findings, we found that naïve bovine CD4+ T cells express TLRs (1-10). Nevertheless, If OO could actually downregulate signaling via TLRs/MYD88 signaling during the T cell activation is a subject of future exploration.

The dissertation project has provided crucial insights in the differentiation of bovine Th2 cells in the beef cattle. Nevertheless, we speculate that the profile might be different in

the dairy cattle, and other genetically distant breeds [352]. As dairy cattle physiology is driven by changes in hormonal conditions [527], their CD4+ T cell profile could be influenced by these factors, so the field must attempt to discover nuances across these breeds. Additionally, although the Wye Angus breed that we used for studying bovine Th2 differentiation are genetically closely related cattle, so are a good model for conducting experiment, cross-bred cattle developed from breeding of genetically distant cattle might exhibit breed-specific differences. So, although the project has generated several important information, and has laid a foundation in studying bovine CD4+ T cell differentiation, the research is bound by some limitations. We recommend further research extending to dairy and other breeds of cattle to generate comprehensive understanding of CD4+ T cell differentiation in the bovine species.

5.4: Significance and contribution to the bovine immunology field:

Overall, the research project has reported several pieces of cattle-specific information regarding differentiation and regulation of bovine Th2 cells, laying a strong foundation for future studies in this direction. Previously, Xiao lab published a manuscript demonstrating that bovine CD4+ T cells can be activated by three upstream signals, mirroring processes observed in mice and humans [44]. Plus, neutrophil could synergize with IL12 that acts as third signal to enhance the activation process [44]. Building on this work, we studied the process of bovine CD4+ T cell differentiation under Th1- and Th2- polarizing conditions, uncovering cattle species-specific information that counteracted classical paradigm adopted from murine and human data. Plus, OO-specific CD4+ single T cell clones generated in this research will serve as a crucial platform for screening the effective OO immunogens, facilitating development of more effective vaccine against this pathogen in the future.

Appendix A: Appendices for chapter 2

Figure A.1: CD45RO is not expressed by lymphocytes in some cattle

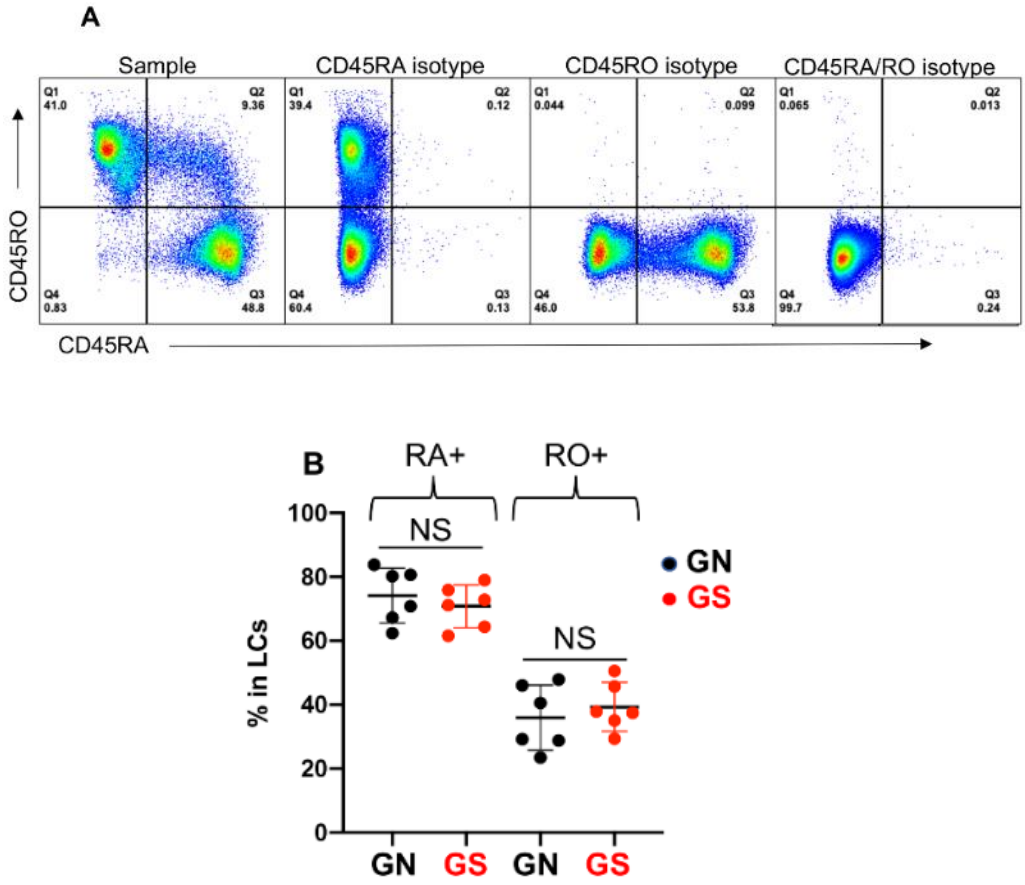

Figure A.1. CD45RO is not expressed by lymphocytes in some cattle. Lymphocytes were gated in PBMCs, and examined for expression of CD45RO and CD45RA. (A) Representative dot plots of samples and CD45RA or CD45RO isotype controls in lymphocytes. (A). (B) Comparison of CD45RO (RO+) and CD45RA (RA+) expression in lymphocytes from Grain-fed (GN) and Grass-fed (GS) cattle. Data were the same as in Fig.1, and reanalyzed by one-way ANOVA with Tukey's Multiple Comparisons Test. Asterisks indicate statistical significance. *P < 0.05; **P < 0.01; ***P < 0.001. NS: not significant.

Figure A.2: CD45RO and CD45RA are expressed differentially in T cell subtypes

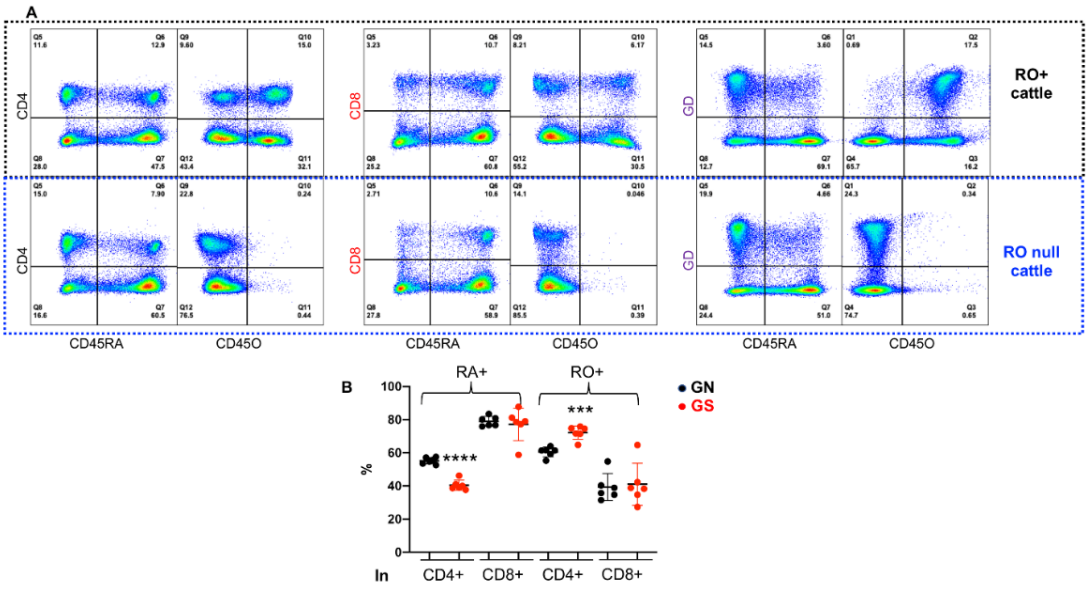

Figure A.2. CD45RO and CD45RA are expressed differentially in T cell subtypes. Lymphocytes were gated in PBMC, and examined for the expression of CD45RO and CD45RA. (A) Representative dot plots of samples and CD45RA or CD45RO isotype controls in CD4+, CD8 and γδ T cells in both CD45-expressing and CD45RO null cattle (A). B) Comparison of CD45RO and CD45RA expression in CD4+ and CD8+ T cells from Grain-fed (GN) and Grass-fed (GS) cattle. Data were the same as in Fig.1, and reanalyzed by one-way ANOVA with Tukey's Multiple Comparisons Test. Asterisks indicate statistical significance. *P < 0.05; **P < 0.01; ***P < 0.001. NS: not significant.

Figure A.3: Activation status varies between CD45RO+ and CD45RA+ sub-populations in CD4+, but not in CD8+ T cells

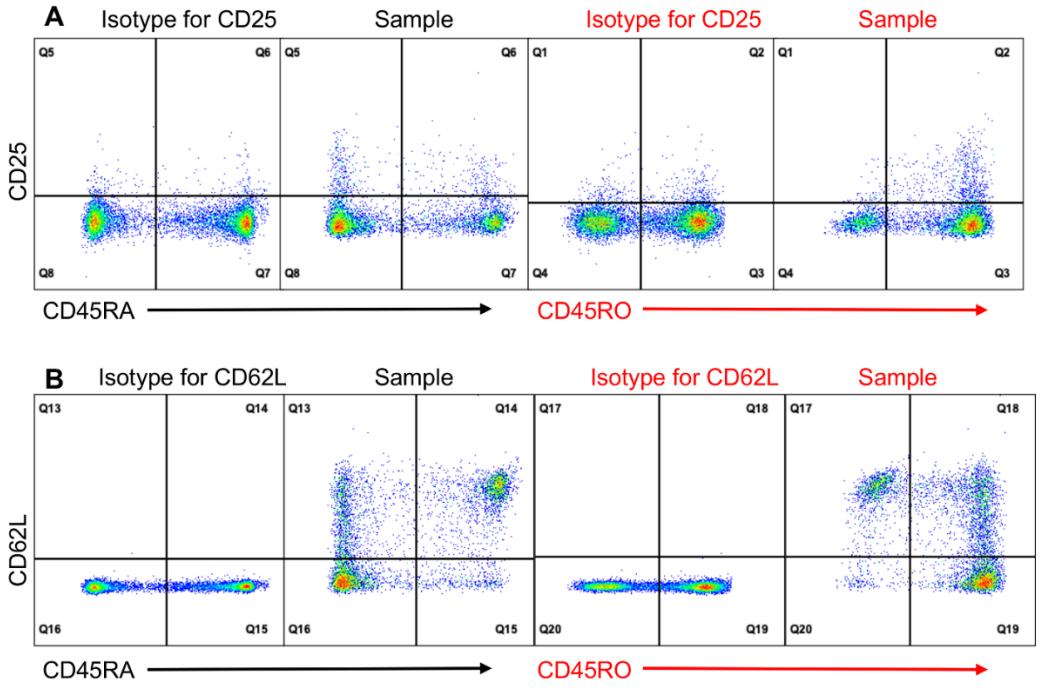

Figure A.3 Activation status varies between CD45RO+ and CD45RA+ sub-populations in CD4+, but not in CD8+ T cells. Lymphocytes were gated on CD4+ or CD8+ T cells in PBMCs, and examined for the expression of CD25 and CD62L in either RO+ or RA+ subpopulations. (A-B) Representative dot plots of samples and isotypes for CD25 (A) or CD62L (B) in RA+ or RO+ gated on CD4+ T cells

Figure A.4: Expression of IFNγ and IL-4 is not associated with CD45RO in CD4+ T cells

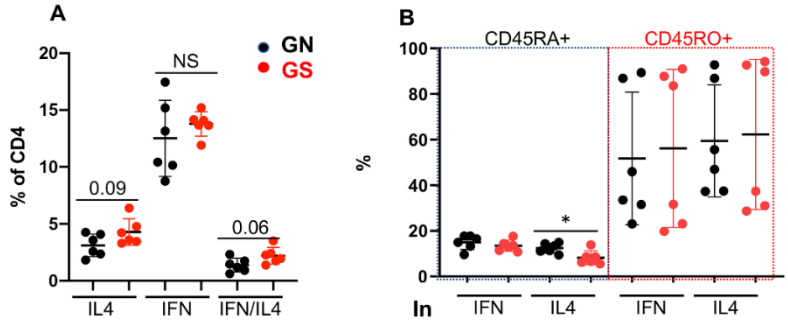

Figure A.4: Expression of IFNγ and IL-4 is not associated with CD45RO in CD4+ T cells. Purified PBMCs were stimulated with activation cocktail for 4 hours before intracellular staining for IFNγ and IL4, plus surface staining with antibodies to identify CD4 T cells, and their expression of CD45RO and CD45RA. (A) Comparison of IFNγ+ and IL4+ in CD4+ T cells from Grain-fed (GN) and Grass-fed (GS) cattle. (B) Comparison of CD45RO+ or CD45RA+ in IFNγ+ or IL4+ CD4+ T cells from GN and GS cattle. Data were the same as in Fig.4, and reanalyzed by one-way ANOVA with Tukey's Multiple Comparisons Test. Asterisks indicate statistical significance. *P < 0.05; **P < 0.01; ***P < 0.001. NS: not significant.

Figure A.5: Expression of IFNγ is not associated with CD45RO in CD8+ T cells

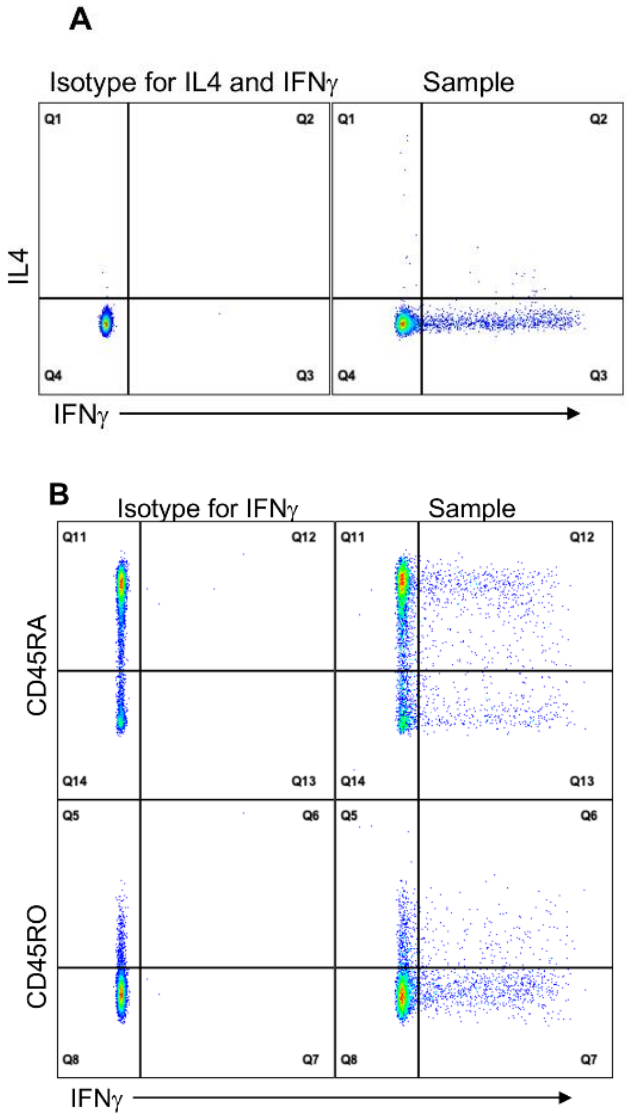

Figure A.5: Expression of IFNγ is not associated with CD45RO in CD8+ T cells. The same samples and treatment were applied as in Figure 4, but the analysis was gated on CD8+ T cells. (A) Representative dot plots of samples and isotypes for IFNγ and IL4 in CD8+ T cells. (A) Representative dot plots of samples and isotypes for IFNγ and IL4 in CD45RA+ or CD45RO+ CD8+ T cells. (B) Representative dot plots of samples and isotypes for IFNγ and CD45RA or CD45RO expressions on gated CD8+ T cells.

Figure A.6: Expression of IFNγ and IL4 is not associated with CD45RO in GD T cells

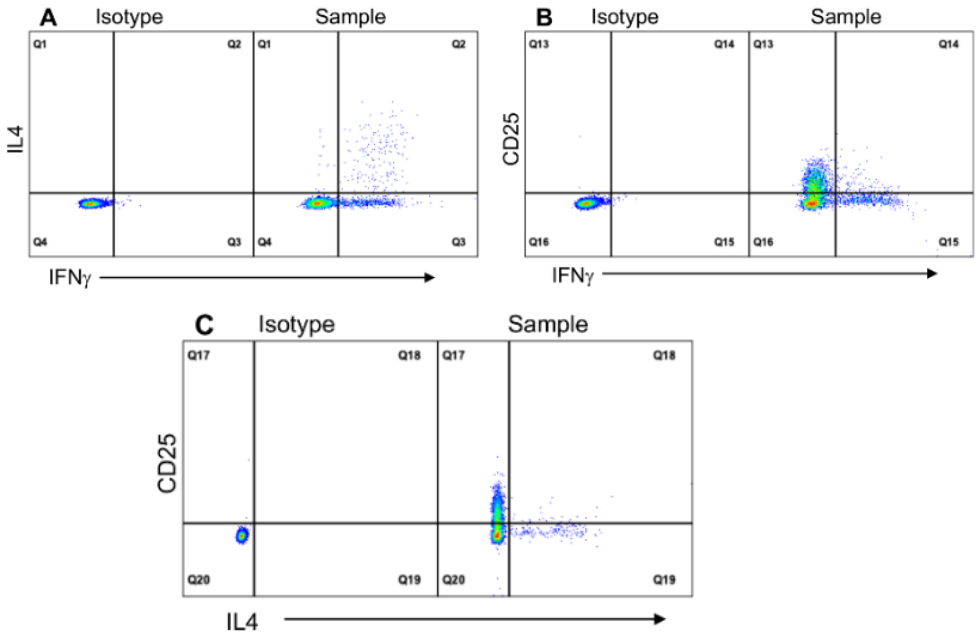

Figure A.6: Expression of IFNγ and IL4 is not associated with CD45RO in GD T cells. PBMCs were isolated from 12 cattle, which were tested for expression of CD45RO, CD62L, IFNγ and IL4 in a way similar to that in figures 4. (A) Representative dot plots of samples and isotypes for IFNγ and IL4 in γδ T cells. (B) Representative dot plots of samples and isotypes for IFNγ and CD25 in γδ T cells. (C) Representative dot plots of samples and isotypes for CD25 and IL4 in γδ T cells.

Appendix B: Appendices for Chapter 3

Figure B.1: Network analysis based on the predicted activation and inhibition states of the upstream regulators identified in the Th1 versus naïve group

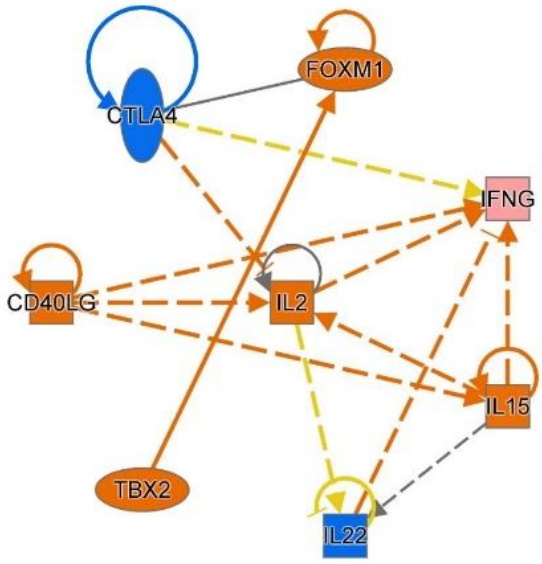

Figure B.1: Network analysis based on the predicted activation and inhibition states of the upstream regulators identified in the Th1 versus naïve group. The differentially expressed proteins were analyzed in Ingenuity Pathway Analysis software. Activation of upstream signaling though IL2, IL15, CD40LG, TBX2 and FOXM1, and inhibition of those via IL22 and CTLA4 was predicted. While statistical relationship between upstream regulator and molecular targets were calculated by the Fisher's exact test with significance ($P < 0.05$), activation (orange color) and inhibition (blue color) states were determined based on their positive or negative z-score values (cut off score = 2). Z score is the number of standard deviations between the obtained value and the average mean of the dataset.

Table B.1: Predicted activation or inhibition states of key signaling molecules obtained from the upstream analysis in the Th2 versus naïve group.

Table B.2 (Th2 vs naïve): Predicted activation or inhibition states of key signaling molecules obtained from the upstream analysis in the Th2 versus naïve group. While the activation and inhibition states were predicted based on positive or negative z-score values (cut off value = 2), the statistical relationship between upstream regulator and molecular targets in the dataset was calculated by the Fisher's exact test with significance ($P < 0.05$). Z score is the number of standard deviations between the obtained value and the average mean of the dataset. The list of the protein has been attached to a separate pdf file.

Upstream regulator	**Molecular Type**	**Predicted Activation state**	**Activation Z score**	**P-value of overlap**	**Number of targeted molecules in Dataset**
CD3	complex	Activated	2.652	1.60E-14	41
CD28	Transmembrane receptor	Activated	3.153	9.58E-15	31
IL1	cytokine	Activated	2.299	4.44E-05	17
IL2	cytokine	Activated	2.715	1.02E-07	30
IL4	cytokine	Activated	2.678	2.35E-14	56
IL7	cytokine	Activated	2.012	2.11E-05	11
IL33	cytokine	Activated	2.559	5.85E-05	17

Table B.2: Predicted activation or inhibition states of major upstream molecules differentially expressed in the Th1 versus naïve group (Th1 vs naïve)

Table B.2: Predicted activation or inhibition states of major upstream molecules differentially expressed in the Th1 versus naïve group (Th1 vs naïve). While the activation and inhibition states were predicted based on positive or negative z-score values (cut off value = 2), the statistical relationship between upstream regulator and molecular targets in the dataset was calculated by the Fisher's exact test with significance ($P < 0.05$). Z score is the number of standard deviations between the obtained value and the average mean of the dataset. The list of protein has been attached in a separate pdf file.

Upstream regulator	**Molecular Type**	**Predicted Activation state**	**Activation Z score**	**P-value of overlap**	**Number of targeted molecules in Dataset**
IL2	cytokine	Activated	2.302	4.03E-03	19
IL15	cytokine	Activated	2.023	5.63E-05	19
IL4	cytokine	-	1.526	6.25E-07	40
TBX2	Transcription regulator	Activated	2.499	9.78E-04	6
FOXM1	Transcription regulator	Activated	2.470	2.30E-06	12
CD40LG	Surface molecule	Activated	2.226	1.84E-02	12
IL-22	cytokine	inhibited	-2.380	1.79E-02	6
CTLA-4	Transmembrane receptor	inhibited	-2.111	6.32E-04	6

Table B.3: Fold changes in the expression of molecular targets in the presence of IL4

Table B.3: Fold changes in the expression of molecular targets in the presence of IL4. Upstream analysis of rbIL4+Th2 versus Th2 group reveals involvement of IL4 in the regulation of molecules, whose statistical relationship ($P < 0.05$) was calculated by the Fisher's exact test. Positive and negative expression fold change values respectively indicate upregulation (↑) and downregulation (↓) of the molecules.

Genes in dataset	**Expr Fold Change**	**Comparison with Literature**	**References**
POSTN	2.085 (↑)	Consistent	[528-530]
ITGB1	1.505 (↑)	Consistent	[531-534]
CTLA4	-2 (↓)	Consistent	[535-541],
PRF1	2.014 (↓)	Consistent	[542]
CCL5	-8.112 (↓)	Consistent	[543-546]
IL2RA	-1.905 (↓)	Inconsistent	[464, 465, 547]
JUNB	-1.591(↓)	Inconsistent	[466, 467]
GZMA	-2 (↓)	Inconsistent	[548]
CD83	-2.028 (↓)	Inconsistent	[464, 468-471, 549, 550]

Table B.4: Predicted activation states of major cytokines differentially expressed in the rbIL4+Th2 versus Th2 group.

Table B.4: Predicted activation states of major cytokines differentially expressed in the rbIL4+Th2 versus Th2 group. While the activation and inhibition states were predicted based on positive or negative z-score values (cut off value = 2), the statistical relationship between upstream regulator and molecular targets in the dataset was calculated by the Fisher's exact test with significance (P <0.05). Z score is the number of standard deviations between the obtained value and the average mean of the dataset.

Th1 versus naïve					**Th2 versus naïve**			
Upstream regulator	Predicted activation	Z score	P value	Targeted molecule	Predicted activation state	Z score	P value	Targeted molecule
GATA3	-	-	-	-	-	-0.237	5.68E-03	10
FOXP3	-	-	6.29E-02	8	-	-0.608	1.80E-07	15
RUNX1	-	-	1.58E-02	9	-	-0.961	6.44E-07	17
STAT-1	-	-	-	-	-	-0.760	6.19E-06	19
STAT-3	-	-0.144	5.07E-06	26	-	0.962	5.83E-10	34
STAT-4	-	-0.097	2.05E-02	8	-	-1.088	1.26E-05	14
STAT-5A	-	2.449	2.93E-03	7	Activated	2.183	3.21E-06	17
STAT-6	-	1.402	6.87E-04	17	Activated	2.087	6.30E-03	6

Table B.5*:* List of proteins regulated by bovine IL4

Table B.5*:* List of proteins regulated by bovine IL4.While the activation and inhibition states were predicted based on positive or negative z-score values (cut off value = 2), the statistical relationship between upstream regulator and molecular targets in the dataset was calculated by the Fisher's exact test with significance (P <0.05). Z score is the number of standard deviations between the obtained value and the average mean of the dataset.

Upstream	**Molecule type**	**Activation z score**	**Activation**	**P-value of overlap**
IL2	cytokine	-1.851	-	7.19E-04
IL12A	cytokine	-	-	3.35E-02
IL4	cytokine	0.219	-	3.01E-05

Table B.6: Predicted inhibition states of molecules obtained from the upstream analysis of Th2+OO versus Th2 group.

Table B.6: Predicted inhibition states of molecules obtained from the upstream analysis of Th2+OO versus Th2 group. While the inhibition state was indicated due to the negative z-score values (cut off value = 2), the statistical relationship between upstream regulator and molecular targets were calculated by the Fisher's exact test with significance (P <0.05). Z score is the number of standard deviations between the obtained value and the average mean of the dataset. List of proteins has been

Upstream regulator	**Predicted activation/inhibition**	**Z score**	**P value of overlap**	**Target molecule in dataset**
CD3	inhibited	-3.247	6.42E-08	13
CD28	inhibited	-2.579	1.11E-04	7
CD40	inhibited	-2.598	8.43E-11	11
IL2	inhibited	-2.370	8.05E-11	16
IL7	inhibited	-2.194	9.34E-05	5
IL15	inhibited	-2.156	1.23E-07	11
NFĶB1	inhibited	-2.415	3.39E-06	8

Appendix C: Appendices for Chapter 4

Figure C.1: CD4 versus CD8 expression in the gated lymphoblast in five CD4+ single T cell clones from 5 different cattle

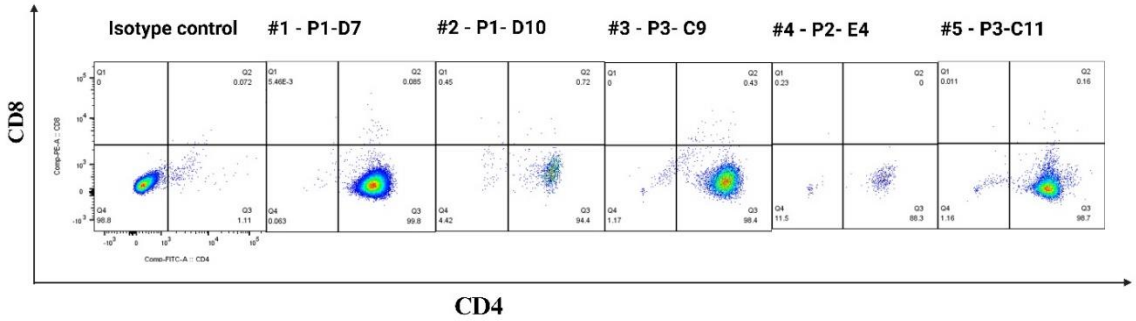

Figure C.1: CD4 versus CD8 expression in the gated lymphoblast in five CD4+ single T cell clones from 5 different cattle. When contrasted with the isotype control, CD4+ single T cell clones from all the cattle were found positive for CD4 expression but strictly negative for the CD8 expression.

Bibliography

1. M Ibrahim, Scozzi D, et al. Naive CD4(+) T Cells Carrying a TLR2 Agonist Overcome TGF-beta-Mediated Tumor Immune Evasion. J Immunol. 2018;200(2):847-856.

2. JJP van Beek, Florez-Grau G, et al. Human pDCs Are Superior to cDC2s in Attracting Cytolytic Lymphocytes in Melanoma Patients Receiving DC Vaccination. Cell Rep. 2020;30(4):1027-1038.e4.

3. A Kandel, Li L, et al. Differential Expression of CD45RO and CD45RA in Bovine T Cells. Cells. 2022;11(11).

4. TR Mosmann, Fowell DJ. The Th1/Th2 paradigm in infections. Immunology of infectious diseases. 2001:161-174.

5. J Zhu, Paul WE. Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors. Immunological reviews. 2010;238(1):247-262.

6. TK Starr, Jameson SC, et al. Positive and negative selection of T cells. Annual review of immunology. 2003;21(1):139-176.

7. C Infante-Duarte, Kamradt T, editors. Th1/Th2 balance in infection. Springer seminars in immunopathology; 1999: Springer.

8. M Tomura, Itoh K, et al. Naive CD4+ T lymphocytes circulate through lymphoid organs to interact with endogenous antigens and upregulate their function. The Journal of Immunology. 2010;184(9):4646-4653.

9. J Saravia, Chapman NM, et al. Helper T cell differentiation. Cellular & Molecular Immunology. 2019;16(7):634-643.

10. B Spellberg, Edwards JE, Jr. Type 1/Type 2 immunity in infectious diseases. Clin Infect Dis. 2001;32(1):76-102.

11. GE Kaiko, Horvat JC, et al. Immunological decision-making: how does the immune system decide to mount a helper T-cell response? Immunology. 2008;123(3):326-338.

12. T Rivera-Hernandez, Rhyme MS, et al. Vaccine-induced Th1-type response protects against invasive group A Streptococcus infection in the absence of opsonizing antibodies. Mbio. 2020;11(2).

13. L Casciotti, Ely KH, et al. CD8+-T-cell immunity against Toxoplasma gondii can be induced but not maintained in mice lacking conventional CD4+ T cells. Infection and Immunity. 2002;70(2):434-443.

14. KA Jordan, Hunter CA. Regulation of CD8+ T cell responses to infection with parasitic protozoa. Experimental parasitology. 2010;126(3):318-325.

15. SL Swain, McKinstry KK, et al. Expanding roles for CD4+ T cells in immunity to viruses. Nature Reviews Immunology. 2012;12(2):136-148.

16. K Jones, Fischetti V. The importance of the location of antibody binding on the M6 protein for opsonization and phagocytosis of group A M6 streptococci. The Journal of experimental medicine. 1988;167(3):1114-1123.

17. J Punt, Stranford SA, et al. Kuby immunology. Eighth edition. ed. New York: Macmillan Education; 2019.

18. NM Blackwell, Else KJ. B cells and antibodies are required for resistance to the parasitic gastrointestinal nematode Trichuris muris. Infect Immun. 2001;69(6):3860-8.

19. Q Liu, Kreider T, et al. B cells have distinct roles in host protection against different nematode parasites. Journal of Immunology. 2010;184(9):5213-23.

20. JU Igietseme, Eko FO, et al. Antibody regulation of T-cell immunity: implications for vaccine strategies against intracellular pathogens. Expert review of vaccines. 2004;3(1):23-34.

21. DM Estes, Closser NM, et al. IFN-gamma stimulates IgG2 production from bovine B cells costimulated with anti-mu and mitogen. Cell Immunol. 1994;154(1):287-95.

22. A Casadevall. Antibody-mediated protection against intracellular pathogens. Trends in microbiology. 1998;6(3):102-107.

23. D Agnew, Corbeil L, et al. A pregnant mouse model for bovine Tritrichomonas foetus infection. Veterinary pathology. 2008;45(6):849-864.

24. S Notebaert, Meyer E. Mouse models to study the pathogenesis and control of bovine mastitis. A review. Veterinary quarterly. 2006;28(1):2-13.

25. W Brown, Rice-Ficht AC, et al. Bovine type 1 and type 2 responses. Veterinary immunology and immunopathology. 1998;63(1-2):45-55.

26. E Claerebout, Vercruysse J. The immune response and the evaluation of acquired immunity against gastrointestinal nematodes in cattle: a review. Parasitology. 2000;120(7):25-42.

27. CR Mackay, Hein WR. A large proportion of bovine T cells express the gamma delta T cell receptor and show a distinct tissue distribution and surface phenotype. Int Immunol. 1989;1(5):540-5.

28. E Guzman, Hope J, et al. Bovine γδ T cells are a major regulatory T cell subset. The Journal of Immunology. 2014;193(1):208-222.

29. L Li, Si H, et al. Characterization of IL-10-producing neutrophils in cattle infected with Ostertagia ostertagi. Scientific reports. 2019;9(1):1-14.

30. TR Mosmann, Cherwinski H, et al. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. The Journal of Immunology. 1986;136(7):2348-2357.

31. S Romagnani. Human TH1 and TH2 subsets: regulation of differentiation and role in protection and immunopathology. International archives of allergy and immunology. 1992;98(4):279-285.

32. J Jores, Baldwin C, et al. Contagious Bovine and Caprine Pleuropneumonia: a research community's recommendations for the development of better vaccines. NPJ vaccines. 2020;5(1):1-9.

33. F Gomes, Henriques M. Control of Bovine Mastitis: Old and Recent Therapeutic Approaches. Curr Microbiol. 2016;72(4):377-82.

34. V Marugan-Hernandez. Neospora caninum and bovine neosporosis: current vaccine research. Journal of Comparative Pathology. 2017;157(2-3):193-200.

35. AN Vlasova, Saif LJ. Bovine Immunology: Implications for Dairy Cattle. Frontiers in immunology. 2021;12.

36. F Runte, Renner IV P, et al. Kuby immunology. 2019.

37. BK Surmi, Hasty AH. The role of chemokines in recruitment of immune cells to the artery wall and adipose tissue. Vascul Pharmacol. 2010;52(1-2):27-36.

38. G Gasteiger, D'osualdo A, et al. Cellular innate immunity: an old game with new players. Journal of innate immunity. 2017;9(2):111-125.

39. H Kumar, Kawai T, et al. Pathogen recognition by the innate immune system. International reviews of immunology. 2011;30(1):16-34.

40. CA Janeway Jr, Medzhitov R. Innate immune recognition. Annual review of immunology. 2002;20(1):197-216.

41. A Weiss, Littman DR. Signal transduction by lymphocyte antigen receptors. Cell. 1994;76(2):263-274.

42. JP Allison. CD28-B7 interactions in T-cell activation. Current opinion in immunology. 1994;6(3):414-419.

43. T Kambayashi, Laufer TM. Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell? Nature Reviews Immunology. 2014;14(11):719-730.

44. Z Xiao, Kandel A, et al. Synergistic Activation of Bovine CD4+ T Cells by Neutrophils and IL-12. Pathogens. 2021;10(6):694.

45. H-G Lee, Cho M-Z, et al. Bystander CD4+ T cells: crossroads between innate and adaptive immunity. Experimental & Molecular Medicine. 2020;52(8):1255-1263.

46. C Alvarez-Dominguez, Calderón-Gonzalez R, et al. Dendritic cell therapy in melanoma. Annals of translational medicine. 2017;5(19).

47. LJ Eggermont, Paulis LE, et al. Towards efficient cancer immunotherapy: advances in developing artificial antigen-presenting cells. Trends in biotechnology. 2014;32(9):456-465.

48. SJ Szabo, Kim ST, et al. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. 2000;100(6):655-669.

49. SJ Szabo, Sullivan BM, et al. Distinct effects of T-bet in TH1 lineage commitment and IFN-γ production in CD4 and CD8 T cells. Science. 2002;295(5553):338-342.

50. M Afkarian, Sedy JR, et al. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. Nature immunology. 2002;3(6):549-557.

51. J Zhu, Jankovic D, et al. The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. Immunity. 2012;37(4):660-673.

52. C Nobile, Lind M, et al. Cognate CD4+ T-cell–dendritic cell interactions induce migration of immature dendritic cells through dissolution of their podosomes. Blood, The Journal of the American Society of Hematology. 2008;111(7):3579-3590.

53. JP Ridge, Di Rosa F, et al. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature. 1998;393(6684):474-8.

54. A Le Bon, Durand V, et al. Direct stimulation of T cells by type I IFN enhances the CD8+ T cell response during cross-priming. The Journal of Immunology. 2006;176(8):4682-4689.

55. A Le Bon, Etchart N, et al. Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. Nature immunology. 2003;4(10):1009-1015.

56. MJ Bevan. Helping the CD8(+) T-cell response. Nat Rev Immunol. 2004;4(8):595-602.

57. JA Trapani. Target cell apoptosis induced by cytotoxic T cells and natural killer cells involves synergy between the pore-forming protein, perforin, and the serine protease, granzyme B. Aust N Z J Med. 1995;25(6):793-9.

58. JT Harty, Bevan MJ. Responses of CD8+ T cells to intracellular bacteria. Current opinion in immunology. 1999;11(1):89-93.

59. GH Pritchard, Kedl RM, et al. The evolving role of T-bet in resistance to infection. Nature Reviews Immunology. 2019;19(6):398-410.

60. A O'Garra. T-cell differentiation: Commitment factors for T helper cells. Current Biology. 2000;10(13):R492-R494.

61. T Biedermann, Röcken M, et al., editors. TH1 and TH2 Lymphocyte development and regulation of TH Cell–mediated immune responses of the skin. Journal of Investigative Dermatology Symposium Proceedings; 2004: Elsevier.

62. CA Thaiss, Semmling V, et al. Chemokines: a new dendritic cell signal for T cell activation. Frontiers in immunology. 2011;2:31.

63. SR Sarawar, Shen J, et al. Insights into CD8 T Cell Activation and Exhaustion from a Mouse Gammaherpesvirus Model. Viral immunology. 2020;33(3):215-224.

64. J Borst, Ahrends T, et al. CD4+ T cell help in cancer immunology and immunotherapy. Nature Reviews Immunology. 2018;18(10):635-647.

65. G Kak, Raza M, et al. Interferon-gamma (IFN-γ): Exploring its implications in infectious diseases. Biomolecular Concepts. 2018;9(1):64-79.

66. S Kang, Brown HM, et al. Direct antiviral mechanisms of interferon-gamma. Immune network. 2018;18(5).

67. AJ Casbon, Long ME, et al. Effects of IFN-γ on intracellular trafficking and activity of macrophage NADPH oxidase flavocytochrome b558. Journal of leukocyte biology. 2012;92(4):869-882.

68. HM Johnson. Gamma interferon: from antimicrobial activity to immune regulation. Frontiers in immunology. 2015;5:667.

69. CE Samuel. Antiviral actions of interferons. Clin Microbiol Rev. 2001;14(4):778-809, table of contents.

70. JK Whitmire, Tan JT, et al. Interferon-γ acts directly on CD8+ T cells to increase their abundance during virus infection. The Journal of experimental medicine. 2005;201(7):1053-1059.

71. P Bhat, Leggatt G, et al. Interferon-γ derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity. Cell death & disease. 2017;8(6):e2836-e2836.

72. SG Zakroff, Beck L, et al. The IgE and IgG subclass responses of mice to four helminth parasites. Cellular immunology. 1989;119(1):193-201.

73. Y Kawano, Noma T. Role of interleukin-2 and interferon-γ in inducing production of IgG subclasses in lymphocytes of human newborns. Immunology. 1996;88(1):40-48.

74. JG Shannon, Cockrell DC, et al. Antibody-mediated immunity to the obligate intracellular bacterial pathogen Coxiella burnetii is Fc receptor-and complement-independent. BMC immunology. 2009;10(1):1-8.

75. W-p Zheng, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell. 1997;89(4):587-596.

76. J Zhu, Yamane H, et al. GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. Cell Res. 2006;16(1):3-10.

77. JA Walker, McKenzie ANJ. TH2 cell development and function. Nature Reviews Immunology. 2018;18(2):121-133.

78. J Zhu. Transcriptional regulation of Th2 cell differentiation. Immunol Cell Biol. 2010;88(3):244-9.

79. J Zhu, Min B, et al. Conditional deletion of Gata3 shows its essential function in TH 1-TH 2 responses. Nature immunology. 2004;5(11):1157-1165.

80. S-Y Pai, Truitt ML, et al. GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells. Proceedings of the National Academy of Sciences. 2004;101(7):1993-1998.

81. MH Kaplan, Schindler U, et al. Stat6 is required for mediating responses to IL-4 and for the development of Th2 cells. Immunity. 1996;4(3):313-319.

82. K Takeda, Tanaka T, et al. Essential role of Stat6 in IL-4 signalling. Nature. 1996;380(6575):627-630.

83. HJ Lee, Takemoto N, et al. GATA-3 induces T helper cell type 2 (Th2) cytokine expression and chromatin remodeling in committed Th1 cells. The Journal of experimental medicine. 2000;192(1):105-116.

84. R Yagi, Zhu J, et al. An updated view on transcription factor GATA3-mediated regulation of Th1 and Th2 cell differentiation. International immunology. 2011;23(7):415-420.

85. C Soliman, Pier GB, et al. Antibody recognition of bacterial surfaces and extracellular polysaccharides. Current opinion in structural biology. 2020;62:48-55.

86. A Cortés, Muñoz-Antoli C, et al. Th2 and Th1 responses: clear and hidden sides of immunity against intestinal helminths. Trends in parasitology. 2017;33(9):678-693.

87. S Crotty. A brief history of T cell help to B cells. Nat Rev Immunol. 2015;15(3):185-9.

88. RA Elsner, Hastey CJ, et al. CD4+ T cells promote antibody production but not sustained affinity maturation during Borrelia burgdorferi infection. Infect Immun. 2015;83(1):48-56.

89. SJ Klaus, Pinchuk LM, et al. Costimulation through CD28 enhances T cell-dependent B cell activation via CD40-CD40L interaction. Journal of Immunology. 1994;152(12):5643-52.

90. J Stavnezer. Immunoglobulin class switching. Current opinion in immunology. 1996;8(2):199-205.

91. B Pulendran, Kumar P, et al. Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo. The Journal of Immunology. 2001;167(9):5067-5076.

92. HM Aboshady, Stear M, et al. immunoglobulins as Biomarkers for Gastrointestinal nematodes Resistance in Small Ruminants: A systematic review. Scientific reports. 2020;10(1):1-14.

93. M Yousefi, Tahmasebi F, et al. Characterization of neutralizing monoclonal antibodies directed against tetanus toxin fragment C. Journal of immunotoxicology. 2014;11(1):28-34.

94. S Johnson. Antibody responses to clostridial infection in humans. Clinical Infectious Diseases. 1997:S173-S177.

95. EV Wenzel, Bosnak M, et al. Human antibodies neutralizing diphtheria toxin in vitro and in vivo. Scientific reports. 2020;10(1):1-21.

96. KP van Kessel, Bestebroer J, et al. Neutrophil-Mediated Phagocytosis of Staphylococcus aureus. Front Immunol. 2014;5:467.

97. Z Xiang, Block M, et al. IgE-mediated mast cell degranulation and recovery monitored by time-lapse photography. Journal of allergy and clinical immunology. 2001;108(1):116-121.

98. DM Segal, Taurog JD, et al. Dimeric immunoglobulin E serves as a unit signal for mast cell degranulation. Proceedings of the National Academy of Sciences. 1977;74(7):2993-2997.

99. AM Dvorak, Massey W, et al. IgE-mediated anaphylactic degranulation of isolated human skin mast cells. Blood. 1991;77(3):569-578.

100. EB Thangam, Jemima EA, et al. The role of histamine and histamine receptors in mast cell-mediated allergy and inflammation: the hunt for new therapeutic targets. Frontiers in immunology. 2018;9:1873.

101. R Goswami, Kaplan MH. A brief history of IL-9. The Journal of Immunology. 2011;186(6):3283-3288.

102. H Faulkner, Renauld J-C, et al. Interleukin-9 enhances resistance to the intestinal nematode Trichuris muris. Infection and Immunity. 1998;66(8):3832-3840.

103. A Sahoo, Wali S, et al. T helper 2 and T follicular helper cells: regulation and function of interleukin-4. Cytokine & growth factor reviews. 2016;30:29-37.

104. P Wang, Zhang Q, et al. The regulatory effects of mTOR complexes in the differentiation and function of CD4+ T cell subsets. Journal of immunology research. 2020;2020.

105. CM Cabrera, Urra JM. Food allergy and the oral immunotherapy approach. Archivum immunologiae et therapiae experimentalis. 2015;63(1):31-39.

106. Chapter 5 - B Cell Development, Activation and Effector Functions. In: Mak TW, Saunders ME, et al., editors. Primer to the Immune Response (Second Edition). Boston: Academic Cell; 2014. p. 111-142.

107. A Zhao, McDermott J, et al. Dependence of IL-4, IL-13, and nematode-induced alterations in murine small intestinal smooth muscle contractility on Stat6 and enteric nerves. The Journal of Immunology. 2003;171(2):948-954.

108. HW Schroeder Jr, Cavacini L. Structure and function of immunoglobulins. Journal of allergy and clinical immunology. 2010;125(2):S41-S52.

109. J Stavnezer, Radcliffe G, et al. Immunoglobulin heavy-chain switching may be directed by prior induction of transcripts from constant-region genes. Proceedings of the National Academy of Sciences. 1988;85(20):7704-7708.

110. P Renner IV, Hoppe M. Kuby immunology. 2019.

111. C Firacative, Gressler AE, et al. Identification of T helper (Th)1- and Th2-associated antigens of Cryptococcus neoformans in a murine model of pulmonary infection. Sci Rep. 2018;8(1):2681.

112. JE Uzonna, Bretscher PA. Anti-IL-4 antibody therapy causes regression of chronic lesions caused by medium-dose Leishmania major infection in BALB/c mice. Eur J Immunol. 2001;31(11):3175-84.

113. TL Stevens, Bossie A, et al. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature. 1988;334(6179):255-258.

114. A Bossie, Vitetta ES. IFN-γ enhances secretion of IgG2a from IgG2a-committed LPS-stimulated murine B cells: implications for the role of IFN-γ in class switching. Cellular immunology. 1991;135(1):95-104.

115. J-F Gauchat, Henchoz S, et al. Induction of human IgE synthesis in B cells by mast cells and basophils. Nature. 1993;365(6444):340-343.

116. C King, Nutman T. IgE and IgG subclass regulation by IL-4 and IFN-gamma in human helminth infections. Assessment by B cell precursor frequencies. The Journal of Immunology. 1993;151(1):458-465.

117. J Punnonen, Aversa G, et al. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. Proceedings of the National Academy of Sciences. 1993;90(8):3730-3734.

118. M Lundgren, Persson U, et al. Interleukin 4 induces synthesis of IgE and IgG4 in human B cells. European journal of immunology. 1989;19(7):1311-1315.

119. A Hjelholt, Christiansen G, et al. IgG subclass profiles in normal human sera of antibodies specific to five kinds of microbial antigens. Pathogens and disease. 2013;67(3):206-213.

120. C Wongkham, Tantrawatpan C, et al. Evaluation of immunoglobulin G subclass antibodies against recombinant Fasciola gigantica cathepsin L1 in an enzyme-linked immunosorbent assay for serodiagnosis of human fasciolosis. Clinical and diagnostic laboratory immunology. 2005;12(10):1152-1156.

121. A Heriazon, Hamilton K, et al. Immunoglobulin isotypes of lactating Holstein cows classified as high, average, and low type-1 or-2 immune responders. Veterinary immunology and immunopathology. 2011;144(3-4):259-269.

122. DM Estes, Brown WC. Type 1 and type 2 responses in regulation of Ig isotype expression in cattle. Veterinary immunology and immunopathology. 2002;90(1-2):1-10.

123. J Rengarajan, Szabo SJ, et al. Transcriptional regulation of Th1/Th2 polarization. Immunology today. 2000;21(10):479-483.

124. MA Fishman, Perelson AS. Th1/Th2 cross regulation. Journal of theoretical biology. 1994;170(1):25-56.

125. O Wurtz, Bajénoff M, et al. IL-4-mediated inhibition of IFN-γ production by CD4+ T cells proceeds by several developmentally regulated mechanisms. International immunology. 2004;16(3):501-508.

126. TF Gajewski, Fitch FW. Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. The Journal of Immunology. 1988;140(12):4245-4252.

127. E Maggi, Parronchi P, et al. Reciprocal regulatory effects of IFN-gamma and IL-4 on the in vitro development of human Th1 and Th2 clones. The Journal of Immunology. 1992;148(7):2142-2147.

128. T Usui, Nishikomori R, et al. GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rβ2 chain or T-bet. Immunity. 2003;18(3):415-428.

129. IA Ferber, Lee H-J, et al. GATA-3 significantly downregulates IFN-γ production from developing Th1 cells in addition to inducing IL-4 and IL-5 levels. Clinical Immunology. 1999;91(2):134-144.

130. R Yagi, Junttila IS, et al. The transcription factor GATA3 actively represses RUNX3 protein-regulated production of interferon-γ. Immunity. 2010;32(4):507-517.

131. MD Welsh, Cunningham RT, et al. Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. Immunology. 2005;114(1):101-111.

132. P Couissinier-Paris, Dessein AJ. Schistosoma-specific helper T cell clones from subjects resistant to infection by Schistosoma mansoni are Th0/2. Eur J Immunol. 1995;25(8):2295-302.

133. C Della Bella, Benagiano M, et al. T-cell clones in human trichinellosis: Evidence for a mixed Th1/Th2 response. Parasite immunology. 2017;39(3):e12412.

134. GS Firestein, Roeder WD, et al. A new murine CD4+ T cell subset with an unrestricted cytokine profile. Journal of Immunology. 1989;143(2):518-25.

135. A Kelso, Troutt AB, et al. Heterogeneity in lymphokine profiles of CD4+ and CD8+ T cells and clones activated in vivo and in vitro. Immunological reviews. 1991;123:85-114.

136. P Openshaw, Murphy EE, et al. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. J Exp Med. 1995;182(5):1357-67.

137. X Paliard, de Waal Malefijt R, et al. Simultaneous production of IL-2, IL-4, and IFN-gamma by activated human CD4+ and CD8+ T cell clones. The Journal of Immunology. 1988;141(3):849-855.

138. KT Miner, Croft M. Generation, persistence, and modulation of Th0 effector cells: role of autocrine IL-4 and IFN-γ. The Journal of Immunology. 1998;160(11):5280-5287.

139. A Vella, Pearce E. CD4+ Th2 response induced by Schistosoma mansoni eggs develops rapidly, through an early, transient, Th0-like stage. The Journal of Immunology. 1992;148(7):2283-2290.

140. C Infante-Duarte, Kamradt T. Th1/Th2 balance in infection. Springer Semin Immunopathol. 1999;21(3):317-38.

141. M Peine, Rausch S, et al. Stable T-bet+ GATA-3+ Th1/Th2 hybrid cells arise in vivo, can develop directly from naive precursors, and limit immunopathologic inflammation. PLoS biology. 2013;11(8):e1001633.

142. P Burt, Peine M, et al. Dissecting the dynamic transcriptional landscape of early T helper cell differentiation into Th1, Th2, and Th1/2 hybrid cells. Frontiers in immunology. 2022;13:4407.

143. CN Bock, Babu S, et al. Th2/1 hybrid cells occurring in murine and human strongyloidiasis share effector functions of Th1 cells. Frontiers in cellular and infection microbiology. 2017;7:261.

144. WC Brown, McElwain TF, et al. Bovine CD4(+) T-lymphocyte clones specific for rhoptry-associated protein 1 of Babesia bigemina stimulate enhanced immunoglobulin G1 (IgG1) and IgG2 synthesis. Infect Immun. 1999;67(1):155-64.

145. WC Brown, Zhu D, et al. The repertoire of Anaplasma marginale antigens recognized by CD4+ T-lymphocyte clones from protectively immunized cattle is diverse and includes major surface protein 2 (MSP-2) and MSP-3. Infection and Immunity. 1998;66(11):5414-5422.

146. D Clery, Torgerson P, et al. Immune responses of chronically infected adult cattle to Fasciola hepatica. Veterinary parasitology. 1996;62(1-2):71-82.

147. SG Rhodes, Graham SP. Is ‘timing’important for cytokine polarization? Trends in immunology. 2002;23(5):246-249.

148. TG Kimman, Westenbrink F, et al. Local and systemic antibody response to bovine respiratory syncytial virus infection and reinfection in calves with and without maternal antibodies. J Clin Microbiol. 1987;25(6):1097-106.

149. LJ Gershwin. Immunology of bovine respiratory syncytial virus infection of cattle. Comparative immunology, microbiology and infectious diseases. 2012;35(3):253-257.

150. LJ Gershwin, Anderson ML, et al. Assessment of IgE response and cytokine gene expression in pulmonary efferent lymph collected after ovalbumin inhalation during experimental infection of calves with bovine respiratory syncytial virus. American journal of veterinary research. 2011;72(1):134-145.

151. RA Palomares, Brock KV, et al. Differential expression of pro-inflammatory and anti-inflammatory cytokines during experimental infection with low or high virulence bovine viral diarrhea virus in beef calves. Veterinary immunology and immunopathology. 2014;157(3-4):149-154.

152. B Mihi, Van Meulder F, et al. Analysis of the mucosal immune responses induced by single and trickle infections with the bovine abomasal nematode O stertagia ostertagi. Parasite immunology. 2014;36(4):150-156.

153. V Weynants, Walravens K, et al. Quantitative assessment by flow cytometry of T-lymphocytes producing antigen-specific γ-interferon in Brucella immune cattle. Veterinary immunology and immunopathology. 1998;66(3-4):309-320.

154. J Herderschee, Fenwick C, et al. Emerging single-cell technologies in immunology. Journal of leukocyte biology. 2015;98(1):23-32.

155. O Estévez, Anibarro L, et al. Multi-parameter flow cytometry immunophenotyping distinguishes different stages of tuberculosis infection. Journal of Infection. 2020;81(1):57-71.

156. G Xu, Qi F, et al. The differential immune responses to COVID-19 in peripheral and lung revealed by single-cell RNA sequencing. Cell discovery. 2020;6(1):1-14.

157. M-L Alegre, Shiels H, et al. Expression and function of CTLA-4 in Th1 and Th2 cells. The Journal of Immunology. 1998;161(7):3347-3356.

158. HJ van den Ham, Binai NA, et al. Proteomic Profiling of Mouse Helper T Cell Differentiation. Proteomics. 2019;19(7):1800045.

159. Y Becker. Respiratory syncytial virus (RSV) evades the human adaptive immune system by skewing the Th1/Th2 cytokine balance toward increased levels of Th2 cytokines and IgE, markers of allergy—a review. Virus genes. 2006;33(2):235-252.

160. U Seitzer, Ahmed J. Tropical theileriosis: cytotoxic T lymphocyte response to vaccination. Vaccine. 2008;26:G24-G28.

161. S Nazifi, Razavi S, et al. Acute phase response in cattle infected with Anaplasma marginale. Veterinary microbiology. 2012;155(2-4):267-271.

162. U Rauf, Suleman M, et al. Humoral and Cell-Mediated Immune Response Validation in Calves after a Live Attenuated Vaccine of Babesia bigemina. Pathogens. 2020;9(11):936.

163. SG Rhodes, Cocksedge JM, et al. Differential cytokine responses of CD4+ and CD8+ T cells in response to bovine viral diarrhoea virus in cattle. Journal of General Virology. 1999;80(7):1673-1679.

164. J Stabel. Transitions in immune responses to Mycobacterium paratuberculosis. Veterinary microbiology. 2000;77(3-4):465-473.

165. M Samad, Dhar S, et al. Effect of humoral antibodies on Theileria annulata infection of cattle. Haryana Agricultural University Journal of Research (India). 1984.

166. P Preston, Brown C, et al. Cell-mediated cytotoxicity in Theileria annulata infection of cattle with evidence for BoLA restriction. Clinical and experimental immunology. 1983;53(1):88.

167. J Ahmed, Rothert M, et al. In vitro proliferative and cytotoxic responses of PBL from Theileria annulata-immune cattle. Journal of Veterinary Medicine, Series B. 1989;36(1-10):584-592.

168. N MacHugh, Burrells A, et al. Demonstration of strain-specific CD8 T cell responses to Theileria annulata. Parasite immunology. 2008;30(8):385-393.

169. JDM Campbell, Brown D, et al. A non-protective T helper 1 response against the intra-macrophage protozoan Theileria annulata. Clinical & Experimental Immunology. 1997;108(3):463-470.

170. R Barigye, GARCÍA-ORTIZ M, et al. Identification of IgG2-Specific Antigens in Mexican Anaplasma marginale Strains. Annals of the New York Academy of Sciences. 2004;1026(1):84-94.

171. WC Brown, Shkap V, et al. CD4+ T-lymphocyte and immunoglobulin G2 responses in calves immunized with Anaplasma marginale outer membranes and protected against homologous challenge. Infection and Immunity. 1998;66(11):5406.

172. J Pollock, McNair J, et al. Immune responses in bovine tuberculosis. Tuberculosis. 2001;81(1-2):103-107.

173. J Pollock, Pollock D, et al. Dynamic changes in circulating and antigen-responsive T-cell subpopulations post-Mycobacterium bovis infection in cattle. Immunology. 1996;87(2):236-241.

174. S Widdison, Schreuder L, et al. Cytokine expression profiles of bovine lymph nodes: effects of Mycobacterium bovis infection and bacille Calmette–Guérin vaccination. Clinical & Experimental Immunology. 2006;144(2):281-289.

175. R van Crevel, Karyadi E, et al. Increased production of interleukin 4 by CD4+ and CD8+ T cells from patients with tuberculosis is related to the presence of pulmonary cavities. The Journal of infectious diseases. 2000;181(3):1194-1197.

176. L Heitmann, Abad Dar M, et al. The IL-13/IL-4R α axis is involved in tuberculosis-associated pathology. The Journal of pathology. 2014;234(3):338-350.

177. GA Rook, Hernandez-Pando R, et al. IL-4 in tuberculosis: implications for vaccine design. Trends in immunology. 2004;25(9):483-488.

178. S Buccheri, Reljic R, et al. IL-4 depletion enhances host resistance and passive IgA protection against tuberculosis infection in BALB/c mice. European journal of immunology. 2007;37(3):729-737.

179. DA Dargatz, Byrum BA, et al. Evaluation of a commercial ELISA for diagnosis of paratuberculosis in cattle. Journal of the American Veterinary Medical Association. 2001;218(7):1163-1166.

180. H Billman-Jacobe, Carrigan M, et al. A comparison of the interferon gamma assay with the absorbed ELISA for the diagnosis of Johne's disease in cattle. Aust Vet J. 1992;69(2):25-8.

181. R Sweeney, Jones D, et al. Interferon-gamma and interleukin 4 gene expression in cows infected with Mycobacterium paratuberculosis. American journal of veterinary research. 1998;59(7):842-847.

182. PM Coussens. Mycobacterium paratuberculosis and the bovine immune system. Animal Health Research Reviews. 2001;2(2):141.

183. SL Mundo, Fontanals AM, et al. Bovine IgG1 antibodies against Mycobacterium avium subsp. paratuberculosis protein p34-cx improve association of bacteria and macrophages. Veterinary research. 2008;39(1):1-12.

184. B Hoebee, Rietveld E, et al. Association of severe respiratory syncytial virus bronchiolitis with interleukin-4 and interleukin-4 receptor α polymorphisms. The Journal of infectious diseases. 2003;187(1):2-11.

185. Y Fu, He J, et al. Intranasal immunization with a replication-deficient adenoviral vector expressing the fusion glycoprotein of respiratory syncytial virus elicits protective immunity in BALB/c mice. Biochemical and biophysical research communications. 2009;381(4):528-532.

186. RA Tripp, Moore D, et al. Peripheral Blood Mononuclear Cells from Infants Hospitalized Owing to Respiratory Syncytial Virus Infection Express T Helper-1 and T Helper-2 Cytokines and CC Chemokine Messenger RNA. Journal of Infectious Diseases. 2002;185(10):1388-1394.

187. AF Antonis, Claassen EA, et al. Kinetics of antiviral CD8 T cell responses during primary and post-vaccination secondary bovine respiratory syncytial virus infection. Vaccine. 2006;24(10):1551-1561.

188. T Kimman, Westenbrink F, et al. Local and systemic antibody response to bovine respiratory syncytial virus infection and reinfection in calves with and without maternal antibodies. Journal of clinical microbiology. 1987;25(6):1097.

189. E Guzman, Taylor G. Immunology of bovine respiratory syncytial virus in calves. Molecular immunology. 2015;66(1):48-56.

190. D Mahoney. Studies on the protection of cattle against Babesia bovis infection. Parasite lives: papers on parasites, their hosts and their associations to honour JFA Sprent/edited by Mary Cremin, Colin Dobson and Douglas E. Moorehouse. 1986.

191. SE Wilkowsky, Farber M, et al. Babesia bovis merozoite surface protein-2c (MSA-2c) contains highly immunogenic, conserved B-cell epitopes that elicit neutralization-sensitive antibodies in cattle. Molecular and biochemical parasitology. 2003;127(2):133-141.

192. V Molina, Risalde M, et al. Cell-Mediated Immune Response During Experimental Acute Infection with Bovine Viral Diarrhoea Virus: Evaluation of Blood Parameters. Transboundary and emerging diseases. 2014;61(1):44-59.

193. MK Rajput, Darweesh MF, et al. Comparative humoral immune response against cytopathic or non-cytopathic bovine viral diarrhea virus infection. Research in veterinary science. 2020;129:109-116.

194. RW Fulton, Saliki J, et al. Bovine viral diarrhea virus cytopathic and noncytopathic biotypes and type 1 and 2 genotypes in diagnostic laboratory accessions: clinical and necropsy samples from cattle. Journal of Veterinary Diagnostic Investigation. 2000;12(1):33-38.

195. G Palmer, Rurangirwa F, et al. Molecular basis for vaccine development against the ehrlichial pathogen Anaplasma marginale. Parasitology Today. 1999;15(7):281-286.

196. MD Welsh, Cunningham RT, et al. Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. Immunology. 2005;114(1):101-11.

197. HC Koo, Park YH, et al. Analysis of the immune response to Mycobacterium avium subsp. paratuberculosis in experimentally infected calves. Infection and Immunity. 2004;72(12):6870-6883.

198. AJ Allen, Park K-T, et al. Experimental infection of a bovine model with human isolates of Mycobacterium avium subsp. paratuberculosis. Veterinary immunology and immunopathology. 2011;141(3-4):258-266.

199. J Mehrzad, Janssen D, et al. Increase in Escherichia coli inoculum dose accelerates CD8+ T-cell trafficking in the primiparous bovine mammary gland. Journal of dairy science. 2008;91(1):193-201.

200. A Corbishley, Ahmad NI, et al. Strain-dependent cellular immune responses in cattle following Escherichia coli O157: H7 colonization. Infection and Immunity. 2014;82(12):5117-5131.

201. G Steinbach, Dinjus U, et al. Course of infection and humoral immune reaction in calves infected orally with different salmonella serovars. Journal of Veterinary Medicine, Series B. 1993;40(1-10):515-521.

202. R Ing, Su Z, et al. Suppressed T helper 2 immunity and prolonged survival of a nematode parasite in protein-malnourished mice. Proceedings of the National Academy of Sciences. 2000;97(13):7078-7083.

203. A González-Hernández, Borloo J, et al. Comparative analysis of the immune responses induced by native versus recombinant versions of the ASP-based vaccine against the bovine intestinal parasite Cooperia oncophora. International journal for parasitology. 2018;48(1):41-49.

204. F Van Meulder, Ratman D, et al. Analysis of the protective immune response following intramuscular vaccination of calves against the intestinal parasite Cooperia oncophora. International journal for parasitology. 2015;45(9-10):637-646.

205. H Parmentier, Ploeger H, et al. Low molecular weight Cooperia oncophora antigens: characterization and humoral immune responses in calves mono-infected with 100 000 infective larvae. Veterinary parasitology. 1995;59(3-4):219-230.

206. K Kanobana, Koets A, et al. T-cell mediated immune responses in calves primary-infected or re-infected with Cooperia oncophora: similar effector cells but different timing. International journal for parasitology. 2003;33(13):1503-1514.

207. P Bricarello, Zaros L, et al. Immunological responses and cytokine gene expression analysis to Cooperia punctata infections in resistant and susceptible Nelore cattle. Veterinary parasitology. 2008;155(1-2):95-103.

208. RW Li, Gasbarre LC. A temporal shift in regulatory networks and pathways in the bovine small intestine during Cooperia oncophora infection. International journal for parasitology. 2009;39(7):813-824.

209. FNJ Kooyman, Yatsuda AP, et al. Serum immunoglobulin E response in calves infected with the lungworm Dictyocaulus viviparus and its correlation with protection. Parasite immunology. 2002;24(1):47-56.

210. CA Scott, McKeand JB, et al. A longitudinal study of local and peripheral isotype/subclass antibodies in Dictyocaulus viviparus-infected calves. Veterinary immunology and immunopathology. 1996;53(3-4):235-247.

211. D Johnson, Sales J, et al. Local cytokine responses in Dictyocaulus viviparus infection. Veterinary parasitology. 2005;128(3-4):309-318.

212. S Holmgren, Hagberg Gustavsson M, et al. Cytokine m RNA expression in bronchoalveolar lavage cells during D ictyocaulus viviparus infection in calves. Parasite immunology. 2014;36(2):78-86.

213. D McCole, Doherty M, et al. T cell subset involvement in immune responses to Fasciola hepatica infection in cattle. Parasite immunology. 1999;21(1):1-8.

214. G Mulcahy, O'connor F, et al. Immune responses of cattle to experimental anti-Fasciola hepaticavaccines. Research in veterinary science. 1999;67(1):27-33.

215. D Clery, Mulcahy G. Lymphocyte and cytokine responses of young cattle during primary infection with Fasciola hepatica. Research in veterinary science. 1998;65(2):169-171.

216. G Oldham, WILLIAMS L. Cell mediated immunity to liver fluke antigens during experimental Fasciola hepatica infection of cattle. Parasite immunology. 1985;7(5):503-516.

217. A Garcia-Campos, Correia CN, et al. Fasciola hepatica infection in cattle: analyzing responses of peripheral blood mononuclear cells (PBMC) using a transcriptomics approach. Frontiers in immunology. 2019;10:2081.

218. DV Hoyle, Taylor DW. The immune response of regional lymph nodes during the early stages of Fasciola hepatica infection in cattle. Parasite Immunol. 2003;25(4):221-9.

219. EA Mendes, de Oliveira Mendes TA, et al. Expression of IL-4, IL-10 and IFN-γ in the liver tissue of cattle that are naturally infected with Fasciola hepatica. Veterinary parasitology. 2013;195(1-2):177-182.

220. J Graham-Brown, Hartley C, et al. Dairy heifers naturally exposed to Fasciola hepatica develop a type 2 immune response and concomitant suppression of leukocyte proliferation. Infection and Immunity. 2018;86(1).

221. A Canals, Zarlenga DS, et al. Cytokine profile induced by a primary infection with Ostertagia ostertagi in cattle. Vet Immunol Immunopathol. 1997;58(1):63-75.

222. J Michel, Lancaster M, et al. Ostertagia ostertagi: protective immunity in calves: the development in calves of a protective immunity to infection with Ostertagia ostertagi. Experimental parasitology. 1973;33(1):179-186.

223. H Hilderson, De Graaf D, et al. Characterisation of Ostertagia ostertagi antigens by the different bovine immunoglobulin isotypes. Research in veterinary science. 1993;55(2):203-208.

224. E Claerebout, Vercauteren I, et al. Cytokine responses in immunized and non-immunized calves after Ostertagia ostertagi infection. Parasite immunology. 2005;27(9):325-331.

225. E Claerebout, Vercauteren I, et al. Cytokine responses in immunized and non-immunized calves after Ostertagia ostertagi infection. Parasite Immunol. 2005;27(9):325-31.

226. P Soto, Parma A. The immune response in cattle infected with Tritrichomonas foetus. Veterinary parasitology. 1989;33(3-4):343-348.

227. S Skirrow, BonDurant R. Immunoglobulin isotype of specific antibodies in reproductive tract secretions and sera in Tritrichomonas foetus-infected heifers. American journal of veterinary research. 1990;51(4):645-653.

228. JM Voyich, Ansotegui R, et al. Antibody responses of cattle immunized with the Tf190 adhesin of Tritrichomonas foetus. Clinical and diagnostic laboratory immunology. 2001;8(6):1120-1125.

229. R BonDurant, Corbeil R, et al. Immunization of virgin cows with surface antigen TF1. 17 of Tritrichomonas foetus. Infection and Immunity. 1993;61(4):1385.

230. L Gómez-Gascón, Cardoso-Toset F, et al. Characterization of the immune response and evaluation of the protective capacity of rSsnA against Streptococcus suis infection in pigs. Comp Immunol Microbiol Infect Dis. 2016;47:52-9.

231. A Donlan, Petri Jr WA. The inflammasome and type-2 immunity in Clostridium difficile infection. Clinics in colon and rectal surgery. 2020;33(02):067-072.

232. MA Hoffman, Menge C, et al. Bovine immune response to Shiga-toxigenic Escherichia coli O157: H7. Clinical and Vaccine Immunology. 2006;13(12):1322-1327.

233. K Kanobana, Ploeger H, et al. Immune expulsion of the trichostrongylid Cooperia oncophora is associated with increased eosinophilia and mucosal IgA. International journal for parasitology. 2002;32(11):1389-1398.

234. M Hagberg, Wattrang E, et al. Mononuclear cell subsets in bronchoalveolar lavage fluid during Dictyocaulus viviparus infection of calves: a potential role for γ/δ TCR-expressing cells in airway immune responses? Parasite immunology. 2005;27(5):151-161.

235. FN Kooyman, de Vries E, et al. Antibodies elicited by the bovine lungworm, Dictyocaulus viviparus, cross-react with platelet-activating factor. Infect Immun. 2007;75(9):4456-62.

236. SZ Skirrow, BonDurant RH. Immunoglobulin isotype of specific antibodies in reproductive tract secretions and sera in Tritrichomonas foetus-infected heifers. Americal Journal of Veterinary Research. 1990;51(4):645-53.

237. R Gault, Kvasnicka W, et al. Specific antibodies in serum and vaginal mucus of heifers inoculated with a vaccine containing Tritrichomonas foetus. American journal of veterinary research. 1995;56(4):454-459.

238. K Bossaert, Farnir F, et al. Humoral immune response in calves to single-dose, trickle and challenge infections with Fasciola hepatica. Veterinary parasitology. 2000;87(2-3):103-123.

239. D Hughes, Hanna R, et al. Fasciola hepatica: IgG and IgA levels in the serum and bile of infected cattle. Experimental parasitology. 1981;52(2):271-279.

240. EK Mitchell, Mastroeni P, et al. Inhibition of cell surface MHC class II expression by Salmonella. European journal of immunology. 2004;34(9):2559-2567.

241. CV Harding, Boom WH. Regulation of antigen presentation by Mycobacterium tuberculosis: a role for Toll-like receptors. Nature reviews microbiology. 2010;8(4):296-307.

242. VK Kuchroo, Das MP, et al. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. Cell. 1995;80(5):707-718.

243. F Sallusto, Lanzavecchia A. The instructive role of dendritic cells on T-cell responses. Arthritis research & therapy. 2002;4(3):1-6.

244. C Nataraj, Eidmann S, et al. Bovine herpesvirus 1 downregulates the expression of bovine MHC class I molecules. Viral immunology. 1997;10(1):21-34.

245. EH Araibi, Marchetti B, et al. Downregulation of major histocompatibility complex class I in bovine papillomas. J Gen Virol. 2004;85(Pt 10):2809-2814.

246. DJ Weiss, Evanson OA, et al. Regulation of Expression of Major Histocompatibility Antigens by Bovine Macrophages Infected withMycobacterium avium subsp. paratuberculosis orMycobacterium avium subsp. avium. Infection and Immunity. 2001;69(2):1002-1008.

247. T Okagawa, Konnai S, et al. Cooperation of PD-1 and LAG-3 contributes to T-cell exhaustion in Anaplasma marginale-infected cattle. Infection and Immunity. 2016;84(10):2779-2790.

248. T Okagawa, Konnai S, et al. Cooperation of PD-1 and LAG-3 in the exhaustion of CD4+ and CD8+ T cells during bovine leukemia virus infection. Veterinary research. 2018;49(1):1-12.

249. L Chen, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nature Reviews Immunology. 2013;13(4):227-242.

250. DE de Almeida, Colvin CJ, et al. Antigen-specific regulatory T cells in bovine paratuberculosis. Veterinary immunology and immunopathology. 2008;125(3-4):234-245.

251. MP Sheridan, Browne JA, et al. IL-10 suppression of IFN-γ responses in tuberculin-stimulated whole blood from Mycobacterium bovis infected cattle. Vet Immunol Immunopathol. 2017;189:36-42.

252. L Eskra, Splitter GA. Bovine herpesvirus-1 infects activated CD4+ lymphocytes. Journal of General Virology. 1997;78(9):2159-2166.

253. C Chase, Elmowalid G, et al. The immune response to bovine viral diarrhea virus: a constantly changing picture. The Veterinary clinics of North America. Food animal practice. 2004;20(1):95-114.

254. S Han, Norimine J, et al. Rapid deletion of antigen-specific CD4+ T cells following infection represents a strategy of immune evasion and persistence for Anaplasma marginale. The Journal of Immunology. 2008;181(11):7759-7769.

255. X Xu, Wen X, et al. Activation-induced T helper cell death contributes to Th1/Th2 polarization following murine Schistosoma japonicum infection. Journal of Biomedicine and Biotechnology. 2010;2010.

256. S Berger, Ballo H, et al. Distinct antigen-induced cytokine pattern upon stimulation with antibody-complexed antigen consistent with a Th1→ Th2-shift. Research in virology. 1996;147(2-3):103-108.

257. LJ Picker, Singh MK, et al. Direct Demonstration of Cytokine Synthesis Heterogeneity among Human Memory/Effector T-Cells by Flow-Cytometry. Blood. 1995;86(4):1408-1419.

258. J Baran, Kowalczyk D, et al. Three-color flow cytometry detection of intracellular cytokines in peripheral blood mononuclear cells: comparative analysis of phorbol myristate acetate-ionomycin and phytohemagglutinin stimulation. ASM Journals/Clinical Diagnostic laboratory Immunology. 2001;8(2):303-13.

259. C Phetsouphanh, Zaunders JJ, et al. Detecting Antigen-Specific T Cell Responses: From Bulk Populations to Single Cells. International Journal of Molecular Sciences. 2015;16(8):18878-93.

260. MA Suni, Picker LJ, et al. Detection of antigen-specific T cell cytokine expression in whole blood by flow cytometry. Journal of immunological methods. 1998;212(1):89-98.

261. MF Maggioli, Palmer MV, et al. Characterization of effector and memory T cell subsets in the immune response to bovine tuberculosis in cattle. PLoS One. 2015;10(4):e0122571.

262. A Akbar, Terry L, et al. Unidirectional phenotypic changes within the T200 complex during activation of T cells. The Journal of Immunology. 1988;140:2171.

263. ME Sanders, Makgoba MW, et al. Human naive and memory T cells: reinterpretation of helper-inducer and suppressor-inducer subsets. Immunology today. 1988;9(7-8):195-9.

264. G Bembridge, MacHugh N, et al. CD45RO expression on bovine T cells: relation to biological function. Immunology. 1995;86(4):537.

265. JR Stabel, Kimura K, et al. Augmentation of secreted and intracellular gamma interferon following johnin purified protein derivative sensitization of cows naturally infected with Mycobacterium avium subsp. paratuberculosis. Journal of Veterinary Diagnostic Investigation. 2007;19(1):43-51.

266. E McInnes, Sopp P, et al. Phenotypic analysis of local cellular responses in calves infected with bovine respiratory syncytial virus. Immunology. 1999;96(3):396.

267. A Duni, Markopoulos GS, et al. The Humoral Immune Response to BNT162b2 Vaccine Is Associated With Circulating CD19+ B Lymphocytes and the Naïve CD45RA to Memory CD45RO CD4+ T Helper Cells Ratio in Hemodialysis Patients and Kidney Transplant Recipients. Frontiers in immunology. 2021;12:760249-760249.

268. CA Michie, McLean A, et al. Lifespan of human lymphocyte subsets defined by CD45 isoforms. Nature. 1992;360(6401):264-5.

269. MR Wills, Carmichael AJ, et al. Human virus-specific CD8+ CTL clones revert from CD45ROhigh to CD45RAhigh in vivo: CD45RAhighCD8+ T cells comprise both naive and memory cells. The Journal of Immunology. 1999;162(12):7080-7087.

270. MF Callan, Tan L, et al. Direct visualization of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus In vivo. Journal of Experimental Medicine. 1998;187(9):1395-402.

271. N Khan, Shariff N, et al. Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. The Journal of Immunology. 2002;169(4):1984-92.

272. MR Wills, Okecha G, et al. Identification of naive or antigen-experienced human CD8+ T cells by expression of costimulation and chemokine receptors: analysis of the human cytomegalovirus-specific CD8+ T cell response. The Journal of Immunology. 2002;168(11):5455-5464.

273. MR Wills, Carmichael AJ, et al. Human virus-specific CD8+ CTL clones revert from CD45ROhigh to CD45RAhigh in vivo: CD45RAhighCD8+ T cells comprise both naive and memory cells. J Immunol. 1999;162(12):7080-7.

274. L Gattinoni, Lugli E, et al. A human memory T cell subset with stem cell-like properties. Nature medicine. 2011;17(10):1290-7.

275. R Ahmed, Roger L, et al. Human Stem Cell-like Memory T Cells Are Maintained in a State of Dynamic Flux. Cell reports. 2016;17(11):2811-2818.

276. H Lee, Stabel JR, et al. Cytokine gene expression in ileal tissues of cattle infected with Mycobacterium paratuberculosis. Veterinary immunology and immunopathology. 2001;82(1-2):73-85.

277. H Hong, Gu Y, et al. The Distribution of Human Stem Cell-like Memory T Cell in Lung Cancer. Journal of Immunotherapy. 2016;39(6):233-240.

278. JH Jung, Rha MS, et al. SARS-CoV-2-specific T cell memory is sustained in COVID-19 convalescent patients for 10 months with successful development of stem cell-like memory T cells. Nature communications. 2021;12(1):1-12.

279. P Sopp, Howard CJ. IFN gamma and IL-4 production by CD4, CD8 and WC1 gamma delta TCR(+) T cells from cattle lymph nodes and blood. Veterinary immunology and immunopathology. 2001;81(1-2):85-96.

280. MC Frie, Sporer KRB, et al. T and B cell activation profiles from cows with and without Johne's disease in response to in vitro stimulation with Mycobacterium avium subspecies paratuberculosis. Veterinary immunology and immunopathology. 2017;193:50-56.

281. E Machura, Mazur B, et al. Expression of naive/memory (CD45RA/CD45RO) markers by peripheral blood CD4+ and CD8+ T cells in children with asthma. Archivum immunologiae et therapiae experimentalis. 2008;56(1):55-62.

282. ML Hermiston, Xu Z, et al. CD45: a critical regulator of signaling thresholds in immune cells. Annual review of immunology. 2003;21(1):107-137.

283. NK Tonks, Charbonneau H, et al. Demonstration that the leukocyte common antigen CD45 is a protein tyrosine phosphatase. Biochemistry. 1988;27(24):8695-701.

284. IS Trowbridge, Thomas ML. Cd45 - an Emerging Role as a Protein-Tyrosine-Phosphatase Required for Lymphocyte-Activation and Development. Annual review of immunology. 1994;12(1):85-116.

285. KW Lynch. Consequences of regulated pre-mRNA splicing in the immune system. Nature Reviews Immunology. 2004;4(12):931-40.

286. ML Hermiston, Xu Z, et al. CD45: a critical regulator of signaling thresholds in immune cells. Annual review of immunology. 2003;21(1):107-37.

287. NN Jonsson, Cox DK, et al. Allelic Variation in Protein Tyrosine Phosphatase Receptor Type-C in Cattle Influences Erythrocyte, Leukocyte and Humoral Responses to Infestation With the Cattle Tick Rhipicephalus australis. Frontiers in immunology. 2021;12.

288. A Williams. Glycoprotein antigens of the lymphocyte surface and their purification by antibody affinity chromatography. Handbook of experimental immunology. 1986;22.

289. KT Ballingall, Waibochi L, et al. The CD45 locus in cattle: allelic polymorphism and evidence for exceptional positive natural selection. Immunogenetics. 2001;52(3-4):276-83.

290. L Blunt, Hogarth PJ, et al. Phenotypic characterization of bovine memory cells responding to mycobacteria in IFNgamma enzyme linked immunospot assays. Vaccine. 2015;33(51):7276-7282.

291. AE Hogg, Parsons K, et al. Characterization of age-related changes in bovine CD8+ T-cells. Veterinary immunology and immunopathology. 2011;140(1-2):47-54.

292. MM Elnaggar, Knowles DP, et al. Flow Cytometric Analysis of the Cytotoxic T-Cell Recall Response to Theileria parva in Cattle Following Vaccination by the Infection and Treatment Method. Veterinary Sciences. 2021;8(6):114.

293. JJ Endsley, Endsley MA, et al. Bovine natural killer cells acquire cytotoxic/effector activity following activation with IL-12/15 and reduce Mycobacterium bovis BCG in infected macrophages. Journal of leukocyte biology. 2006;79(1):71-9.

294. M Denis, Lacy-Hulbert SJ, et al. Streptococcus uberis-specific T cells are present in mammary gland secretions of cows and can be activated to kill S. uberis. Veterinary research communications. 2011;35(3):145-156.

295. S Mitoma, Carr BV, et al. The detection of long-lasting memory foot-and-mouth disease (FMD) virus serotype O-specific CD4(+) T cells from FMD-vaccinated cattle by bovine major histocompatibility complex class II tetramer. Immunology. 2021;164(2):266-278.

296. NN Jonsson, Cox DK, et al. Allelic Variation in Protein Tyrosine Phosphatase Receptor Type-C in Cattle Influences Erythrocyte, Leukocyte and Humoral Responses to Infestation With the Cattle Tick Rhipicephalus australis. Frontiers in immunology. 2021;12:675979.

297. RM Silflow, Degel PM, et al. Bronchoalveolar immune defense in cattle exposed to primary and secondary challenge with bovine viral diarrhea virus. Veterinary immunology and immunopathology. 2005;103(1-2):129-39.

298. M Hagberg, Lundén A, et al. Characterization of bovine lymphocytes stimulated in vitro by Dictyocaulus viviparus homogenate. Parasite immunology. 2008;30(6-7):342-353.

299. M Hagberg. Immune cell responses to the cattle lungworm, Dictyocaulus viviparus. Department of Biomedical Sciences and Veterinary Public Health. 2008(2008: 37):52.

300. AE Hogg, Worth A, et al. The antigen-specific memory CD8+ T-cell response induced by BCG in cattle resides in the CD8+ γ/δTCR− CD45RO+ T-cell population. Vaccine. 2009;27(2):270-279.

301. CJ Howard, Sopp P, et al. Distinction of naive and memory BoCD4 lymphocytes in calves with a monoclonal antibody, CC76, to a restricted determinant of the bovine leukocyte-common antigen, CD45. European journal of immunology. 1991;21(9):2219-26.

302. M Guerra-Maupome, Palmer MV, et al. Characterization of gammadelta T Cell Effector/Memory Subsets Based on CD27 and CD45R Expression in Response to Mycobacterium bovis Infection. Immunohorizons. 2019;3(6):208-218.

303. C Zhao, Tian F, et al. Muscle transcriptomic analyses in Angus cattle with divergent tenderness. Molecular biology reports. 2012;39(4):4185-4193.

304. W Tuo, Li L, et al. Abomasal mucosal immune responses of cattle with limited or continuous exposure to pasture-borne gastrointestinal nematode parasite infection. Veterinary parasitology. 2016;229:118-125.

305. J Mendez, Sun D, et al. Bovine neutrophils form extracellular traps in response to the gastrointestinal parasite Ostertagia ostertagi. Scientific reports. 2018;8(1):1-12.

306. JA Carrillo, He Y, et al. Integrated metabolomic and transcriptome analyses reveal finishing forage affects metabolic pathways related to beef quality and animal welfare. Scientific reports. 2016;6(1):1-16.

307. E Panagioti, Klenerman P, et al. Features of effective T cell-inducing vaccines against chronic viral infections. Frontiers in immunology. 2018;9:276.

308. A Flaxman, Ewer KJ. Methods for Measuring T-Cell Memory to Vaccination: From Mouse to Man. Vaccines (Basel). 2018;6(3):43.

309. W Craig, Poppema S, et al. CD45 isoform expression on human haemopoietic cells at different stages of development. British journal of haematology. 1994;88(1):24-30.

310. E Machura, Mazur B, et al. Expression of naive/memory (CD45RA/CD45RO) markers by peripheral blood CD4+ and CD8 + T cells in children with asthma. Archivum immunologiae et therapiae experimentalis. 2008;56(1):55-62.

311. LT Clement. Isoforms of the CD45 common leukocyte antigen family: markers for human T-cell differentiation. Journal of clinical immunology. 1992;12(1):1-10.

312. M Valentine, Song K, et al. Expression of the memory marker CD45RO on helper T cells in macaques. PLoS One. 2013;8(9):e73969.

313. N Kumar, Rao TKS, et al. Internal parasite management in grazing livestock. Journal of parasitic diseases. 2013;37(2):151-157.

314. SL Blumerman, Herzig CT, et al. WC1+ γδ T cell memory population is induced by killed bacterial vaccine. European journal of immunology. 2007;37(5):1204-1216.

315. S Kulberg, Boysen P, et al. Reference values for relative numbers of natural killer cells in cattle blood. Developmental & Comparative Immunology. 2004;28(9):941-948.

316. W Liao, Lin J-X, et al. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. Immunity. 2013;38(1):13-25.

317. BH Nelson. IL-2, regulatory T cells, and tolerance. The Journal of Immunology. 2004;172(7):3983-3988.

318. K Schuster, Gadiot J, et al. Homeostatic proliferation of naïve CD8+ T cells depends on CD62L/L-selectin-mediated homing to peripheral LN. European journal of immunology. 2009;39(11):2981-2990.

319. SL Waldrop, Pitcher CJ, et al. Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. The Journal of Clinical Investigations. 1997;99(7):1739-50.

320. P Totté, Duperray C, et al. CD62L defines a subset of pathogen-specific bovine CD4 with central memory cell characteristics. Developmental & Comparative Immunology. 2010;34(2):177-182.

321. Z Xiao, Casey KA, et al. Programming for CD8 T cell memory development requires IL-12 or type I IFN. The Journal of Immunology. 2009;182(5):2786-2794.

322. MF Mescher, Curtsinger JM, et al. Signals required for programming effector and memory development by CD8+ T cells. Immunological reviews. 2006;211(1):81-92.

323. A Kandel, Masello M, et al. CD4+ T Cell Responses to Pathogens in Cattle [Book chapter]. London: IntechOpen; 2021. Available from: https://www.intechopen.com/chapters/78918

324. W Holtmeier, Kabelitz D. gammadelta T cells link innate and adaptive immune responses. Chem Immunol Allergy. 2005;86:151-183.

325. CL Baldwin, Yirsaw A, et al. γδ T cells in livestock: Responses to pathogens and vaccine potential. Transboundary and emerging diseases. 2020;67:119-128.

326. W Waters, Rahner T, et al. Expression of L-selectin (CD62L), CD44, and CD25 on activated bovine T cells. Infection and Immunity. 2003;71(1):317-326.

327. FN Toka, Kenney MA, et al. Rapid and transient activation of γδ T cells to IFN-γ production, NK cell-like killing, and antigen processing during acute virus infection. Journal of Immunology. 2011;186(8):4853-61.

328. SP Phalke, Chiplunkar SV. Activation status of γδ T cells dictates their effect on osteoclast generation and bone resorption. Bone reports. 2015;3:95-103.

329. M Hagberg, Lunden A, et al. Characterization of bovine lymphocytes stimulated in vitro by Dictyocaulus viviparus homogenate. Parasite immunology. 2008;30(6-7):342-53.

330. Y Tian, Babor M, et al. Unique phenotypes and clonal expansions of human CD4 effector memory T cells re-expressing CD45RA. Nature communications. 2017;8(1):1-13.

331. J Carrasco, Godelaine D, et al. CD45RA on human CD8 T cells is sensitive to the time elapsed since the last antigenic stimulation. Blood. 2006;108(9):2897-2905.

332. JM Faint, Annels NE, et al. Memory T cells constitute a subset of the human CD8+ CD45RA+ pool with distinct phenotypic and migratory characteristics. The Journal of Immunology. 2001;167(1):212-220.

333. D Hamann, Baars PA, et al. Phenotypic and functional separation of memory and effector human CD8+ T cells. Journal of Experimental Medicine. 1997;186(9):1407-18.

334. JP Deans, Boyd AW, et al. Transitions from high to low molecular weight isoforms of CD45 (T200) involve rapid activation of alternate mRNA splicing and slow turnover of surface CD45R. The Journal of Immunology. 1989;143(4):1233-8.

335. ML Birkeland, Johnson P, et al. Changes in CD45 isoform expression accompany antigen-induced murine T-cell activation. proceedings of the National Academy of Sciences of United States of America. 1989;86(17):6734-8.

336. A Akbar, Terry L, et al. Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T cells. The Journal of Immunology. 1988;140(7):2171-2178.

337. H Zola, Flego L, et al. The CD45RO (p180, UCHL1) marker: complexity of expression in peripheral blood. Cellular immunology. 1992;145(1):175-186.

338. HE Prince, York J, et al. Phenotypic comparison of the three populations of human lymphocytes defined by CD45RO and CD45RA expression. Cellular immunology. 1992;145(2):254-262.

339. T Sathaliyawala, Kubota M, et al. Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. Immunity. 2013;38(1):187-97.

340. W Yang, Jia X, et al. Immunophenotypic characterization of CD45RO+ and CD45RA+ T cell subsets in peripheral blood of peripheral T cell lymphoma patients. Cell biochemistry and biophysics. 2014;70(2):993-7.

341. Y Qin, Van Den Noort S, et al. Dual expression of CD45RA and CD45RO isoforms on myelin basic protein-specific CD4+ T-cell lines in multiple sclerosis. Journal of clinical immunology. 1993;13(2):152-61.

342. A Kandel, Li L, et al. Differential Expression of CD45RO and CD45RA in Bovine T Cells. Cells. 2022;11(11):1844.

343. GP Bembridge, MacHugh ND, et al. CD45RO expression on bovine T cells: relation to biological function. Immunology. 1995;86(4):537-44.

344. H Zola, Flego L, et al. The CD45RO (p180, UCHL1) marker: complexity of expression in peripheral blood. Cellular immunology. 1992;145(1):175-86.

345. HE Prince, York J, et al. Phenotypic comparison of the three populations of human lymphocytes defined by CD45RO and CD45RA expression. Cellular immunology. 1992;145(2):254-62.

346. A Cossarizza, Ortolani C, et al. CD45 isoforms expression on CD4+ and CD8+ T cells throughout life, from newborns to centenarians: implications for T cell memory. Mechanisms of ageing and development. 1996;86(3):173-95.

347. RR Chowdhury, Valainis JR, et al. The role of antigen recognition in the γδ T cell response at the controlled stage of M. tuberculosis infection. bioRxiv. 2021.

348. F Sallusto, Geginat J, et al. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annual Review of Immunology 2004;22:745-763.

349. A Gaballa, Arruda L, et al. CD8+ γδ T cells are more frequent in CMV seropositive bone marrow grafts and display phenotype of an adaptive immune response. Stem cells international. 2019;2019.

350. O Lecomte, Fischer A. Antigen-independent adhesion of CD45RA (naive) and CD45RO (memory) CD4 T cells to B cells. International immunology. 1992;4(2):191-196.

351. E Frolova, Scott C, et al. CD45RO+ T-cells immunoregulate spontaneous in vitro immunoglobulin production by normal and chronic lymphocytic leukaemia B-cells. Leukemia & Lymphoma. 1995;18(1-2):103-111.

352. AN Vlasova, Saif LJ. Bovine Immunology: Implications for Dairy Cattle. Front Immunol. 2021;12:643206.

353. E Claerebout, Geldhof P. Helminth vaccines in ruminants: from development to application. Veterinary Clinics: Food Animal Practice. 2020;36(1):159-171.

354. B Kebede, Sori T, et al. Review on current status of vaccines against parasitic diseases of animals. J Veterinar Sci Techno. 2016;7(327):2.

355. W Smith, Zarlenga D. Developments and hurdles in generating vaccines for controlling helminth parasites of grazing ruminants. Veterinary parasitology. 2006;139(4):347-359.

356. CC Motran, Silvane L, et al. Helminth infections: recognition and modulation of the immune response by innate immune cells. Frontiers in immunology. 2018;9:664.

357. PH Gazzinelli-Guimaraes, Nutman TB. Helminth parasites and immune regulation. F1000Research. 2018;7.

358. RM Anthony, Urban Jr JF, et al. Memory TH2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. Nature medicine. 2006;12(8):955-960.

359. PM Boggiatto, Schaut RG, et al. Enhancing the Detection of Brucella-Specific CD4(+) T Cell Responses in Cattle via in vitro Antigenic Expansion and Restimulation. Front Immunol. 2020;11:1944.

360. BV Carr, Lefevre EA, et al. CD4+ T-cell responses to foot-and-mouth disease virus in vaccinated cattle. J Gen Virol. 2013;94(Pt 1):97-107.

361. P Totté, Rodrigues V, et al. Analysis of cellular responses to Mycoplasma mycoides subsp. mycoides small colony biotype associated with control of contagious bovine pleuropneumonia. 2008.

362. E Bassey, Collins MT. Study of T-lymphocyte subsets of healthy and Mycobacterium avium subsp. paratuberculosis-infected cattle. Infection and Immunity. 1997;65(11):4869-4872.

363. B Charleston, Brackenbury LS, et al. Alpha/beta and gamma interferons are induced by infection with noncytopathic bovine viral diarrhea virus in vivo. J Virol. 2002;76(2):923-7.

364. JE Allen, Sutherland TE, editors. Host protective roles of type 2 immunity: parasite killing and tissue repair, flip sides of the same coin. Seminars in immunology; 2014: Elsevier.

365. C Strube, Haake C, et al. Vaccination with recombinant paramyosin against the bovine lungworm Dictyocaulus viviparus considerably reduces worm burden and larvae shedding. Parasites & vectors. 2015;8(1):1-12.

366. TR Mosmann, Cherwinski H, et al. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. Journal of Immunology. 1986;136(7):2348-57.

367. S Romagnani. Human TH1 and TH2 subsets: doubt no more. Immunology today. 1991;12(8):256-257.

368. S Romagnani. Human TH1 and TH2 subsets: regulation of differentiation and role in protection and immunopathology. Int Arch Allergy Immunol. 1992;98(4):279-85.

369. S Romagnani. T-cell subsets (Th1 versus Th2). Ann Allergy Asthma Immunol. 2000;85(1):9-18; quiz 18, 21.

370. A Mena, Ioannou XP, et al. Th1/Th2 biasing effects of vaccination in cattle as determined by real-time PCR. Journal of immunological methods. 2002;263(1-2):11-21.

371. S Rhodes, Buddle B, et al. Bovine tuberculosis: immune responses in the peripheral blood and at the site of active disease. Immunology. 2000;99(2):195-202.

372. M Mohrs, Shinkai K, et al. Analysis of type 2 immunity in vivo with a bicistronic IL-4 reporter. Immunity. 2001;15(2):303-311.

373. S Kim, Prout M, et al. Cutting edge: basophils are transiently recruited into the draining lymph nodes during helminth infection via IL-3, but infection-induced Th2 immunity can develop without basophil lymph node recruitment or IL-3. The Journal of Immunology. 2010;184(3):1143-1147.

374. Z Liu, Liu Q, et al. Nippostrongylus brasiliensis can induce B7-independent antigen-specific development of IL-4-producing T cells from naive CD4 T cells in vivo. The Journal of Immunology. 2002;169(12):6959-6968.

375. MJ Holland, Harcus YM, et al. Proteins secreted by the parasitic nematode Nippostrongylus brasiliensis act as adjuvants for Th2 responses. European journal of immunology. 2000;30(7):1977-1987.

376. J Osborne, Devaney E. The L3 of Brugia induces a Th2-polarized response following activation of an IL-4-producing CD4-CD8-alphabeta T cell population. International immunology. 1998;10(10):1583-1590.

377. W Tuo, Li L, et al. Abomasal mucosal immune responses of cattle with limited or continuous exposure to pasture-borne gastrointestinal nematode parasite infection. Vet Parasitol. 2016;229:118-125.

378. M Fang, Xie H, et al. Stochastic cytokine expression induces mixed T helper cell states. PLoS biology. 2013;11(7):e1001618.

379. J Mestas, Hughes CC. Of mice and not men: differences between mouse and human immunology. The Journal of Immunology. 2004;172(5):2731-2738.

380. A Iwasaki, Omer SB. Why and How Vaccines Work. Cell. 2020;183(2):290-295.

381. CM Lau, Sun JC. The widening spectrum of immunological memory. Current opinion in immunology. 2018;54:42-49.

382. D Maura, Elmekki N, et al. The ammonia oxidizing bacterium Nitrosomonas eutropha blocks T helper 2 cell polarization via the anti-inflammatory cytokine IL-10. Sci Rep. 2021;11(1):14162.

383. CA Anderson, Patel P, et al. A degradatory fate for CCR4 suggests a primary role in Th2 inflammation. Journal of leukocyte biology. 2020;107(3):455-466.

384. W Mandala, Harawa V, et al. Optimization of stimulation and staining conditions for intracellular cytokine staining (ICS) for determination of cytokine-producing T cells and monocytes. Curr Res Immunol. 2021;2:184-193.

385. W Ai, Li H, et al. Optimal Method to Stimulate Cytokine Production and Its Use in Immunotoxicity Assessment. International Journal of Environmental Research and Public Health. 2013;10(9):3834-3842.

386. MF Mescher, Curtsinger JM, et al. Signals required for programming effector and memory development by CD8+ T cells. Immunol Rev. 2006;211:81-92.

387. JT Harty, Badovinac VP. Shaping and reshaping CD8+ T-cell memory. Nat Rev Immunol. 2008;8(2):107-19.

388. M Pepper, Jenkins MK. Origins of CD4(+) effector and central memory T cells. Nat Immunol. 2011;12(6):467-71.

389. TR Mosmann, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol. 1989;7:145-73.

390. B Pulendran. Modulating TH1/TH2 responses with microbes, dendritic cells, and pathogen recognition receptors. Immunol Res. 2004;29(1-3):187-96.

391. YJ Liu. Thymic stromal lymphopoietin: master switch for allergic inflammation. J Exp Med. 2006;203(2):269-73.

392. S Ochiai, Jagot F, et al. Thymic stromal lymphopoietin drives the development of IL-13(+) Th2 cells. Proc Natl Acad Sci U S A. 2018;115(5):1033-1038.

393. J Elhmouzi-Younes, Storset AK, et al. Bovine neonate natural killer cells are fully functional and highly responsive to interleukin-15 and to NKp46 receptor stimulation. Vet Res. 2009;40(6):54.

394. MF Ethier, Madison JM. IL-4 inhibits calcium transients in bovine trachealis cells by a ryanodine receptor-dependent mechanism. Faseb j. 2006;20(1):154-6.

395. WL Goff, Storset AK, et al. Bovine splenic NK cells synthesize IFN-gamma in response to IL-12-containing supernatants from Babesia bovis-exposed monocyte cultures. Parasite Immunol. 2006;28(5):221-8.

396. K Anmol, Akanksha H, et al. Are CD45RO+ and CD45RA-genuine markers for bovine memory T cells? Animal Diseases. 2022;2(1):1-9.

397. T Sekiya, Yoshimura A. In Vitro Th Differentiation Protocol. Methods Mol Biol. 2016;1344:183-91.

398. L Li, Si H, et al. Characterization of IL-10-producing neutrophils in cattle infected with Ostertagia ostertagi. Sci Rep. 2019;9(1):20292.

399. J Zhu, Yamane H, et al. GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. Cell research. 2006;16(1):3-10.

400. M Rincón, Dérijard B, et al. Reprogramming the signalling requirement for AP-1 (activator protein-1) activation during differentiation of precursor CD4+ T-cells into effector Th1 and Th2 cells. Genes and function. 1997;1(1):51-68.

401. B Li, Tournier C, et al. Regulation of IL-4 expression by the transcription factor JunB during T helper cell differentiation. The EMBO journal. 1999;18(2):420-432.

402. B Hartenstein, Teurich S, et al. Th2 cell-specific cytokine expression and allergen-induced airway inflammation depend on JunB. The EMBO journal. 2002;21(23):6321-6329.

403. D Fang, Elly C, et al. Dysregulation of T lymphocyte function in itchy mice: a role for Itch in TH2 differentiation. Nature immunology. 2002;3(3):281-287.

404. B Li, Tournier C, et al. Regulation of IL-4 expression by the transcription factor JunB during T helper cell differentiation. Embo j. 1999;18(2):420-32.

405. G Seumois, Chavez L, et al. Epigenomic analysis of primary human T cells reveals enhancers associated with TH2 memory cell differentiation and asthma susceptibility. Nature immunology. 2014;15(8):777-788.

406. RJ Lund, Löytömäki M, et al. Genome-wide identification of novel genes involved in early Th1 and Th2 cell differentiation. The Journal of Immunology. 2007;178(6):3648-3660.

407. J Voice, Donnelly S, et al. c-Maf and JunB mediation of Th2 differentiation induced by the type 2 G protein-coupled receptor (VPAC2) for vasoactive intestinal peptide. The Journal of Immunology. 2004;172(12):7289-7296.

408. I-C Ho, Lo D, et al. c-maf promotes T helper cell type 2 (Th2) and attenuates Th1 differentiation by both interleukin 4–dependent and–independent mechanisms. The Journal of experimental medicine. 1998;188(10):1859-1866.

409. GR Lee. Transcriptional regulation of T helper type 2 differentiation. Immunology. 2014;141(4):498-505.

410. T Kozuka, Sugita M, et al. c-Myb and GATA-3 cooperatively regulate IL-13 expression via conserved GATA-3 response element and recruit mixed lineage leukemia (MLL) for histone modification of the IL-13 locus. The Journal of Immunology. 2011;187(11):5974-5982.

411. SP Khare, Shetty A, et al. NF-κB signaling and IL-4 signaling regulate SATB1 expression via alternative promoter usage during Th2 differentiation. Frontiers in immunology. 2019;10:667.

412. V Santarlasci, Cosmi L, et al. IL-1 and T Helper Immune Responses. Front Immunol. 2013;4:182.

413. SM Caucheteux, Hu-Li J, et al. IL-1β enhances inflammatory TH2 differentiation. Journal of allergy and clinical immunology. 2016;138(3):898-901. e4.

414. C Cayrol, Girard JP. Interleukin-33 (IL-33): A nuclear cytokine from the IL-1 family. Immunol Rev. 2018;281(1):154-168.

415. T Leal-Silva, Vieira-Santos F, et al. Detrimental role of IL-33/ST2 pathway sustaining a chronic eosinophil-dependent Th2 inflammatory response, tissue damage and parasite burden during Toxocara canis infection in mice. PLoS Negl Trop Dis. 2021;15(7):e0009639.

416. SM Yeon, Halim L, et al. IL-7 plays a critical role for the homeostasis of allergen-specific memory CD4 T cells in the lung and airways. Sci Rep. 2017;7(1):11155.

417. B Bošnjak, Kazemi S, et al. Th2-T(RMs) Maintain Life-Long Allergic Memory in Experimental Asthma in Mice. Front Immunol. 2019;10:840.

418. NJ Tubo, Jenkins MK. TCR signal quantity and quality in CD4+ T cell differentiation. Trends in immunology. 2014;35(12):591-596.

419. T Nakayama, Yamashita M, editors. The TCR-mediated signaling pathways that control the direction of helper T cell differentiation. Seminars in immunology; 2010: Elsevier.

420. ND Bhattacharyya, Feng CG. Regulation of T helper cell fate by TCR signal strength. Frontiers in immunology. 2020;11:624.

421. CS Guy, Vignali KM, et al. Distinct TCR signaling pathways drive proliferation and cytokine production in T cells. Nature immunology. 2013;14(3):262-270.

422. JL Brogdon, Leitenberg D, et al. The potency of TCR signaling differentially regulates NFATc/p activity and early IL-4 transcription in naive CD4+ T cells. The Journal of Immunology. 2002;168(8):3825-3832.

423. KD Cook, Miller J. TCR-dependent translational control of GATA-3 enhances Th2 differentiation. The Journal of Immunology. 2010;185(6):3209-3216.

424. T Meningher, Barsheshet Y, et al. Schistosomal extracellular vesicle-enclosed miRNAs modulate host T helper cell differentiation. EMBO Rep. 2020;21(1):e47882.

425. J Mendez, Sun D, et al. Bovine neutrophils form extracellular traps in response to the gastrointestinal parasite Ostertagia ostertagi. Sci Rep. 2018;8(1):17598.

426. M Bakshi, Hebert D, et al. Ostertagia ostertagi Mediates Early Host Immune Responses via Macrophage and Toll-Like Receptor Pathways. Infection and Immunity. 2021;89(6):e00017-21.

427. T Seya, Funami K, et al. Antibodies against human Toll-like receptors (TLRs): TLR distribution and localization in human dendritic cells. Journal of endotoxin research. 2005;11(6):369-374.

428. H Kumar, Kawai T, et al. Toll-like receptors and innate immunity. Biochem Biophys Res Commun. 2009;388(4):621-5.

429. N Mojtabavi, Dekan G, et al. Long-lived Th2 memory in experimental allergic asthma. The Journal of Immunology. 2002;169(9):4788-4796.

430. S Steinfelder, Rausch S, et al. Intestinal helminth infection induces highly functional resident memory CD4+ T cells in mice. European journal of immunology. 2017;47(2):353-363.

431. JA Lederer, Perez VL, et al. Cytokine transcriptional events during helper T cell subset differentiation. The Journal of experimental medicine. 1996;184(2):397-406.

432. JL Grogan, Mohrs M, et al. Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. Immunity. 2001;14(3):205-215.

433. RG Jenner, Townsend MJ, et al. The transcription factors T-bet and GATA-3 control alternative pathways of T-cell differentiation through a shared set of target genes. Proceedings of the National Academy of Sciences. 2009;106(42):17876-17881.

434. DJ Cousins, Lee TH, et al. Cytokine coexpression during human Th1/Th2 cell differentiation: direct evidence for coordinated expression of Th2 cytokines. The Journal of Immunology. 2002;169(5):2498-2506.

435. M Messi, Giacchetto I, et al. Memory and flexibility of cytokine gene expression as separable properties of human TH1 and TH2 lymphocytes. Nature immunology. 2003;4(1):78-86.

436. SP Collier, Henderson MA, et al. Regulation of the Th1 genomic locus from Ifng through Tmevpg1 by T-bet. The Journal of Immunology. 2014;193(8):3959-3965.

437. WE Paul. What determines Th2 differentiation, in vitro and in vivo? Immunology and cell biology. 2010;88(3):236-239.

438. A Taylor-Robinson, Phillips R. Expression of the IL-1 receptor discriminates Th2 from Th1 cloned CD4+ T cells specific for Plasmodium chabaudi. Immunology. 1994;81(2):216.

439. LA Greenbaum, Horowitz J, et al. Autocrine growth of CD4+ T cells. Differential effects of IL-1 on helper and inflammatory T cells. Journal of immunology (Baltimore, Md.: 1950). 1988;140(5):1555-1560.

440. AH Lichtman, Chin J, et al. Role of interleukin 1 in the activation of T lymphocytes. Proceedings of the National Academy of Sciences. 1988;85(24):9699-9703.

441. H Helmby, Grencis RK. Interleukin 1 plays a major role in the development of Th2-mediated immunity. European journal of immunology. 2004;34(12):3674-3681.

442. J Zhu, Guo L, et al. Growth factor independent-1 induced by IL-4 regulates Th2 cell proliferation. Immunity. 2002;16(5):733-744.

443. J Cote-Sierra, Foucras G, et al. Interleukin 2 plays a central role in Th2 differentiation. Proc Natl Acad Sci U S A. 2004;101(11):3880-5.

444. J Zhu, Jankovic D, et al. Gfi-1 plays an important role in IL-2-mediated Th2 cell expansion. Proceedings of the National Academy of Sciences. 2006;103(48):18214-18219.

445. H Yamane, Zhu J, et al. Independent roles for IL-2 and GATA-3 in stimulating naive CD4+ T cells to generate a Th2-inducing cytokine environment. The Journal of experimental medicine. 2005;202(6):793-804.

446. L Guo, Huang Y, et al. Innate immunological function of TH2 cells in vivo. Nature immunology. 2015;16(10):1051-1059.

447. AL Wurster, Rodgers VL, et al. Interleukin 21 is a T helper (Th) cell 2 cytokine that specifically inhibits the differentiation of naive Th cells into interferon gamma-producing Th1 cells. J Exp Med. 2002;196(7):969-77.

448. A Fröhlich, Marsland BJ, et al. IL-21 receptor signaling is integral to the development of Th2 effector responses in vivo. Blood. 2007;109(5):2023-31.

449. IV Davydov, Krammer PH, et al. Nuclear factor-IL6 activates the human IL-4 promoter in T cells. The Journal of Immunology. 1995;155(11):5273-5279.

450. J Park, Chung SW, et al. Up-regulation of interleukin-4 production via NF-AT/AP-1 activation in T cells by biochanin A, a phytoestrogen and its metabolites. Toxicol Appl Pharmacol. 2006;212(3):188-99.

451. Y Zhang, Zhang Y, et al. TH1/TH2 cell differentiation and molecular signals. T helper cell differentiation and their function. 2014:15-44.

452. J Zhu, Cote-Sierra J, et al. Stat5 activation plays a critical role in Th2 differentiation. Immunity. 2003;19(5):739-748.

453. W Liao, Schones DE, et al. Priming for T helper type 2 differentiation by interleukin 2–mediated induction of interleukin 4 receptor α-chain expression. Nature immunology. 2008;9(11):1288-1296.

454. J Zhu, Cote-Sierra J, et al. Stat5 activation plays a critical role in Th2 differentiation. Immunity. 2003;19(5):739-48.

455. VT Heussler, Eichhora M, et al. Cloning of a full-length cDNA encoding bovine interleukin 4 by the polymerase chain reaction. Gene. 1992;114(2):273-278.

456. T Prashanth, Reddy GR, et al. Cloning and expression of Bos indicus interleukin-4 in mammalian cells. Indian J Exp Biol. 2013;51(5):352-6.

457. S Olsen, Stevens M. Effects of recombinant human cytokines on mitogen-induced bovine peripheral blood mononuclear cell proliferation. Cytokine. 1993;5(5):498-505.

458. W Tuo, Estes DM, et al. Comparative effects of interleukin-12 and interleukin-4 on cytokine responses by antigen-stimulated memory CD4+ T cells of cattle: IL-12 enhances IFN-gamma production, whereas IL-4 has marginal effects on cytokine expression. Journal of Interferon & Cytokine Research. 1999;19(7):741-749.

459. P Parronchi, De Carli M, et al. IL-4 and IFN (alpha and gamma) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones. Journal of Immunology. 1992;149(9):2977-83.

460. TO Yarovinsky, Butler NS, et al. Early exposure to IL-4 stabilizes IL-4 mRNA in CD4+ T cells via RNA-binding protein HuR. The Journal of Immunology. 2006;177(7):4426-4435.

461. H Renz, Domenico J, et al. IL-4-dependent up-regulation of IL-4 receptor expression in murine T and B cells. Journal of Immunology. 1991;146(9):3049-55.

462. H Imrie, Williams DJ. Stimulation of bovine monocyte-derived macrophages with lipopolysaccharide, interferon-ɣ, Interleukin-4 or Interleukin-13 does not induce detectable changes in nitric oxide or arginase activity. BMC veterinary research. 2019;15(1):1-8.

463. A Nuntaprasert, Mori Y, et al. The effect of recombinant swine interleukin-4 on swine immune cells and on pro-inflammatory cytokine productions in pigs. Comparative immunology, microbiology and infectious diseases. 2005;28(2):83-101.

464. S Ebner, Hofer S, et al. A novel role for IL-3: human monocytes cultured in the presence of IL-3 and IL-4 differentiate into dendritic cells that produce less IL-12 and shift Th cell responses toward a Th2 cytokine pattern. Journal of Immunology. 2002;168(12):6199-207.

465. N Selliah, Finkel TH. Cutting edge: JAK3 activation and rescue of T cells from HIV gp120-induced unresponsiveness. The Journal of Immunology. 1998;160(12):5697-5701.

466. Q Liang, Guo L, et al. IL-2 and IL-4 Stimulate MEK1 Expression and Contribute to T Cell Resistance against Suppression by TGF-β and IL-10 in Asthma. The Journal of Immunology. 2010;185(10):5704-5713.

467. Y Ohkubo, Arima M, et al. A role for c-fos/activator protein 1 in B lymphocyte terminal differentiation. Journal of Immunology. 2005;174(12):7703-10.

468. LF Zhang, Okuma K, et al. Generation of mature dendritic cells with unique phenotype and function by in vitro short-term culture of human monocytes in the presence of interleukin-4 and interferon-beta. Exp Biol Med (Maywood). 2008;233(6):721-31.

469. M Dauer, Obermaier B, et al. Mature dendritic cells derived from human monocytes within 48 hours: a novel strategy for dendritic cell differentiation from blood precursors. Journal of Immunology. 2003;170(8):4069-76.

470. K Liedtke, Alter C, et al. Endogenous CD83 Expression in CD4(+) Conventional T Cells Controls Inflammatory Immune Responses. Journal of Immunology. 2020;204(12):3217-3226.

471. B Hock, Fernyhough L, et al. Release and clinical significance of soluble CD83 in chronic lymphocytic leukemia. Leukemia research. 2009;33(8):1089-1095.

472. RM Maizels, McSorley HJ. Regulation of the host immune system by helminth parasites. Journal of allergy and clinical immunology. 2016;138(3):666-675.

473. RM Maizels, Smits HH, et al. Modulation of host immunity by helminths: the expanding repertoire of parasite effector molecules. Immunity. 2018;49(5):801-818.

474. MD Taylor, van der Werf N, et al. T cells in helminth infection: the regulators and the regulated. Trends in immunology. 2012;33(4):181-189.

475. T Bouchery, Kyle R, et al. The differentiation of CD4+ T-helper cell subsets in the context of helminth parasite infection. Frontiers in immunology. 2014;5:487.

476. C Wiggin, Gibbs H. Studies of the immunomodulatory effects of low-level infection with Ostertagia ostertagi in calves. American journal of veterinary research. 1989;50(10):1764-1770.

477. C Wiggin, Gibbs H. Adverse immune reactions and the pathogenesis of Ostertagia ostertagi infections in calves. American journal of veterinary research. 1990;51(5):825-832.

478. T De Marez, Cox E, et al. Induction and suppression of lymphocyte proliferation by antigen extracts of Ostertagia ostertagi. Veterinary immunology and immunopathology. 1997;57(1-2):69-77.

479. M Hagberg, Lundén A, et al. Characterization of bovine lymphocytes stimulated in vitro by Dictyocaulus viviparus homogenate. Parasite Immunol. 2008;30(6-7):342-53.

480. J Campbell, Howie S, et al. Theileria annulata induces abberrant T cell activation in vitro and in vivo. Clinical & Experimental Immunology. 1995;99(2):203-210.

481. TN McNeilly, Nisbet AJ. Immune modulation by helminth parasites of ruminants: implications for vaccine development and host immune competence. Parasite. 2014;21:51.

482. GS Noland, Chowdhury DR, et al. Helminth infection impairs the immunogenicity of a Plasmodium falciparum DNA vaccine, but not irradiated sporozoites, in mice. Vaccine. 2010;28(17):2917-23.

483. HJ van der Zande, Zawistowska-Deniziak A, et al. Immune regulation of metabolic homeostasis by helminths and their molecules. Trends in parasitology. 2019;35(10):795-808.

484. RR White, Artavanis-Tsakonas K. How helminths use excretory secretory fractions to modulate dendritic cells. Virulence. 2012;3(7):668-677.

485. JK Nono, Ndlovu H, et al. Interleukin-4 receptor alpha is still required after Th2 polarization for the maintenance and the recall of protective immunity to Nematode infection. PLoS neglected tropical diseases. 2017;11(6):e0005675.

486. K Bian, Zhong M, et al. Helminth regulation of host IL-4Rα/Stat6 signaling: Mechanism underlying NOS-2 inhibition by Trichinella spiralis. Proceedings of the National Academy of Sciences. 2005;102(11):3936-3941.

487. S Babu, Blauvelt CP, et al. Cutting edge: diminished T cell TLR expression and function modulates the immune response in human filarial infection. The Journal of Immunology. 2006;176(7):3885-3889.

488. D Chen, Zhao Y, et al. Expression of TLR2, TLR3, TLR4, and TLR7 on pulmonary lymphocytes of Schistosoma japonicum-infected C57BL/6 mice. Innate immunity. 2019;25(4):224-234.

489. MA Pineda, McGrath MA, et al. The parasitic helminth product ES-62 suppresses pathogenesis in collagen-induced arthritis by targeting the interleukin-17–producing cellular network at multiple sites. Arthritis & Rheumatism. 2012;64(10):3168-3178.

490. D Schenten, Nish SA, et al. Signaling through the adaptor molecule MyD88 in CD4+ T cells is required to overcome suppression by regulatory T cells. Immunity. 2014;40(1):78-90.

491. Y Nouri, Weinkove R, et al. T-cell intrinsic Toll-like receptor signaling: implications for cancer immunotherapy and CAR T-cells. Journal for Immunotherapy of Cancer. 2021;9(11).

492. A Cottalorda, Verschelde C, et al. TLR2 engagement on CD8 T cells lowers the thresholdfor optimal antigen-induced T cell activation. European journal of immunology. 2006;36(7):1684-1693.

493. M Komai-Koma, Jones L, et al. TLR2 is expressed on activated T cells as a costimulatory receptor. Proceedings of the National Academy of Sciences. 2004;101(9):3029-3034.

494. AE Gelman, Zhang J, et al. Toll-like receptor ligands directly promote activated CD4+ T cell survival. The Journal of Immunology. 2004;172(10):6065-6073.

495. A Mansson, Adner M, et al. Toll-like receptors in cellular subsets of human tonsil T cells: altered expression during recurrent tonsillitis. Respiratory research. 2006;7(1):1-10.

496. AH Rahman, Taylor DK, et al. The contribution of direct TLR signaling to T cell responses. Immunologic research. 2009;45(1):25-36.

497. J Norimine, Suarez CE, et al. Immunodominant epitopes in Babesia bovis rhoptry-associated protein 1 that elicit memory CD4(+)-T-lymphocyte responses in B-bovis-immune individuals are located in the amino-terminal domain. Infection and Immunity. 2002;70(4):2039-2048.

498. WC Brown, Davis WC, et al. CD4+ T-cell clones obtained from cattle chronically infected with Fasciola hepatica and specific for adult worm antigen express both unrestricted and Th2 cytokine profiles. Infection and Immunity. 1994;62(3):818-27.

499. A Rosbottom, Guy C, et al. Peripheral immune responses in pregnant cattle following Neospora caninum infection. Parasite immunology. 2007;29(4):219-228.

500. E Muraille, Leo O. Revisiting the TH1/TH2 paradigm. Scandinavian journal of immunology. 1998;47(1):1-9.

501. S Rotmagnani. The Th1/Th2 paradigm and allergic disorders. Allergy. 1998;53:12-15.

502. A Hertweck, Vila de Mucha M, et al. The TH1 cell lineage-determining transcription factor T-bet suppresses TH2 gene expression by redistributing GATA3 away from TH2 genes. Nucleic Acids Research. 2022;50(8):4557-4573.

503. S Mariotti, Nisini R. Generation of human T cell clones. Methods Mol Biol. 2009;514:65-93.

504. H Takamatsu, Collen T, et al. Characterization of long-term cultured bovine CD4-positive and CD8-positive T-cell lines and clones. Immunology. 1990;69(2):293-7.

505. BM Goddeeris, Morrison WI, et al. Bovine cytotoxic T-cell clones specific for cells infected with the protozoan parasite Theileria parva: parasite strain specificity and class I major histocompatibility complex restriction. Proceedings of the National Academy of Sciences. 1986;83(14):5238-5242.

506. WC Brown, Woods VM, et al. Heterogeneity in cytokine profiles of Babesia bovis-specific bovine CD4+ T cells clones activated in vitro. Infect Immun. 1993;61(8):3273-81.

507. WC Brown, McElwain TF, et al. Bovine CD4+ T-lymphocyte clones specific for rhoptry-associated protein 1 of Babesia bigemina stimulate enhanced immunoglobulin G1 (IgG1) and IgG2 synthesis. Infection and Immunity. 1999;67(1):155-164.

508. WC Brown, Davis W, et al. CD4+ T-cell clones obtained from cattle chronically infected with Fasciola hepatica and specific for adult worm antigen express both unrestricted and Th2 cytokine profiles. Infection and Immunity. 1994;62(3):818-827.

509. WC Brown, Woods V, et al. Heterogeneity in cytokine profiles of Babesia bovis-specific bovine CD4+ T cells clones activated in vitro. Infection and Immunity. 1993;61(8):3273-3281.

510. CL Baldwin, Iams KP, et al. Theileria parva: CD4+ helper and cytotoxic T-cell clones react with a schizont-derived antigen associated with the surface of Theileria parva-infected lymphocytes. Experimental parasitology. 1992;75(1):19-30.

511. CL Baldwin, Goddeeris B, et al. Bovine helper T-cell clones specific for lymphocytes infected with Theileria parva (Muguga). Parasite immunology. 1987;9(4):499-513.

512. MA Neller, Lai MH-L, et al. High efficiency ex vivo cloning of antigen-specific human effector T cells. PLoS One. 2014;9(11):e110741.

513. W Tuo, Zarlenga D, et al. Repeated, drug-truncated infections with Ostertagia ostertagi elicit strong humoral and cell-mediated immune responses and confer partial protection in cattle. Veterinary parasitology. 2021;296:109510.

514. G Qu, Fetterer R, et al. Ostertagia ostertagi macrophage migration inhibitory factor is present in all developmental stages and may cross-regulate host functions through interaction with the host receptor. International journal for parasitology. 2014;44(6):355-367.

515. P Mendoza, Jackson JR, et al. Antigen-responsive CD4+ T cell clones contribute to the HIV-1 latent reservoir. Journal of Experimental Medicine. 2020;217(7).

516. S Nikiforow, Bottomly K, et al. Cytolytic CD4+-T-cell clones reactive to EBNA1 inhibit Epstein-Barr virus-induced B-cell proliferation. Journal of virology. 2003;77(22):12088-12104.

517. HM Long, Zuo J, et al. CD4+ T-cell clones recognizing human lymphoma-associated antigens: Generation by in vitro stimulation with autologous Epstein-Barr virus–transformed B cells. Blood, The Journal of the American Society of Hematology. 2009;114(4):807-815.

518. T De Marez, Cox E, et al. Identification of Ostertagia ostertagi specific cells in bovine abomasal lymph nodes. Veterinary immunology and immunopathology. 2000;73(2):145-154.

519. J Sprent, Surh CD. Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells. Nature immunology. 2011;12(6):478-484.

520. A Harari, Vallelian F, et al. Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. The Journal of Immunology. 2005;174(2):1037-1045.

521. E Glass, Spooner R. Generation and characterisation of bovine antigen-specific T cell lines. Journal of immunological methods. 1990;128(2):267-275.

522. D Vokurková, Sinkora J, et al. CD8+ natural killer cells have a potential of a sensitive and reliable biodosimetric marker in vitro. Physiol Res. 2006;55(6):689-698.

523. A Kandel, Masello M, et al. CD4+ T Cell Responses to Pathogens in Cattle. 2021.

524. H Zhao, Zhuang Y, et al. Effects of different doses of X-ray irradiation on cell apoptosis, cell cycle, DNA damage repair and glycolysis in HeLa cells. Oncology letters. 2019;17(1):42-54.

525. S AlmerÂõa, Canals A, et al. Characterization of protective immune responses in local lymphoid tissues after drug-attenuated infections with Ostertagia ostertagi in calves. 1998.

526. A Hada, Li L, et al. Characterization of Bovine Intraepithelial T Lymphocytes in the Gut. Pathogens. 2023;12(9):1173.

527. L Yang, Wang Y, et al. Changes in expression of Th1 and Th2 cytokines in bovine peripheral blood mononuclear cells during early pregnancy. Indian Journal of Animal Research. 2016;50(4):466-470.

528. E Sonnenberg-Riethmacher, Miehe M, et al. Periostin in allergy and inflammation. Frontiers in immunology. 2021;12:722170.

529. M Nakajima, Honda T, et al. Th2 cytokines efficiently stimulate periostin production in gingival fibroblasts but periostin does not induce an inflammatory response in gingival epithelial cells. Archives of oral biology. 2014;59(2):93-101.

530. G Takayama, Arima K, et al. Periostin: a novel component of subepithelial fibrosis of bronchial asthma downstream of IL-4 and IL-13 signals. Journal of allergy and clinical immunology. 2006;118(1):98-104.

531. I Striz, Mio T, et al. IL-4 induces ICAM-1 expression in human bronchial epithelial cells and potentiates TNF-α. American Journal of Physiology-Lung Cellular and Molecular Physiology. 1999;277(1):L58-L64.

532. TH Piela-Smith, Broketa G, et al. Regulation of ICAM-1 expression and function in human dermal fibroblasts by IL-4. Journal of immunology (Baltimore, Md.: 1950). 1992;148(5):1375-1381.

533. T Jinquan, Quan S, et al. Eotaxin activates T cells to chemotaxis and adhesion only if induced to express CCR3 by IL-2 together with IL-4. The Journal of Immunology. 1999;162(7):4285-4292.

534. P Valent, Bevec D, et al. Interleukin 4 promotes expression of mast cell ICAM-1 antigen. Proceedings of the National Academy of Sciences. 1991;88(8):3339-3342.

535. R Khattri, Auger JA, et al. Lymphoproliferative disorder in CTLA-4 knockout mice is characterized by CD28-regulated activation of Th2 responses. The Journal of Immunology. 1999;162(10):5784-5791.

536. T Kato, Nariuchi H. Polarization of naive CD4+ T cells toward the Th1 subset by CTLA-4 costimulation. The Journal of Immunology. 2000;164(7):3554-3562.

537. K Bratke, Goettsching H, et al. Interleukin-4 suppresses the cytotoxic potential of in vitro generated, adaptive regulatory CD4+ T cells by down-regulation of granzyme B. Immunology. 2009;127(3):338-344.

538. W-C Yang, Hwang Y-S, et al. Interleukin-4 supports the suppressive immune responses elicited by regulatory T cells. Frontiers in immunology. 2017;8:1508.

539. N Kienzle, Olver S, et al. Progressive differentiation and commitment of CD8+ T cells to a poorly cytolytic CD8low phenotype in the presence of IL-4. The Journal of Immunology. 2005;174(4):2021-2029.

540. C Riou, Dumont AR, et al. IL-4 influences the differentiation and the susceptibility to activation-induced cell death of human naive CD8+ T cells. International immunology. 2006;18(6):827-835.

541. T Mutis, Cornelisse YE, et al. Mycobacteria induce CD4+ T cells that are cytotoxic and display Th1-like cytokine secretion profile: heterogeneity in cytotoxic activity and cytokine secretion levels. European journal of immunology. 1993;23(9):2189-2195.

542. J Hao, Dong S, et al. Regulatory role of Vγ1 γδ T cells in tumor immunity through IL-4 production. Journal of Immunology. 2011;187(10):4979-86.

543. T Kawai, Seki M, et al. Selective diapedesis of Th1 cells induced by endothelial cell RANTES. The Journal of Immunology. 1999;163(6):3269-3278.

544. TJ Schall, Bacon K, et al. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. Nature. 1990;347(6294):669-671.

545. M Odaka, Matsukura S, et al. Differential regulation of chemokine expression by Th1 and Th2 cytokines and mechanisms of eotaxin/CCL-11 expression in human airway smooth muscle cells. International archives of allergy and immunology. 2007;143(Suppl. 1):84-88.

546. LM Teran, Mochizuki M, et al. Th1-and Th2-type cytokines regulate the expression and production of eotaxin and RANTES by human lung fibroblasts. American Journal of Respiratory Cell and Molecular Biology. 1999;20(4):777-786.

547. RD Butcher, McGarvie GM, et al. Recombinant interleukin-4 promotes expression of the CD25 (Tac) antigen at the plasma membrane of high-density human tonsillar B lymphocytes. Immunology. 1990;69(1):57-64.

548. C Bouffi, Rochman M, et al. IL-33 markedly activates murine eosinophils by an NF-κB-dependent mechanism differentially dependent upon an IL-4-driven autoinflammatory loop. Journal of Immunology. 2013;191(8):4317-25.

549. T Beyer, Herrmann M, et al. Bacterial carriers and virus-like-particles as antigen delivery devices: role of dendritic cells in antigen presentation. Current Drug Targets-Infectious Disorders. 2001;1(3):287-302.

550. P Brossart, Grünebach F, et al. Generation of functional human dendritic cells from adherent peripheral blood monocytes by CD40 ligation in the absence of granulocyte-macrophage colony-stimulating factor. Blood. 1998;92(11):4238-47.