REGULATORY B CELLS IN THE JEJUNAL PEYER'S PATCHES OF BOVINE AND SHEEP

A thesis submitted to

College of Graduate Studies and Research

in partial fulfilment of requirements for the

degree of Doctor of Philosophy in the

Vaccinology and Immunotherapeutics Program,

School of Public Health,

University of Saskatchewan

By

Steve Odera Jimbo

PERMISSION TO USE POSTGRADUATE THESIS

In presenting this thesis in partial fulfilment of the requirements for a postgraduate degree from the University of Saskatchewan, the author agrees that the libraries of this University may make it freely available for inspection. I further agree that permission for copying this thesis in any manner, in whole or in parts, for scholarly purpose may be granted by the professor or professors who supervised my thesis work. In their absence, permission may be granted from the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying, publication, or use of this thesis or part of it for financial gains shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in this thesis. Requests for permission to copy or to make others use of materials in this thesis in whole or in part should be addressed to:

Executive Director, School of Public Health,

Program Director, Vaccinology and Immunotherapeutics

University of Saskatchewan

107 Wiggins Road

Saskatoon, Saskatchewan, S7N 5E5

Canada

DEDICATION

To my Father and Mother

(Eliakim Odera Jimbo and Pamela Odera)

Who brought me into this world, instilled in me the value of education and unconditionally supported me throughout my life

And

To the love of my life, my wonderful wife, Jania (Nyar Tanga)

For her endless love and support

ABSTRACT

Toll-like receptors (TLRs) recognize microbial components as danger signals and induce immune responses. TLR's are expressed in many tissues of the host that are involved in immune responses including the intestines where they are abundantly expressed. This situation presents a challenge in the gastrointestinal tract which is constantly exposed to a wide variety of commensal organisms. Therefore, innate immune recognition in the intestine must be tightly regulated to prevent unwanted inflammation against harmless commensal micro-organisms and yet allow for the induction of protective immunity to invading pathogens. A dysregulation of this balance can result in intestinal inflammation.

Peyer's patches (PP) are the primary site for the induction of immune responses in the intestine and abundantly express TLRs. It is not known how PP regulate microbial signals from commensal bacteria and yet mount vigorous immune responses against dangerous pathogens. CpG DNA, an agonist for TLR9, can strongly activate immune cells in blood, lymph nodes and spleen. However, CpG very poorly activates immune cells from Peyer's patches, although these cells express TLR9 [1, 2]. Understanding how TLR responses are regulated in PP cells will unveil important information on how immune responses are regulated in the intestine.

Investigations from our laboratory have revealed a B cell population ($CD5^{-}CD11c^{-}CD21^{+}$) in PP that spontaneously secrete high levels of IL-10 which in turn down regulates TLR9 induced IFN and IL-12 production. These IL-10-secreting PP B cells represent a novel subset of the recently proposed regulatory B cells (B_{regs}) in the intestine [1, 3]. B_{regs}

may have a role in maintaining tolerance to commensal bacteria thereby achieving intestinal homeostasis.

The overall goal of the work described in this thesis was to improve our understanding of the immunobiology of B_{regs}. We performed several experiments to achieve this goal. First, we studied the development of regulatory B cells in lambs of different ages. Jejunal PP were collected from 3-4 month old, neonatal and fetal lambs and the production of IL-10 (the immunoregulatory cytokine secreted by B_{regs}) was assayed. We found that IL-10 was secreted by $CD21^+$ B cells from the PP in all the three age groups, confirming that B_{regs} develop prior to birth. We then wondered whether our $CD21^+$ B cells might be contaminated with other cells or activated when using MACS to enrich B cells. To address this issue, we prepared very highly purified $CD21^+$ B cell population using high speed cell sorting to negatively enrich for B cells. We also sorted DCs and assayed IL-10 production in both cell populations. Only the PP B cells spontaneously secreted IL-10. In contrast, dendritic cells, T cells, macrophages, neutrophils and NK cells did not secrete detectable IL-10.

Since B cells exist as regulatory and effector populations in mice, we wondered whether an effector B cell population also existed in ovine PP that secreted the pro-inflammatory cytokines IFN-α, IFN-γ and IL-12. Therefore, ovine PP B cells were fractionated into $CD72^+CD21^+$and $CD72^+CD21^-$ subpopulations to assess their capacity to secrete pro-inflammatory cytokines. Interestingly, the $CD72^+CD21^-$ B cell population secreted the cytokines IFN-α, IFN-γ and IL-12 suggesting there was an effector population.

We then surveyed for B_{regs} in different mucosal and peripheral tissues in sheep. We observed the B_{regs} frequency varied among the different lymphoid tissues. Finally, we investigated whether B_{regs} were present in PP of other ruminant species. We identified B_{regs} exist in PP of neonatal calves.

In conclusion, our investigations reveal that ovine B_{regs} develop in utero prior to antigen exposure, and are present in a variety of mucosal and peripheral tissues. We also report the novel observation that two distinct B cell sub-populations are present in ovine jejunal PP's: Regulatory and effector B cells.

ACKNOWLEDGEMENTS

First and foremost, I am indebted to my supervisor, Dr George Mutwiri, for giving me the opportunity to work on this project. George, thank you for your support, advice, criticism and encouragement throughout my entire thesis process, for pushing me when I needed it and for making the whole experience rewarding and enjoyable. You have been a true friend, brother, father and more than a mentor. As the African saying goes, “what an old man can see while sitting, a young man cannot see even while standing”. Thank you.

My gratitude also extends to members of my advisory committee, Drs Philip Griebel, Suresh Tikoo, Baljit Singh and Popi Havele for their valuable technical advice, constructive critique and for your willingness to undertake the arduous task of proof reading my thesis.

I would also like to thank Dr. Hugh Townsend for his suggestions and assistance with statistics. I wish to thank my colleagues at VIDO for their continued support. Specifically, Rahwa Osman, Teresia Maina and Lisanework Eshetu. I would also like to thank the many students and staff at VIDO and also at the School of Public Health especially the Vaccinology and Immunotherapeutics programme students who I have shared my experience with over the last few years. This includes the staff in the front office, the ‘techies’ and Donna and Sherri, who did all the hard work to keep the administration part of my work at VIDO running smoothly. Very special thanks to Natasa Arsic for her technical support and all the hours she put in performing and analysing my flow cytometry work.

Thank you Ken Lai and Donna Dent for your patience and technical help with my

laboratory work. My heartfelt thanks go to personnel at the animal care unit for always willing to collect samples. Thank you Jan. Your hard work and caring attitude is very much appreciated. A special word of appreciation for Mrs. Joyce Sander for her willingness to help, taking a keen interest in my studies and for all the advice and being the VIDO mother during the course of my study.

I would also like to thank Natural Sciences and Engineering Research Council of Canada and Vaccinology and Immunotherapeutics program, School of Public Health at the University of Saskatchewan for their financial support

Last but not least, this thesis would never have been completed without the constant encouragement and support of family. To my Canadian family, dad Stew, mum Julie and sisters Xanthe and Sky, I am thankful for the unconditional round the clock love and support. I would like to express my gratitude to my parents, Eliakim Odera Jimbo and Pamela Odera for bringing me to this world and for teaching me right from wrong, my siblings, Mike, Paul, Zeddy, Tom and Edda, and my in-laws, Papa Chilima and Mama Irina and sister Happy for their inestimable support throughout my study and for allowing me to marry their daughter.

To my wife Jania. Without your understanding and patience throughout this project, it may never have come to completion. Thank you for keeping me in check, for the steady food supply and for the constant ‘when will you be finished?’ question, and for your daily and lifelong dedication. I promise to make time for us now that I am done.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

a a	Amino acid
Ab	Antibody
AIS	Adaptive immune system
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APC	Antigen presenting cells
APRIL	A proliferation-inducing ligand
BAFF	B cell activating factor
BCIP/NBT	5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium
BCR	B cell receptor
bIFN-γ	Recombinant bovine IFN-γ
bIFN-α	Bovine IFN-α
Be cells	B-effector cells
B_{regs}	B regulatory cells
BSA	Bovine serum albumin
c.p.m	Counts per minute
cDC	Conventional DC
cDNA	Complimentary Deoxyribonucleic acid
ConA	Concavalin A
CpG	Cytosine phosphate Guanosine

Ct	Cycle threshold
DC	Dendritic cells
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dsRNA	Double stranded RNA
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylene di-amine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay
FACs	Fluorescence activated cell sorting
FAE	Follicle-associated epithelium
FBS	Fetal bovine serum
FO B cells	Follicular B cells
Foxp3	Forkhead box P3
GALT	Gut-associated lymphoid tissues
GC	Guanine Cytosine
GIPLs	Glycosylphosphatidylinositol lipids
GM-CSF	Granulocyte-macrophage colony-stimulating factor
G-ve	Gram-negative
HCl	Hydrochloric acid
HEPES	[N (-2-hydroxyethyl)] piperazine-n'-[2-ethane sulfonic acid]
hrs	Hours
IBD	Inflammatory bowel disease

ICAM-1	Inter-cellular adhesion molecule-1
IEC	Intestinal epithelial cells
IFN	Interferon
IgA	Immunoglobulin A
IIS	Innate immune system
IPP	Ileal Peyer’s patches
IRAK	IL-1R-associated kinase
ISC	IFN-α-secreting cells
IU	International unit
JPP	Jejunal PP
KCl	Potassium chloride
KO	Knock-out
LN	Lymph nodes
LPR	Lymphocyte Proliferative Responses
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LTA	Lipoteichoic acid
M cell	Microfold cell
MACS	Magnetic-activated cell sorting
MAL	MyD88-adaptor-like
MALT	Mucosal-associated lymphoid tissues
MAMP	Microbial-associated molecular pattern
MAPK	Mitogen-activated protein kinase

MDA5	Melanoma Differentiation-Associated protein 5
MEM	Minimum essential medium
MHC	Major Histocompatibility complex
MHC I	Major Histocompatibility complex I
MHC II	Major Histocompatibility complex II
MLN	Mesenteric lymph nodes
mM	Millimolar
Mφ	Macrophages
mRNA	Messenger Ribonucleic acid
MyD88	Myeloid differentiation primary response gene (88**)**
MZ	Marginal zone
NIH	National Institutes of Health
NK	Natural killer
NKT	Natural Killer T cell
NLR	Nucleotide-binding oligomerization domain (NOD) - like receptors
nM	Nanomole
NOD	Nucleotide-binding oligomerization domain
ODN	Oligodeoxynucleotides
Oligo dt	Oligodeoxythymidylic acid
ORN	Oligoribonucleotides
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate-buffered saline
PBSA	Phosphate-buffered saline calcium and magnesium free
PCR	Polymerase Chaín reaction
pDC	Plasmacytoid dendritic cells
PE	phycoerythrin
PKACa	cAMP dependent protein kinase alpha
Poly dI-dC	Polydeoxyinosinic-deoxycytidylic acid
Poly U	Poly (U) polymerase
PP	Peyer's patches
PRR	Pattern recognition receptors
qRT-PCR	Quantitative reverse transcriptase PCR
RA	Rheumatoid arthritis
rHuIL-12	Recombinant human IL-12
RLR	RIG-like receptors
RNA	Ribonucleic acid
RT	Reverse transcriptase
scLN	Superficial cervical lymph node
SED	Subepithelial dome
sIgM	Surface IgM
siRNA	Small interfering RNA
SIGGIRR	Single-immunoglobulin-IL-1R-related-molecule
ssRNA	Single stranded RNA
ST2	Suppression of tumorigenicity 2

TBST	Tris buffered-saline/0.01% Tween 20
TBST-g	TBST/0.5% gelatin
TCR	T cell receptor
TGFβ	Transforming growth factor beta
TICAM1	TIR domain containing adaptor protein inducing IFN-β
TICAM2	TRIF-related adaptor molecule
TIR	Toll/IL-1R
TIRAP (MAL)	MyD88-adaptor-like
TLR	Toll-like receptors
TOLLIP	Toll-interacting protein
Tr1	Type 1 regulatory T cells
TRAF	Tumour-necrosis factor receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAM (TICAM2)	TRIF-related adaptor molecule
T_{regs}	Regulatory T cells

CHAPTER 1: LITERATURE REVIEW

1.1 Overview of innate immunity

1.1.1 Definition

The innate immune system is the body's first line of defence against infection including both soluble factors and cells. Cells of the innate immune system express genetically encoded receptors called pattern recognition receptors (PRR), which can detect pathogen-associated molecular patterns (PAMPs) as danger signals [4]. The innate immune system includes a wide variety of immune cells, including dendritic cells (DCs), macrophages, neutrophils and natural killer (NK) cells.

1.1.2 Pathogen Associated Molecule Patterns (PAMPs)

PAMPs are distinct molecular motifs present on microbial organisms and not in the host. They are recognized by evolutionarily conserved germ-line encoded receptors known as pattern recognition receptors (PRR) which are expressed in host cells [4, 5]. Most PRR detect pathogens regardless of their life cycle with exceptions of ds RNA that are only present during viral replication. PRR and are present intracellularly or extracellularly on the cell surface. Different classes of PRR, such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), scavenger receptors, lectin receptors and DNA receptors (cytosolic sensors for DNA), have been described [4, 6]. These PRR are at the forefront of both extracellular and intracellular pathogen recognition and sense various classes of microbial molecules including proteins, lipids, carbohydrates and nucleic acids [7-10].

1.1.3 Toll Like Receptors (TLR)

TLR are a family of transmembrane receptors homologous to Drosophila toll proteins [6, 9, 11]. They are the most widely studied PRRs and are considered to be the primary sensors of pathogens. TLRs are type I trans-membrane glycoproteins and are made up of extracellular leucine rich repeats (LRRs) and a cytoplasmic Toll/ interleukin-1 receptor (TIR) domain. The LRRs are required for PAMP recognition, and the cytoplasmic Toll/ interleukin-1 receptor (TIR) domain is required for downstream signalling. To date, 13 members of the TLR family have been identified in mammals (10 in humans and 12 in mice), with each receptor recognizing a unique set of PAMP (Table 1)[12]. TLR1, 2, 4, 5, 6 and 10 are primarily expressed on the cell surface and recognize PAMPs derived from bacteria, fungi and protozoa, whereas TLR3, 7, 8 and 9 are exclusively expressed within endocytic compartments and primarily recognize nucleic acid PAMPs derived from various viruses and bacteria [6, 13]. Restricted access to these TLRs may be important to prevent aberrant activation by self-nucleic acids released from apoptotic/ necrotic cells, which could lead to autoimmune diseases.

Most TLRs signal via an adaptor protein called myeloid differentiation factor 88 (MyD88), except for TLR3, which signals through TIR domain-containing adaptor- inducing interferon-b (TRIF), and TLR4, which signals through both. Both MyD88 and TRIF pathways elicit transcriptional alterations through the activation of nuclear factor-k B ($NF_K\beta$) and other transcription factors [6].

Table 1. TLRs and their respective ligands

TLR	Agonist	Cells expressing TLR	Reference
TLR1	Triacyl lipopeptides	Mφ, cDC, neutrophils, B cells, mast cells, epithelial cells	[14]
TLR2	LTA, Zymosan, PamCSK4, MALP2, Lipoarabinomann	Mφ, cDC, neutrophils, mast cells, epithelial cells	[15]
TLR3	dsRNA, poly(I:C)	cDC, Mφ, endo/epithelial cells	[16]
TLR4	LPS, mannan, GIPLs, envelope proteins, Heat shock proteins	cDC, neutrophils, mast cells, eosinophils, epithelial cells	[17]
TLR5	Flagellin	Monocytes, cDC, IEC, epithelial cells	[18]
TLR6	Diacyl lipopetides, LTA, Zymosan	Monocytes, mast cells, cDC, neutrophils, neutrophils, eosinophils, pDC, epithelial cells	[19]
TLR7	ssRNA, Imiquimod,Resiquimod	Monocytes, cDC, mast cells, neutrophils, epithelial cells	[20]
TLR8	(R848), synthetic polyU RNA,	pDC, B cells, NK cells, eosinophils, neutrophils, epithelial cells	[21]

	siRNAs Resiquimod (R848), ssRNA		
TLR9	CpG DNA,	pDC, B cells, NK cells, eosinophils,	[22]
TLR10	hemozoin	neutrophils	[23]
TLR 11	Not known	pDC, B cells, DC	[24]
TRL 12	Profilin	Neurons, pDC, cDC, Mφ	[25, 26]
TLR 13	bacterial ribosomal RNA sequence "CGGAAAGACC"	monocytes/ Mφ	[27, 28]

Mφ – Macrophages, **cDC** - conventional DC, **pDC** - plasmacytoid dendritic cells, **IEC** - intestinal epithelial cells, **NK** - Natural Killer cells, **MALP2** - Macrophage Activating LipoPeptide-2, **GILPS** - glycoinositolphospholipids, **LPS** - Lipopolysaccharides, **PolyU RNA** - polyadenylation uridine ribonucleic acid, **LTA** – Lipoteichic acid, **dsRNA** - Double-stranded RNA, **PamCSK4** - N-palmit-oyl-S-[2-hydroxy-3-(palmitoyloxy)propyl]-(R)-cysteinyl-(lysyl)3-lysine and **Poly(IC)** - Polyinosinic:polycytidylic acid.

1.1.4 TLR9 and CpG

TLR9 recognizes unmethylated CpG motifs of bacterial DNA, as well as synthetic CpG oligodeoxynucleotides (ODNs) [29, 30]. TLR9 is also a sensor of DNA of viruses including HSV-1, HSV-2 and murine cytomegalovirus, and also recognizes the pigment hemozoin from Plasmodium falciparum [31-34]. CpG dinucleotides occur in the expected frequency (1 of 16)

in bacterial DNA, but have a suppressed frequency (1 of 64) in vertebrate DNA [35]. It is thought that the mammalian immune system has evolved to recognize CpG dinucleotides in microbial DNA as a danger signal. Several studies have shown that CpG oligodeoxynucleotides (ODN) are potent activators of the innate immune system in numerous species including humans, non-human primates, sheep, cattle, pigs, chickens, mice, horses, dogs, cats and fish [35-43]. CpG ODN has been shown to induce protective innate immune responses against a variety of pathogens including bacteria, viruses and protozoa in numerous animal models [44-48].

CpG activates a variety of cells including, B-lymphocytes, monocytes, macrophages, dendritic cells, NK cells and even mast cells. Activation of these cells with CpG ODN results in up-regulation of MHC class I and II, B7-1 and B7-2 co-stimulatory molecules, cell proliferation and also induces expression of a broad range of cytokines including IL-1, IL-6, IL10, IL-12, IFN-γ and TNF-α [49-52]. In humans, NK cells, eosinophils, neutrophils, plasmacytoid dendritic cells (pDCs) and B cells express TLR-9 [53]. Plasmacytoid dendritic cells (pDCs) and B cells are directly stimulated by CpG ODN to proliferate, produce cytokines and upregulate co-stimulatory molecules [22, 54, 55].

In-vitro and in-vivo studies have shown that in humans TLR-9 is expressed primarily by plasmacytoid dendritic cells (PDCs) and B cells [51]. These cells produce Th1 like pro-inflammatory cytokines, interferons, and chemokines. Krieg and colleagues have shown that certain CpG motifs (CpG-A) are especially potent at activating NK cells and inducing IFN-α production by PDCs, while other motifs (CpG-B) are especially potent B cell activators [51].

TLR signalling may play a role in the process of negative selection of B cells [56]. Meffre and colleagues found an increased ratio of auto-reactive B cells to naive B cells in patients deficient in MyD88 or IRAK4 (a kinase downstream of MyD88 signalling) compared to healthy controls, suggesting a defect in negative selection [57].

1.2 Mucosal Immunity

The mucosal immune system is at the interface between the internal and external environments and interacts with air, water, microbes and food antigens. It is made up of inductive and effector sites. Inductive sites are anatomically defined lymphoid compartments including Peyer's patches, the mesenteric lymph nodes, appendix and solitary follicles in the intestine, the tonsils and adenoids [29]. These inductive sites are where immune responses are initiated. Immune effector sites are the battlegrounds where neutralization of antigen and protection of the host against invading pathogens takes place. Effector sites are present in all mucosal tissues as disseminated lymphoid tissue diffusely distributed throughout the lamina propria [29].

The mucosal tissues constitute efficient barriers, preventing pathogens from gaining access to the host but they are also a major route of invasion by pathogenic microorganisms [58]. The mucosal immune system has three main functions; (i) to protect the mucosae against colonization and invasion by potentially pathogenic microbes, (ii) to prevent uptake of un-degraded antigens including foreign proteins derived from ingested food and commensal microorganisms, and (iii) to prevent the development of potentially harmful immune responses to harmless antigens [59].

1.2.1 Gut associated lymphoid tissues

In the intestine, a balance between immune responses to harmful (or dangerous) pathogens and tolerance to harmless antigens from commensals and food must be maintained to ensure homeostasis. When this balance is not met or is compromised, the outcome is disease conditions such as inflammatory bowel disease, food allergies and malignancies associated with inflammation [60].

The gut-associated lymphoid tissue (GALT) consists of both isolated and aggregated lymphoid follicles and is one of the largest lymphoid tissues, which is thought to contain up to 70% of the body's total lymphocyte population [61]. It is made up of mesenteric lymph nodes (MLN) and PP, and more diffusely scattered lymphocytes in the intestinal lamina propria (LP) and epithelium [62].

1.2.2 Peyer's patches

Peyer's patches (PP's) were first described in 1673 by Johann Conrad Peyer after whom they are named [63]. They are a group of sub-epithelial lymphoid follicles that are located throughout the small intestines [64]. Morphologically, PPs can be divided into three main sections: the follicular area, the inter-follicular area and the follicle-associated epithelium [65] The PP follicular areas in mice are made up of lymphoid follicles with a germinal center (GC) containing proliferating B-lymphocytes, follicular dendritic cells (FDCs) and macrophages. The follicle is surrounded by the corona, or sub-epithelial dome (SED) containing mixed-cells including B-cells, T-cells, macrophages and dendritic cells (DCs). In sheep, the follicle is surrounded by a capsule and lymphatic sinus [66].

The follicle-associated epithelium (FAE) separates the PP from the intestinal lumen. The FAE facilitates the movement and transport of bacteria and antigens from the lumen to the immune cells underneath it [67]. The FAE also acts as a barrier that prevents interactions between the antigens and the immune cells underneath the epithelial surface, therefore ensuring a state of tolerance. The Peyer's patches serves as an antigenic sampling site [67]. This process involves the complex interplay of a variety of mechanisms that aim to recognize luminal antigens, induce an immunological response and decrease the incidence of antigen translocation across the mucosal epithelium [67]. The majority of the PP are found in the jejunum and a single PP in the ileum [68].

1.2.2.1 Peyer's patches in ruminants

In ruminants, and some other species, there is evidence of two types of Peyer's patches (PPs) within the small intestine, the continuous or ileal PP, which extends 150 cm along the distal intestine, and the discrete or jejunal PPs distributed throughout the rest of the small intestine. These two types of PP differ significantly in their histology, ontogeny and the extent of lymphocyte traffic [69, 70]. Another intriguing difference is that the continuous PP involutes about the time of puberty whereas jejunal PPs persist throughout life [70].

1.2.2.2 Ileal Peyer's patches

In sheep and cattle, the ileal Peyer's patch (PP), is a primary lymphoid organ for B-cell development [71]. B-cell diversity in the ileal PP is thought to develop by combinatorial mechanisms, gene conversion and/or point mutation. During fetal development, Peyer's patches develop first in the jejunum and then the ileum. This happens independent of external

antigens [71]. In fetal lambs, lymphopoiesis in ileal and jejunal PP is not dependent on exogenous antigens [71]. B cells are generated in the ileal PP at a rate of 3.6 x 10^9 cells per hour in fetal ileal PP [72, 73]. However, very few of the B-cells (up to 5%) produced in the ileal PP differentiate and emigrate. Instead the vast majority of the B cells die by apoptosis [73]. During fetal development, the ileal PP is not detected until Day 110 of gestation [72]. Before birth, ileal PP have had no prior contact with exogenous antigen yet B cells proliferate at normal rates and this continues for the first two weeks after birth. From about 12 weeks after birth the IPP begins to involute and only a few PP follicles remain in this region of the intestine by 18 months of age [69, 74]. The ileal PP is made up of about 100,000 closely packed elongated follicles with small domes and little inter-follicular area [74].

1.2.2.3 Jejunal Peyer's patches

The JPP are the primary immune inductive sites in the intestine [69]. In sheep and cattle, discrete PPs are present as 30-40 lymphoid aggregates in the jejunum [74]. During fetal development, ovine JPP develop by Day 65 of the 145-day period of gestation. Each JPP contains approximately 100 pear-shaped follicles with very large domes that are well exposed to the gut lumen and adjacent lymphoid follicles are separated by abundant inter-follicular tissue [74]. Extensive lymphocyte trafficking occurs within the jejunal PP. Lymphocyte extravasation is substantially greater in jejunal PPs than in the ileal PP [73]. B cells account for the majority of cells (up to 70%) in the JPP whereas T cells account for about 10-30% of the cells ([75], [68, 76]). The JPP's have functional lymphopoietic capacity in utero, and reach their maximum size by 2-3 months after birth. The jejunal PP's, as opposed to the IPP that involutes at puberty, remains intact throughout life [70]. What is not known is if the jejunal

PP's function as a primary lymphoid organ in utero but then evolve after birth into a secondary organ.

1.2.2.4 Mechanisms of immune regulation in the intestine

The intestine represents one of the most challenging sites for the immune system as immune cells must be able to mount an efficient response to invading pathogens while tolerating the large number and diverse array of resident commensal bacteria and food antigens. Excessive immune responses lead to uncontrolled inflammation and disease [60]. Intestinal homeostasis is achieved, in part, by the integration of a complex set of mechanisms that eliminate pathogens and tolerates commensal microbiota. The bacterial load is particularly dense in the intestinal tract where trillions of bacteria composed of hundreds of different species reside and are separated from the underlying immune system by a monolayer of epithelial cells. Such an intimate relationship facilitates the dynamic cross talk between host and microbe that underlines the delicate balance between tolerance and immunity in the intestines. Compared to adaptive immunity, there is relatively less information available on how innate immunity is controlled in the intestine.

The mechanisms through which innate immune responses in the intestines maybe controlled include the following:

i) **Activities in the Intestinal epithelial cells (IEC):** Goblet cells produce a thick layer of mucus and paneth cells produce bactericidal proteins that separate the luminal contents from the host. IEC down regulate Toll like receptor expression to remain unresponsive to the vast bacterial load. On the other hand, commensal bacteria enhance innate

immunity to pathogens by providing tonic signals (PAMPS) that are required for optimal immune stimulation [77].

(ii) **Inhibition of TLR signals:** TLR signaling must be tightly regulated in the intestine to prevent chronic stimulation by commensal microorganisms. Negative regulation (inhibition) is achieved at multiple levels.

1) **Interruption of TLR interaction with their ligands**: This is achieved through decoy receptors that prevent a direct interaction between TLRs and their bacterial ligands [78]. For example, soluble TLR2 (sTLR2) could compete with TLR2 for microbial ligands and prevent the interaction between TLR2 and its ligand, and hence blocking TLR2 signaling. Soluble TLR2 are constitutively released by host blood monocytes and are naturally expressed in plasma and breast milk [79].
2) **Intracellular inhibitions:** Intracellular regulators can inhibit TLR signalling pathways. Some of the regulators are present constitutively to control TLR activation at a physiological level, whereas others are up-regulated by TLR signalling during infection to attenuate TLR response in a negative feedback loop. Some of these regulators include MyD88s, IRAKM, SOCS1, and NOD2 [80-83].
3) **Down-regulation of transcription and translation of TLR genes:** These occur through trans-membrane protein regulators for example the suppression of tumorigenicity 2 (ST2), Single-immunoglobulin-IL-1R-related-molecule (SIGGIRR) and TNF-related apoptosis-inducing ligand (TRAIL). The absence of these receptors leads to down-regulation of TLR genes by decreasing the nuclear translocation of NF-κB [84-87].

4) **Reduction of TLR protein:** This is achieved through degradation of TLRs through ubiquitylation or inhibition of TLR expression by anti-inflammatory cytokines such as Transforming Growth Factor β (TGF-β) and IL-10 [88, 89].
5) **Inhibition by apoptosis:** TLR-induced apoptosis might be important in the control of a dysregulated TLR response. It has been shown that interaction of TLR2 with microbial agonists triggered apoptosis in macrophages and epithelial cells. This interaction results in the recruitment of MyD88 and subsequent interaction with FAS-associated death domain (FADD) through its death domain. This allows the recruitment of caspase-8 to form the death-inducing signalling complex, which triggers the apoptotic cascade [90, 91].

(iii) Regulatory Cells: Different regulatory cells including regulatory T cells (T_{regs}), regulatory B cells (B_{regs}) and regulatory DCs have been identified and are thought to contribute to the regulation of immunity in the intestines. These cells exert their influence on other cells through the secretion of the anti-inflammatory cytokine, IL-10 and also by secreting TGFβ. Regulatory T (T_{reg}) cells are known to limit microbe-triggered intestinal inflammation [92]. Also, T_{regs} were shown to maintain intestinal homeostasis by suppressing γδ T cells in PDK1-deficient mice and prevented the development of colitis [93].

1.3 B lymphocyte Development

B cells develop from hematopoietic precursor cells in an ordered maturation and selection process. B cells originate from hematopoietic stem cells (HSCs), and undergo several developmental stages including early lymphoid progenitor, common lymphoid progenitor

(CLP), pro-B and pre-B cells within the bone marrow, and transitional B cells in the peripheral lymphoid organs, before developing into mature B cells [94].

Two schemes of B cell development have been described for different species. In the first scheme, immunoglobulin (Ig) locus rearranges early in ontogeny followed by a definitive pool of B cells that expand by a self-renewing mechanism, and they are mostly B1 type of B cells [95]. In the second scheme, the Ig repertoires are continuously generated from precursors in the bone marrow throughout life and these are composed of mostly B2 types of B cells. During B-cell development, the BCR promotes B-cell maturation while purging auto-reactive clones and sorting mature B cells into follicular I and II (FoBI and FoBII), marginal zone (MZ) and B1 B-cell populations [96]. Mature B cells further differentiate into effector, regulatory, memory and plasma cells [97]. The development of B cells begins in the bone marrow (BM) culminating in surface IgM (sIgM) expressing immature B cells, which then undergo further maturation in the spleen [97]. To safeguard against autoimmunity, immature B cells are subjected to central tolerance mechanisms in the bone marrow. [98]

B cells in mammals are composed of several subsets with different functionalities. In sheep, three major B cell subsets have been identified, namely the follicular B2 cells that are responsible for immune responses against thymus-dependent antigens that undergo germinal center reactions and generate high affinity antibodies with precise antigen specificities [95, 99]. The B1 and marginal zone (MZ) B cells respond to thymus-independent antigens and produce low affinity antibodies with broad re-activity [95, 99].

1.3.1 Activation and maturation of B lymphocytes

Bacterial DNA can trigger direct activation of B cells. In-vivo and in-vitro studies in mice have shown that purified B cells can be activated with microbial PAMPs [100]. When Kurt and colleagues (2000) stimulated B cells with TLR9 ligand, the level of proliferation was less than 2500 cpm but on activation through BCR, the level of proliferation increased to above 15000 cpm [100]. This suggests that BCR signaling may be required for significant activation of B cells through TLR9. Therefore, there is need for 2 signals for optimal CpG B cell activation. This is similar to the two signal model proposed by Bretscher & Cohn over 30 years ago and reaffirmed by Baumgarth and Janeway [101-103].

B cell activation is initiated by the ligation of the B cell receptor (BCR) with antigen and ultimately results in the production of protective antibodies against pathogens [104]. The earliest events in B cells activation include the initiation of BCR triggering, the formation of BCR micro-clusters, and the dynamic regulation of BCR signalling. Antigen engagement of BCR leads to a cascade of events that result in antigens within endosomes being processed, loaded onto the major histocompatibility complex (MHC), and presented on the B cell surface [105, 106]. This antigen presentation allows for the recruitment of cognate $CD4^+$ T helper cells to facilitate the complete activation of B cells through CD40 [105, 106].

1.3.2 Sheep PP B cells

Sheep PP B cells can be divided into two subsets based on cell size and surface expression of CD21 and CD72 molecules [107]. $CD21^+$ B cells, which are a representative of naive B cells, are localized in the B cell follicles of PPs, LNs and the spleen and are actively re-circulating

[107, 108]. We recently reported that within the PP $CD21^+$ B cell population there are cells that produce IL-10 and have a regulatory function [1].

CD72 is a 45-kDa type II membrane protein containing a C-type lectin like domain and includes other related cell surface molecules such as asialoglycoprotein receptors [109]. CD72 antigen is expressed on all cells of B cell lineage with the exception of plasma cells [110].

1.3.3 TLR9 in B cell activation

Expression of Toll-like receptors (TLRs) in B cells provides a cell-intrinsic mechanism for innate signals regulating adaptive immune responses [111]. In combination with other signalling pathways in B cells, including through the B-cell receptor (BCR), TLR signalling plays multiple roles in B-cell differentiation and activation [112]. B-cell-intrinsic TLR signalling regulates antibody responses, including germinal center formation and auto antibody production in autoimmune disease models [112].

TLR signalling also acts on the precursors of B cells, which could influence the immune response of animals by shaping the composition of the immune system. Transitional B cells can be subdivided into two separate subpopulations, termed transitional 1 (T1) and transitional 2 (T2) B cells, based on their surface phenotype and functional characteristics [113]. TLR activation in transitional B cells which is also the stage for negative selection and might change cell differentiation routes. Ueda et al (2007) have shown that T1 B cells could be activated by LPS or CpG to secrete IgM and class-switched Igs [114]. Similarly, it has been shown that stimulation of T1 and T2 B cells with CpG leads to secretion of pro-inflammatory cytokines

[115]. Carsetti and colleagues have recently shown that CpG induced human cord blood transitional B cells to differentiate into plasma cells with both class switching and somatic hyper-mutations [116].

1.3.4 Cytokine secreting B cells

There is substantial evidence that B cells do not act solely through their antibody secretion but also influence the course of immune responses through antibody-independent mechanisms. For example, B cells can be activated by TLR agonists to secrete pro-inflammatory or anti-inflammatory cytokines [51]. It has been proposed that B cells can be subdivided on the basis of the cytokines they produce. B lymphocytes that secrete cytokines can be subdivided in two different subsets: (i) regulatory B cells (B_{reg}), producing IL-10 or TGF, and (ii) Effector B cells (Be), which express distinct arrays of cytokines depending on their maturation stage and environment [3, 117]. B effector cells were initially classified as producing Th1 cytokines (Be1) or Th2 cytokines (Be2) [118, 119]. The Be1 cells secrete IL-12 and IFN-γ whereas Be2 secrete IL-4, IL-5, IL-6, IL-10 and IL-13 [9, 120]. Although it has long been known that B cells produce cytokines, such as IL-10, IL-6 and TNF-α, they also acquire the ability to express IL-2, IFN-γ, IL-4 and IL-12 when stimulated with antigen in the presence of effector Th1 cells [121, 122].

1.3.5 Regulatory B cells

The first evidence that B cells may exert suppressive activity came from early studies in guinea pigs in the mid-1970s. The transfer of total but not B cell-depleted splenocytes inhibited delayed-type hypersensitivity reactions [123]. Regulatory B cells secrete IL-10 or

TGFβ and are analogous to T_{regs} [3, 124]. Janeway and colleagues were the first to provide evidence for a direct regulatory role of B cells in the suppression of a T cell-mediated autoimmune reaction, using a mouse model (μMT – a strain genetically deficient in B cells) of experimentally induced autoimmune encephalomyelitis (EAE) [125, 126]. Furthermore, Fillatreau and colleagues demonstrated that while disease onset and severity were not affected by the μMT mutation, recovery was severely impaired in the absence of IL-10 producing B cells in IL-10 knockout mice [127]. Subsequently, Mizoguchi and colleagues coined the term regulatory B cells (B_{regs}) to designate B cells with regulatory properties, independent of secreted immunoglobulins [128]. These B_{regs} exert their regulatory activities through IL-10 production. While studying spontaneous colitis that develops in TCR knockout mice, Mizoguchi and Bhan also demonstrated that IL-10-producing B cells were responsible for suppressing the T cell-mediated progression of chronic intestinal inflammation [122, 129]. Mann et al (2012) have recently confirmed that a regulatory B cell population plays an essential role in the spontaneous recovery from EAE, an experimental disease model for human multiple sclerosis [130]. Furthermore B_{regs} can have both protective and pathogenic roles as seen in a mouse model of collagen induced arthritis and EAE [122]. The negative role of B cells in down modulating immune responses has then been demonstrated in several murine models, but mainly in autoimmune encephalomyelitis [125, 127, 131].

Regulatory B cells that produce IL-10 are now recognized as an important component of the immune system. However, other cells have been implicated in the production of IL-10 in the intestine, including, follicular DCs and T_{regs} [122]. The identification of regulatory T cells (Tr1, Tr3, $TCD4^{+}CD25^{+}FoxP3^{+}$) suggested the existence of regulatory B cells (B_{regs}). Several

studies have also shown B_{regs} to have a negative role in immune interactions for example a recent study has shown that B_{reg} cell dysfunction contributes pivotally to the progression of adipose tissue inflammation in obesity in mice [132]. B_{regs} are also potent negative regulators of antigen-specific inflammation and T-cell-dependent autoimmune diseases in mice [133-135].

1.3.6 Peyer's patches regulatory B cells

Recently, a novel regulatory B cell that secretes IL-10 and does not secrete IL-12, IFN-α, IFN-γ and IgM and does not proliferate when stimulated with CpG ODN was discovered in sheep Peyer's patches [1, 76]. In chapter 3 of this thesis, we show that these PP B_{regs} develop in utero prior to antigen exposure [76]. In subsequent chapters of this thesis, we show that B_{regs} are present in various tissues and we also show that these B_{regs} are present in the jejunal PP of newborn cattle.

CHAPTER 2: RATIONALE, HYPOTHESIS, OVERALL OBJECTIVE AND AIMS.

2.1 Rationale and hypothesis

The innate immune system uses a variety of germ-line encoded receptors collectively referred to as pathogen recognition receptors (PRR) to detect the presence of microbial agents. The best known of these are Toll-like receptors (TLRs) which play a critical role in the initiation, development and regulation of innate and adaptive immune responses. The gastrointestinal tract expresses these receptors and is constantly exposed to diverse commensal microbiome. Innate immune recognition in the intestine must be well regulated to prevent unwanted inflammation against harmless food antigens and commensal bacteria and yet allow the induction of protective immunity to pathogens. A dysregulation of this balance results in abnormal immune responses leading to intestinal inflammation. Peyer's patches (PP) are the primary site for the induction of immune responses in the intestine and cells in PPs express TLRs. Since the intestine harbours many commensal organisms, it therefore makes sense that TLR responses be appropriately regulated in this tissue.

Regulatory B cells (B_{regs}) are known to regulate immune responses through the secretion of the cytokine, IL-10. B_{regs} have recently been described in jejunal PP of sheep [1]. However it is not known when B_{regs} develop, whether they exist in other lymphoid tissues or whether they exist in other ruminant species.

Hypothesis:

1. B_{regs} in PP develop prior to birth and are distributed in variety of mucosal and systemic lymphoid tissues of ruminants.
2. B cells in PP exist in two distinct populations as Regulatory and Effector B cells.

2.2 Overall Objective

Our overall objective was to characterize regulatory B cells (B_{regs}) by addressing the following specific Aims

2.3 Aims

1. Investigate B_{regs} development in fetal and neonatal lambs.
2. Define functional subsets of B cell populations in PP (Regulatory vs Effector B cells)
3. Determine whether B_{regs} expressing surface IgM^+, IgA^+ and $IgG1^+$ secrete IL-10.
4. Survey distribution of B_{regs} in mucosal and systemic lymphoid tissues.
5. Examine whether B_{regs} exist in other ruminant species.

CHAPTER 3: IL-10 SECRETING CD21+ B CELLS ARE PRESENT IN SHEEP JEJUNAL PEYER'S PATCHES DURING FETAL DEVELOPMENT

(Article published in Cell and Tissue Research, 2014 [76])

Relationship of this study to the dissertation.

Regulatory B cells (B_{regs}) are known to regulate immune responses through the secretion of the cytokine, IL-10. Studies in our laboratory have recently described B_{regs} in jejunal PP of sheep [1]. However it is not known when B_{regs} develop in sheep. We therefore sought to study the development of $CD21^+$ regulatory B cells in Peyer's patches of sheep. These studies suggest that B_{regs} develop in utero prior to birth and are present in young lambs.

3.1 Abstract

We recently reported a novel IL-10-secreting $CD21^+$ B cell population in jejunal Peyer's patches (PP) of sheep with a regulatory function (B_{regs}) which suppresses TLR9-induced cytokine responses. However, little is known about the development of these cells. Therefore, we investigated the existence of these IL-10-secreting B_{regs} in jejunal PP cells from fetal and newborn lambs. $CD21^+$ B cells were purified from jejunal PP cells by magnetic cell sorting and subsequently stimulated with the TLR9-agonist, CpG ODN (CpG oligodeoxynucleotide). Lymphocyte proliferative responses, cytokine production (IL-10, IL-12 and INF γ), and antibody secretion were assayed. We observed that fetal and neonatal $CD21^+$ B cells spontaneously secreted high levels of IL-10 regardless of CpG stimulation, but these cells did not produce any IL-12 or IFN-γ upon stimulation with CpG. These observed responses are consistent with those previously reported for B_{regs} characterized in PP of older lambs. Surprisingly, unlike in older lambs, fetal and neonatal PP $CD21^+$ B cells proliferated in

response to CpG stimulation. Our investigations of fetal and neonatal lambs provide evidence for the development of IL-10-secreting $CD21^+$ B cells in Peyer's patches prior to antigen exposure.

Key words: Immune regulation; regulatory cells; B_{regs}; Peyer's patches; TLR9; CpG ODN; sheep.

3.2 Introduction

B cells can be divided into two classes according to their functional characteristics; the effector and regulatory B cells. Effector B cells are positive regulators of immune responses and can be further subdivided into two distinct cytokine-secreting subsets, B-effector-1 cells (Be1) and B-effector-2 (Be2) [9, 136]. The Be1 cells produce a Th-1-like cytokine pattern (IL-12 and IFN-γ) and Be2 produce a Th-2-like cytokine pattern (IL-4, IL-5, IL-6, IL-10 and IL-13 [9, 120]. These cells function as antibody producing cells, present antigens to T cells and modulate T cell-mediated responses [137]. These cells terminally differentiate into plasma cells that are tasked with the production of different antibodies [138].

B cells with regulatory functions have been recently described. Janeway and colleagues were the first to provide evidence for a direct regulatory role of B cells in the suppression of a T cell-mediated autoimmune reaction, using a mouse model (μMT – a strain genetically deficient in B cells) of experimentally induced autoimmune encephalomyelitis (EAE) [125, 126]. Furthermore, Fillatreau and colleagues demonstrated that while disease onset and severity were not affected by the μMT mutation, recovery was severely impaired in the absence of IL-10 producing B cells in IL-10 knockout mice [127]. Subsequently, Mizoguchi and colleagues coined the term *regulatory B cells* (B_{regs}) to designate B cells with regulatory properties independent of secreted immunoglobulins [128]. These B_{regs} exert their regulatory activities through IL-10 production. While studying spontaneous colitis that develops in TCR knockout mice, Mizoguchi and Bhan also demonstrated that IL-10-producing B cells were responsible for suppressing the T cell-mediated progression of chronic intestinal inflammation [122, 129]. Mann et al (2012) have recently confirmed that a

regulatory B cell population plays an essential role in the spontaneous recovery from EAE, an experimental disease model for human multiple sclerosis [130]. Furthermore, B_{reg} cells can have both protective and pathogenic roles as seen in a mouse model of collagen induced arthritis and EAE [122] . The species of choice for studying these B_{reg} cells has been mice. However, in mice B_{regs} only exist in disease models or are induced but do not occur spontaneously.

Recent investigations in our laboratory with sheep have identified a novel B cell population in jejunal Peyer's patches with regulatory function (B_{regs}) that spontaneously produce IL-10 and suppresses TLR9-induced IFN-γ and IL-12 cytokine responses [1]. This B cell population is $CD5^-$, $CD11c^-$ and $CD21^+$ [1]. The difference between B_{regs} described by others and the B_{regs} described by our group is that sheep Peyer's patch B_{regs} exist in a normal disease-free condition and spontaneously secretes high levels of IL-10, while the B_{regs} reported by others only occur during intestinal inflammation or colitis [128]. However, little is known regarding the development of B_{regs} in sheep and other species. Since, the intestine is subjected to many changes after birth due to exposure to dietary antigens and colonization by the commensal flora, we investigated whether post-natal changes in the intestine influenced B_{regs} development and function in the JPP. Here, we describe investigations regarding the ontogeny of IL-10 producing B cells. In particular, we determined when sheep Peyer's patch B_{regs} develop and whether there are any differences between fetal, neonatal and adult sheep Peyer's patch IL-10- producing B cells.

3.3. Materials and methods

3.3.1 Animals

Suffolk sheep of either sex were obtained from the Department of Animal and Poultry Science (University of Saskatchewan, Saskatoon, SK, Canada). 3rd trimester foetuses, newborn (1-4 days old) and 2-4 months old lambs were used in these studies. Experiments were conducted according to the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care. All experimental protocols were approved by the University of Saskatchewan Animal Care Committee.

3.3.2 CpG Oligodeoxynucleotides

Oligodeoxynucleotides of different classes have been shown to be biologically active in sheep both *in vitro* and *in vivo*. We used B-class CpG ODN 2007 to stimulate cells in our experiments. The CpG ODN 2007 and CpG ODN 2007 GC were obtained from Merial Limited (Lyon, France). They have the following sequences and backbone structures; 2007 B tcgtcgttgtcgttttgtcgtt and 2007GC B tgctgcttgtgcttttgtgctt. The ODN doses were previously optimized and used at a dose of 5ug/ml [1, 139].

3.3.3 Enzyme-linked immunosorbent assay (ELISA) for IFN-γ, IL-12 and IL-10

Polystyrene microtiter plates were coated with capture antibody as follows; for IL-10, Mouse anti-recombinant bovine IL-10 CC318 (Serotec MCA 2110) was diluted to 0.5 μg/mL in coating buffer, for IL-12, mouse anti-recombinant bovine IL-12 antibody (Serotec MCA 1782EL, NC, USA) was diluted to 8 μg/mL in coating buffer. For the IFN-γ ELISA, mouse anti-bovine IFN-γ antibody (clone 2-2-1A) was used in coating plates. Plates were then

incubated overnight at 4°c in the refrigerator. Plates were then washed four times with Tris buffered saline /0.05% Tween 20 (Sigma-Aldrich) (TBST). Recombinant bovine IL-10 [140], recombinant bovine IFN-γ (bIFN-γ) and recombinant human IL-12 (rHuIL-12) (Serotec PHP 100) were used as standards to measure cytokine concentration. Standards and samples were diluted in TBST/0.5% gelatin (Sigma-Aldrich) (TBST-g) and added to the wells at 2 fold dilutions. To detect bound cytokine, Mouse anti bovine IL-10 CC320 biotin (Serotec MCA 2111B) for IL-10, rabbit anti-bovine IFN-γ antisera followed by biotinylated goat anti-rabbit IgG (Zymed, South San Fransisco, CA, USA) for IFN-γ and mouse anti-bovine IL-12 CC326 biotin (Serotec MCA 2173B) were added. Next, streptavidin-alkaline phosphatase (GibcoBRL) was used for detection of the bound biotinylated antibodies. The assay was then developed by using p-nitrophenyl phosphate (Sigma-Aldrich) substrate in 1 % diethanolamine (Sigma-Aldrich) and 0.5 mg/mL magnesium chloride. The reaction was stopped by adding EDTA to each well. Optical density of the reaction product was measured at 405 nm using a 490 nm reference on a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The sample concentrations were calculated using Microplate Manager 5.0.1 version software (Bio-Rad). U/ml (units per ml) for IL-10 was defined previously by Kwong et al [140], as the biological activity of IL-10 with one unit being the reciprocal of the IL-10 dilution that inhibited IFN-γ secretion by 50 % of Cos-7 cells.

3.3.4 Isolation of JPP cells

Lambs were euthanised and JPP tissues immediately removed and placed in ice-cold DMEM (GibcoBRL) containing antibiotics (100 U/mL), Penicillin, 100 µg/mL Streptomycin

sulphate and 0.25 μg/mL Amphotericin B (Sigma-Aldrich). JPP cells were isolated from lambs as described previously [141]. The number of viable cells isolated from all tissues was determined by trypan blue dye exclusion and viable cells were counted with a hemocytometer under a light microscope. Cells were re-suspended at the appropriate concentrations in AIM V medium, (Medium supplemented with 2% FBS, 100U/mL penicillin, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B, 2Mm L-glutamine, 50 μM 2-mercaptoethanol and 10 μg/mL polymyxin B sulfate (Sigma- Aldrich) and added to round bottomed 96-well plates (Nunc, Naperville, IL, USA).

3.3.5 Magnetic activated cell sorting (MACS)

JPP cells were stained with mouse anti-bovine CD21 (IgG1 isotype; AbD Serotec, UK) for 15 min at 4^0C. The JPP cells were then washed twice with MACS buffer (PBSA, 0.5M EDTA and 10% BSA) which was followed by centrifugation for 8 min at 440 x g. The JPP cells were then stained with goat anti-mouse IgG1 phycoerythrin (PE) conjugate (ABD Serotec, UK) for 15 min at 4^0C before washing as above. The JPP cells were then labelled with anti-PE magnetic beads for 15 min at 4^0C and passed through the LC MACS column (Miltenyi Biotec, Bergish Gladbach, Germany) according to manufacturer's instruction. The $CD21^+$ B cell fraction was eluted, washed in PBSA and re-suspended in AIM V medium. Cell purity was assessed by flow cytometry and was consistently greater than 98%.

3.3.6 Tissue culture conditions and stimulation with TLR agonists

Isolated $CD21^+$ cells were re-suspended in AIM V medium. Aliquots of $5x10^5$ cells for $CD21^+$ and 3×10^5 for $CD21^-$ cells were cultured in triplicate wells in a final volume of 200 μL. Cells were stimulated with TLR9 agonists B-class CpG 2007 or GpC 2007 as a control

at 5 μg/mL and then incubated at 37°C in a 5% CO_2 atmosphere and 95% humidity. For optimal detection of cytokines, cells were stimulated for 48 hrs as previously described [141]. Culture supernatants were stored at -20°C until assayed for secreted cytokines.

3.3.7 Lymphocyte Proliferative responses

$CD21^+$ cells isolated by MACS were plated in triplicate cultures at 2.5 x 10^5 cells per well in a final volume of 200 μL. The cells were stimulated and incubated as described above before pulsing each well with 0.4 μCi ^{3}H-Thymidine (Amersham Pharmacia, Piscataway, NJ) during the final 6 hrs of the 72 hrs incubation. Cells were then harvested using standard liquid scintillation protocols. The uptake of ^{3}H-Thymidine was assessed in a beta counter (Topcount, Packard Instrument Company, Meriden, CT). The proliferation of the cells was calculated as the mean counts per minute (cpm) of triplicate cultures and expressed as a stimulation index (cpm in the presence of stimulus/cpm in the absence of stimulus).

3.3.8 Statistical analysis

Data analysis was performed with the statistical software program, Graph Prism 5 (Graphpad software). Data that were not normally distributed were transformed by ranking. Individual group differences were examined by performing one way analysis of variance (ANOVA). Values of $p<0.05$ and $p<0.01$, were considered significant and very significant respectively. Kruskal-Wallis test and Dunn's test was used to perform the post-hoc tests.

3.4 RESULTS

3.4.1 IL-10 production

Previous investigations in our laboratory have shown that a regulatory B cell (B_{regs}) population exists in jejunal PPs of 3-4 month old lambs and when cultured these B_{regs} spontaneously secrete high levels of IL-10 [1]. However, it is not known when these B_{regs} first develop and whether they are present in fetal or neonatal lambs. We therefore carried out experiments to determine whether B_{reg} activity could be detected in fetal and neonatal lambs and to confirm our earlier observations with 3-4 month old lambs.

IL-10 production was assayed in culture supernatants from cells isolated from JPP tissues of 3-4 month old lambs. Spontaneous IL-10 secretion by the total JPP cell population was increased following stimulation with CpG (Fig 1a). Similarly, enriched JPP $CD21^+$ B cells spontaneously secreted IL-10, but IL-10 secretion was not significantly increased following CpG ODN stimulation (Fig 1a). There was, however, no IL-10 secretion by $CD21^-$ cells, with or without CpG stimulation. These results are consistent with our previous observations for PP cells isolated from this age group of lambs [1].

We then investigated IL-10 secretion by PP cells isolated from neonatal lambs (1-4 days old). Both total JPP cells and enriched $CD21^+$ B cells spontaneously produced IL-10 and CpG stimulation increased IL-10 secretion by these two cell populations. Thus, $CD21^+$ B cells secreted IL-10 regardless of stimulation with CpG (Fig 1b). Again, IL-10 secretion was not detected in either the stimulated or unstimulated $CD21^-$ cells.

Similar results were observed following the isolation of jejunal PP cells from third trimester fetal lambs. Unstimulated JPP cells and enriched $CD21^+$ B cells both secreted IL-10 spontaneously and stimulation with CpG ODN did not significantly increase IL-10 secretion (Fig. 1c). Furthermore, no IL-10 secretion was detected in the fetal jejunal PP $CD21^-$ cells (Fig.1c). Therefore, unstimulated $CD21^+$ B cells from fetal, neonatal and 3 month old lambs all secrete IL-10, indicating that IL-10 secreting $CD21^+$ B cells develop prior to birth.

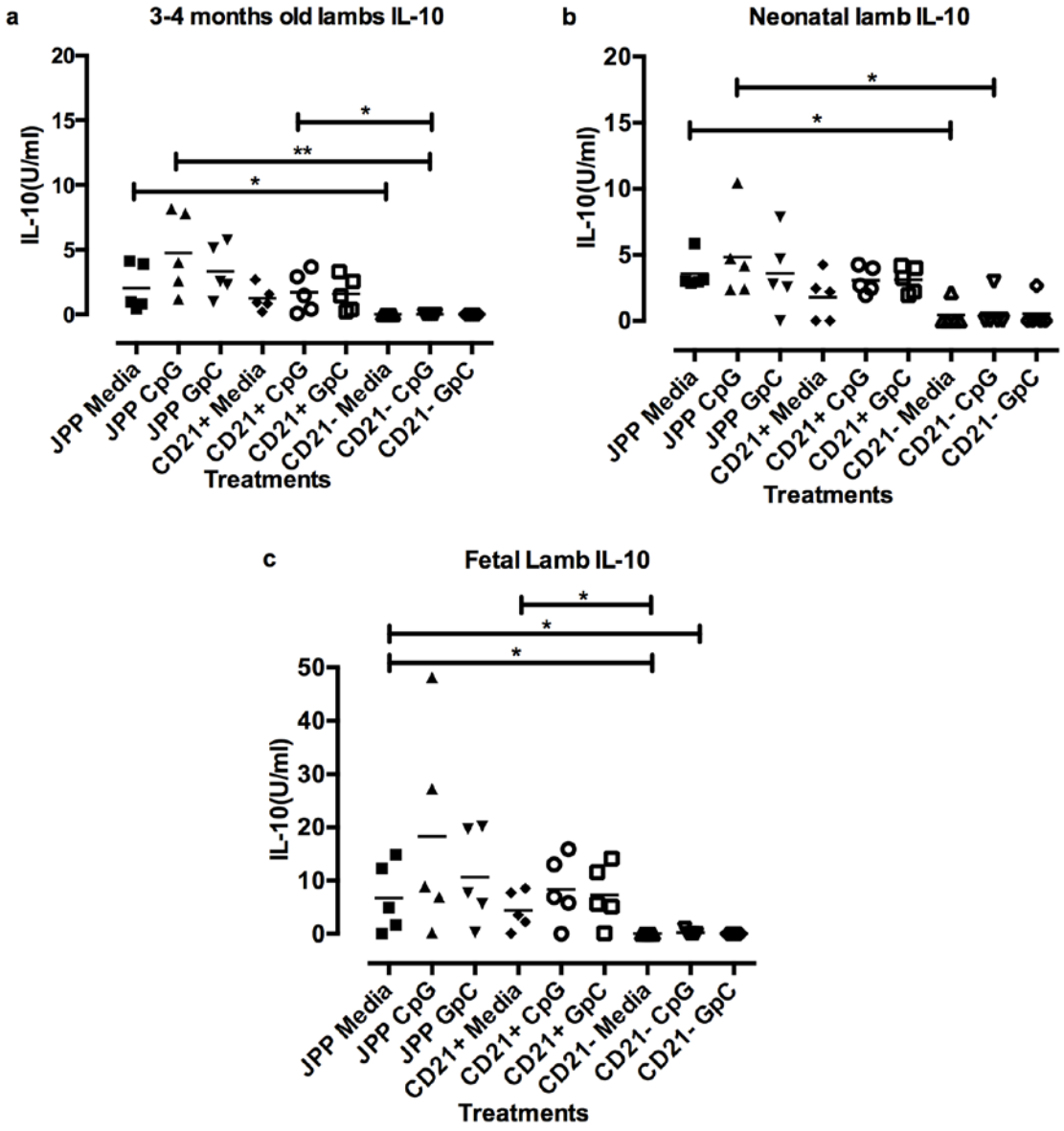


Figure 1. Interleukin (IL)-10 production in JPP cells from adult (a), neonatal (b) and foetal lambs (c). Unfractionated jejunal PP (JPP), JPP $CD21^+$ B cells and JPP $CD21^-$ cells were cultured for 48 hours in medium alone, or in the presence of 5 µg/ml of CpG or GpC ODN. IL-10 production was evaluated in the culture supernatants by IL-10 ELISA. Data for individual animals are presented with mean value indicated by horizontal bar for each group (*n*=5).

3.4.2 IL-12 production

In our previous investigation, B_{regs} from PP from sheep were characterised by the production of IL-10 but not IL-12 or IFN-γ [1]. Therefore, we evaluated the production of the cytokine, IL-12 in $CD21^+$ B cells. In 3-4 month old lambs, there were low levels of spontaneous IL-12 secretion by JPP cells (Fig. 2a) but these levels did not increase significantly following CpG ODN stimulation. There was no detectable IL-12 secretion by the enriched $CD21^+$ B cells with or without CpG ODN stimulation which is consistent with previous observations [1]. In contrast, $CD21^-$ cells increased IL-12 secretion following CpG ODN stimulation but this increase was not significant (Fig. 2a).

Significant IL-12 secretion was also detected in both neonatal JPP cells and $CD21^-$ populations but IL-12 secretion was not significantly changed by CpG ODN stimulation (Fig. 2b). No IL-12 secretion was detected in the enriched $CD21^+$ B cell population, with or without CpG ODN stimulation.

JPP cells isolated from fetal lambs spontaneously secreted IL-12 and the IL-12 secretion was again increased following CpG stimulation. Enriched $CD21^+$ B cells did not secrete detectable IL-12, with or without CpG ODN stimulation. In contrast, $CD21^-$ PP cells spontaneously secreted low but detectable levels of IL-12 and this response increased significantly following CpG ODN stimulation (Fig. 2c). Therefore, $CD21^+$ B cells from fetal, neonatal and 3-4 month old lambs do not secrete IL-12, with or without CpG ODN stimulation.

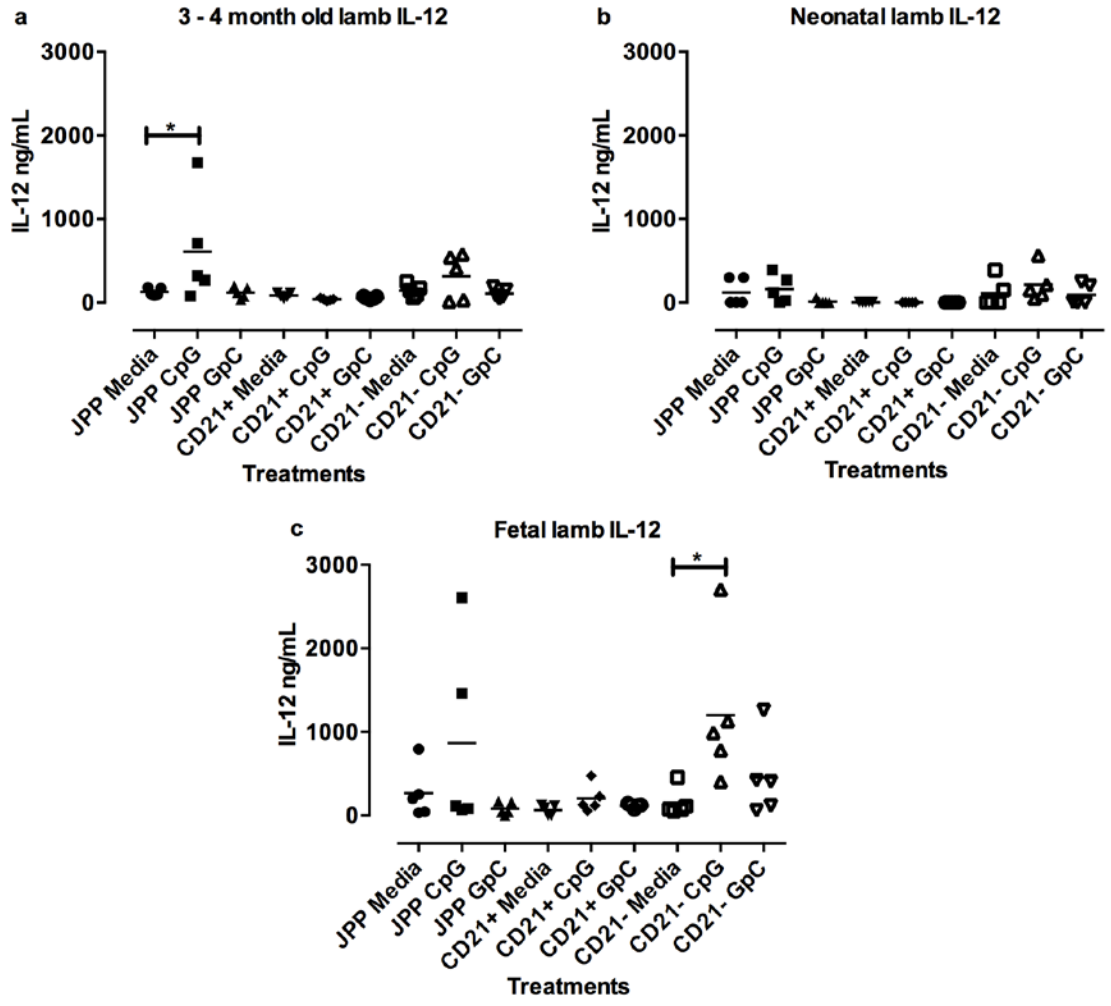


Figure 2. Interleukin (IL)-12 production in JPP cells from adult (a), neonatal (b) and foetal lambs (c). Total jejunal PP (JPP), JPP $CD21^+$ B cells and JPP $CD21^-$ cells were cultured for 48 hours in medium alone, or in the presence of 5 μg/ml of CpG or GpC ODN. IL-12 production was evaluated in the culture supernatants by IL-12 ELISA. Data for individual animals are presented with mean value indicated by horizontal bar for each group (*n*=5).

3.4.3 IFN-γ production

In 3-4 month old lambs, there were low levels of IFN-γ secretion by the unstimulated total JPP population (Fig. 3a). These levels did not significantly change following stimulation with CpG. There was no IFN-γ secretion in the enriched $CD21^+$ B cell population even after stimulation with CpG ODN, which is consistent with our previous observations [1]. There was significant production of IFN-γ in the $CD21^-$ population though these levels did not increase following stimulation with CpG (Fig. 3a).

In neonatal lambs, significant IFN-γ was detected in the total JPP and the $CD21^-$ populations and this response was increased on stimulation with CpG (Fig. 3b). However there was no IFN-γ production in the $CD21^+$ population even after CpG stimulation.

In the fetal lambs, there was essentially no production of IFN-γ in the $CD21^+$ B cells population (Fig. 3c). In contrast, there was significant IFN-γ production by the unstimulated $CD21^-$ cell fraction and this improved after stimulation with CpG. Surprisingly, no IFN-γ was detected in the total JPP cell population.

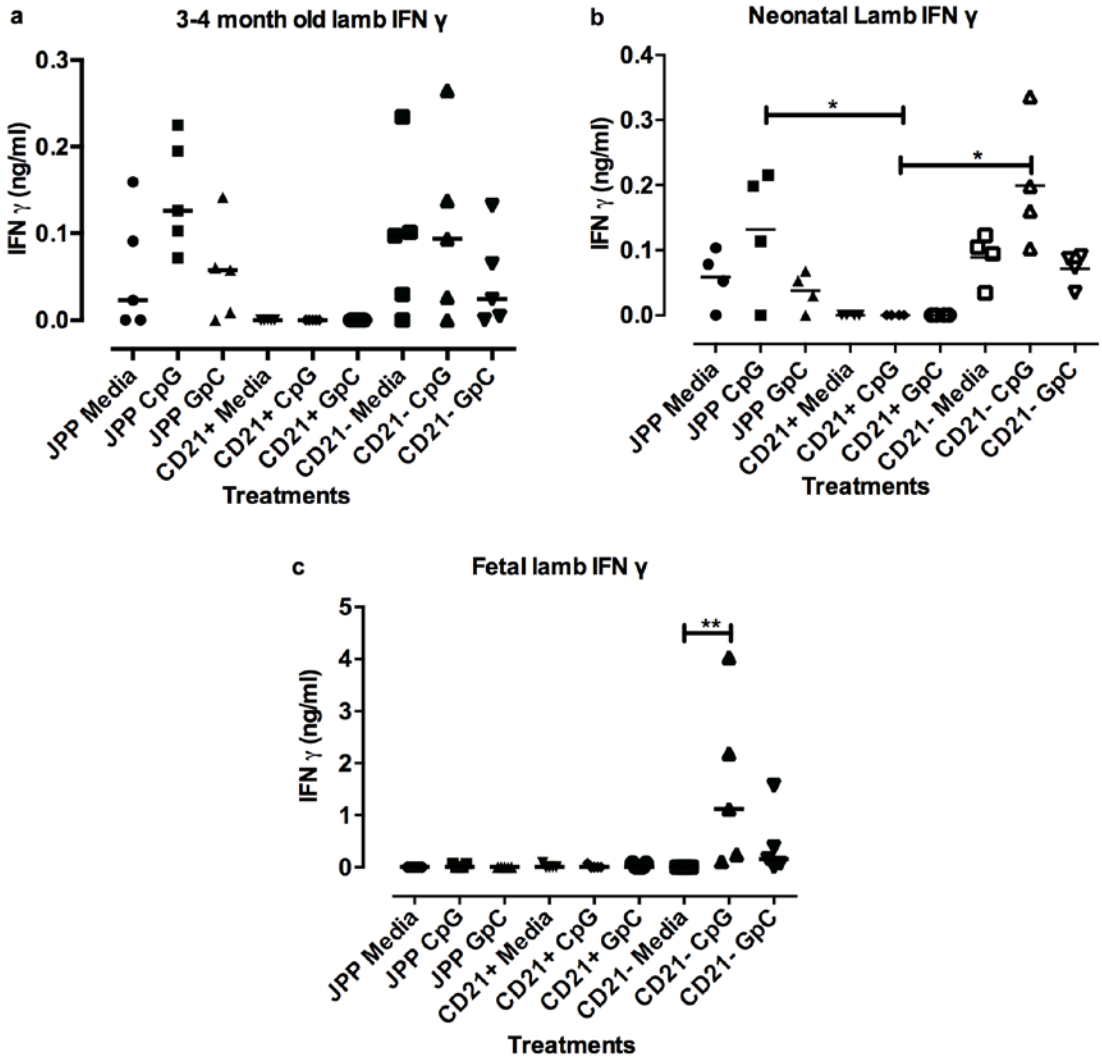


Figure 3. IFN-γ production in JPP cells from adult (a), neonatal (b) and foetal lambs (c). Total jejunal PP (JPP), JPP $CD21^+$ B cells and JPP $CD21^-$ cells were cultured for 48 hours in medium alone, or in the presence of 5 µg/ml of CpG or GpC ODN. IFN-γ production was evaluated in the culture supernatants by IFN-γ ELISA. Data for individual animals are presented with mean value indicated by horizontal bar for each group (*n*=5).

3.4.4 IgM production

We then evaluated IgM secretion from JPP cells of 3-4 month old lambs. JPP cells spontaneously secreted IgM but CpG ODN stimulation did not alter this response (Fig. 4a). There was, however, no detectable IgM secretion by enriched $CD21^+$ cells, either with or without CpG ODN stimulation (Fig. 4a). These observations are consistent with our previous report [1] with the exception that significant spontaneous IgM secretion was detected for the $CD21^-$ cells.

A similar IgM secretion pattern was observed for neonatal JPP cells and enriched $CD21^+$ B cells (Fig. 4b) with the single difference that $CD21^-$ PP cells did secrete significant levels of IgM following CpG ODN stimulation. The pattern of IgM secretion was also repeated with fetal JPP cells and enriched $CD21^+$ B cells (Fig. 4c) and there was significant IgM secretion by the $CD21^-$ cells (Fig. 4b).

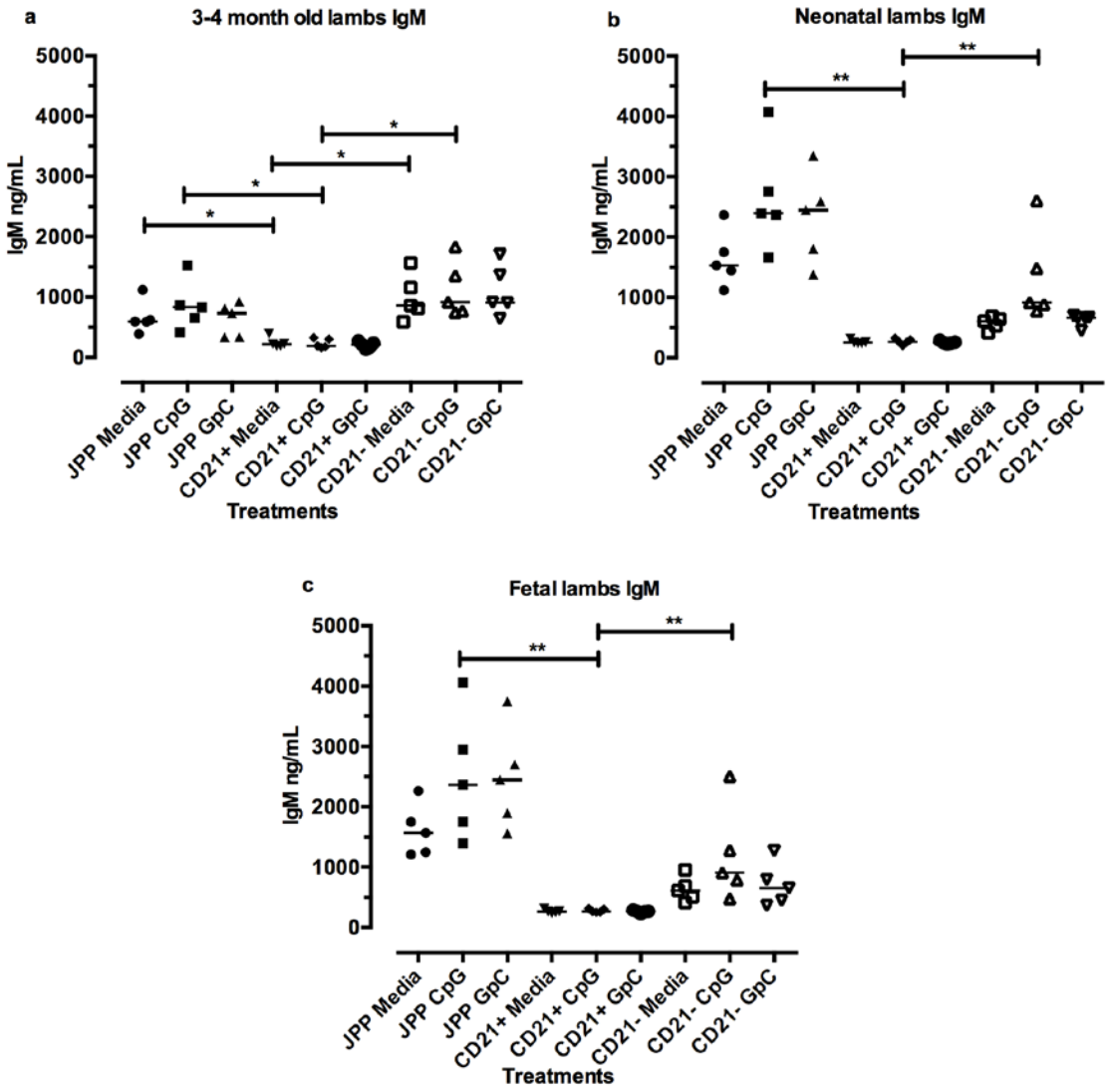


Figure 4. IgM production in JPP cells from adult (a), neonatal (b) and foetal lambs (c). Total jejunal PP (JPP), JPP CD21^{+} B cells and JPP CD21^{-} cells were cultured for 48 hours in medium alone, or in the presence of 5 µg/ml of CpG or GpC ODN. IgM production was evaluated in the culture supernatants by IgM ELISA. Data for individual animals are presented with mean value indicated by horizontal bar for each group (*n*=5).

3.4.5 Lymphocyte Proliferation

Proliferation responses of $CD21^+$ and $CD21^-$ PP cells were assayed with cells isolated from lambs of different ages. As expected, PP cells from 3-4 month old lambs displayed significant proliferation when stimulated with either CpG or GpC control (Table 1). $CD21^-$ PP cells also proliferated significantly following both CpG and GpC ODN stimulation, although this response was low. In contrast, $CD21^+$ B cells did not proliferate following either CpG or GpC stimulation (Table 2).

Neonatal JPP cells also proliferated significantly following CpG ODN stimulation**.** The $CD21^-$ PP cells did not proliferate following stimulation with CpG but, surprisingly, enriched $CD21^+$ B cells displayed significant proliferation following CpG stimulation (Table 2). This B cell response was distinct from earlier observations made with B cells isolated from 2 to 3 month old lambs. We did observe, however, spontaneous proliferation of fetal JPP cells and purified $CD21^+$ B cells displayed CpG ODN-specific proliferation. Thus, unlike B cell responses in 3-4 month old lambs, fetal and neonatal $CD21^+$ B cells proliferated following CpG. ODN stimulation.

	Fetal (Mean ± SD)	Neonatal (Mean ± SD)	3-4 month old (Mean ± SD)
Total JPP media	**1**	**1**	**1**
Total JPP CpG	**6.59 (±7.45)**	**13.12 (±11.65)**	**3.83 (± 0.88)**
Total JPP GpC	**0.974 (±0.42)**	**1.24 (±0.89)**	**4.29 (± 3.23)**
Total $CD21^+$ Media	**1**	**1**	**1**
Total $CD21^+$ CpG	**6.04 (±7.11)**	**10.20 (±12.06)**	**1.41 (± 0.51)**
Total $CD21^+$ GpC	**2.90 (±1.34)**	**0.86 (±0.95)**	**0.70 (± 0.41)**
Total $CD21^-$ Media	**1**	**1**	**1**
Total $CD21^-$ CpG	**3.55 (±2.57)**	**1.10 (±0.67)**	**3.13 (± 1.93)**
Total $CD21^-$ GpC	**1.71 (±1.36)**	**0.77 (±0.47)**	**2.50 (± 0.68)**

S.I values of ≥2.5 are considered to be significant compared to media.

Table 2. Proliferation in JPP cells from adult (a), neonatal (b) and foetal lambs (c). Total jejunal PP (JPP), JPP $CD21^+$ B cells and JPP $CD21^-$ cells were cultured for 66 hours in medium alone, or in the presence of 5 µg/ml of CpG or GpC ODN. Radioactive thymidine was then added in the cultures and cells incubated for an extra 6hours. Proliferation of the different cell groups was then carried out. Data for individual animals are presented with mean value indicated by horizontal bar for each group (*n*=5).

3.5. Discussion

We investigated the development of IL-10 producing regulatory B cells, that we have previously shown to exist in 3-4 months old lambs [1]. These investigations confirmed that IL-10 secreting B_{regs} are also detectable in JPP of fetal and neonatal lambs.

Sheep B cells can be divided into two subsets based on cell size and surface expression of CD21 and CD72 [107]. $CD21^+$ B cells, which are a representative of naive B cells, are localized in the B cell follicles of PPs, LNs and the spleen and are actively re-circulating [107, 108]. We recently reported that within the PP $CD21^+$ B cell population there are cells that produce IL-10 and have a regulatory function [1].

Like their T cell counterparts, B_{regs} cells are capable of inhibiting inflammatory responses and inducing immune tolerance through the production of IL-10 and TGFβ [3, 142]. IL-10 is an immune regulatory cytokine with pleiotropic functions, including suppression of inflammation [143]. IL-10 is produced by different cells including B cells, T_{regs}, monocytes, macrophage, neutrophils and DCs. It has been convincingly demonstrated that IL-10-producing B cells can suppress inflammatory responses in colitis, collagen induced arthritis and in experimental autoimmune encephalomyelitis [122, 127, 131]. IL-10 mediates suppression of inflammation by several mechanisms, including down regulation of the production of proinflammatory cytokines, such as IL-6, IL-12, IL-17, IL-18, IFN-γ, TNFα and the expression of MHC class II [143-145]. We have previously reported that PP B_{regs} from 4 month old lambs secrete IL-10 which suppresses production of IL-12, IFN- γ and IFN-α [1]. In the present investigation, we show that both neonatal and fetal $CD21^+$ B cells also

secrete IL-10 and presumably have a regulatory function. The significance of the presence of IL-10 producing $CD21^+$ B cells is not clear but implies that there is a competent mucosal immune system present at birth to protect the newborn against unnecessary inflammatory responses induced by enteric flora.

The $CD21^-$ cells in this experiment are made up of $CD21^-CD72^+$ B cells, $CD21^-CD72^-$ B cells, T cells, Dendritic cells and Macrophages [146]. Follicular dendritic (FDC) cells are also present in PP. FDC play a role during the selection of memory B lymphocytes during a germinal center reaction [147] [148]. They have been shown to express the CD21 marker [149, 150]. However, FDC express the SCR10a-containing CD21L, while B cells selectively express the short CR2/CD21 lacking exon 10a (CD21S) [150]. It is unlikely that the FDC that expressed CD21 produced IL-10 since we have negatively sorted B cells and shown that only B cells produce IL-10 (Jimbo et al, manuscript in preparation).

$CD21^+$ B cells isolated from fetal PPs would not have been exposed to exogenous antigens and yet they produced detectable levels of IL-10. This implies that PP B_{regs} production of IL-10 is antigen-independent and is therefore spontaneous. Therefore, PP may function as a site for the production of natural B_{regs} analogous to the thymus which functions as a site for natural T_{reg} generation [151]. Peyer's patches are the primary mucosa-associated immune induction sites in the small intestine [69]. We previously reported that jejunal PP cells produced low levels of IFN-γ, IL-12 and IFN-α and when IL-10 was neutralized in-vitro with an anti-IL-10 antibody, then IFN-γ and IL-12 responses were elevated [1]. Presumably the suppressive effects of IL-10 produced by PP $CD21^+$ B cells dampens potentially harmful

inflammatory responses (e.g. IL-12 and IFN-γ) in response to CpG-DNA from commensal microflora in the intestine. This suppressive effect in fetuses may be present to ready the system for the colonization of the gut lumen with different microbiota at birth. We were however not able to carry out further experiments to evaluate the suppressive ability of the PP $CD21^+$ in the fetal lambs due to the limited number of isolated cells. We therefore focussed on assessing their existence in this age group.

IL-12 is a pro-inflammatory cytokine that influences development of Th-1 type responses. It is produced primarily by monocytes, macrophages and dendritic cells following microbial stimulation, whereas T cells and NK cells are the major responders to IL-12. The T cells and NK cells act by up-regulating IFN-γ production, proliferation and cytotoxic activity [152]. In the present study, IL-12 was produced in jejunal PP cell population but not in $CD21^+$ B cells population (B_{regs}). Similarly, B_{regs} did not produce IFN-γ. Therefore, PP B_{regs} secrete immunoregulatory IL-10 but not the pro-inflammatory cytokines, IL-12 and IFN-γ. This observation is consistent with the concept that $CD21^+$ B cells include a regulatory B cell subpopulation.

Sheep B cells from blood produce IgM and proliferate in response to CpG stimulation [2]. However, it has also been shown that $CD21^+$ B_{regs} isolated from sheep PP do not secrete IgM or proliferate following CpG ODN stimulation [2]. This lack of CpG-specific responses was attributed to a unique pattern of TLR9 signal transduction that was attributed to a defect in the signalling transduction pathway downstream of TLR9 [2]. In our present study, we observed that $CD21^+$ B cells isolated from the jejunal PP of older lambs do not proliferate,

results that were consistent with earlier observations [1]. In contrast, we observed that $CD21^+$ B cells isolated from the PPs of fetal and neonatal lambs did proliferate following CpG ODN stimulation. Therefore, we hypothesize that the proliferation of PP B cells in fetal and neonatal lambs may be attributed to the fact that at these early stages of development the B cells do not have a defect in the TLR9 signal transduction pathway as reported for older lambs [2]. The other possibility may be technical; maybe a population of fetal and neonatal $CD21^-$ B cells (capable of proliferating), co-purify with $CD21^+$. Alternatively, there may be insufficient B_{reg} activity to block CpG ODN stimulation. Further investigations are required to determine the reason why fetal and neonatal $CD21^+$ B cells proliferate in response to CpG ODN stimulation and to determine if the naturally occurring B_{regs} have the capacity to influence either T or B cell responses.

In conclusion, the present investigations confirmed that B cells, spontaneously secreting IL-10, are present in both fetal and newborn lamb jejunal PPs. These observations support the conclusion that naturally occurring B_{regs} develop in mucosa-associated lymphoid tissue in an antigen-independent manner.

CHAPTER 4: EVIDENCE FOR THE EXISTENCE OF REGULATORY AND EFFECTOR B CELL POPULATIONS IN PEYER'S PATCHES OF SHEEP

Relationship of this study to the dissertation.

In chapter 3 we showed that B_{regs} develop in utero prior to birth and are present in young lambs of different ages. Hence in this study, we sought to determine whether $CD21^+$ B cells are the only source of IL-10 in Peyer's patches. We also wondered whether an effector B cell population co-existed with regulatory B cells in the PPs. Furthermore, we sought to find out whether IL-10 secretion was limited to naïve $CD21^+IgM^+$ B cells, or B cells that had isotype switched could also secrete IL-10, whether they exist in other lymphoid tissues or whether they exist in other ruminant species.

4.1 ABSTRACT

IL-10 secreting $CD21^+$ B exist in sheep Peyer's patches (PP). It's not known however, whether all PP B are regulatory or whether an effector population also exists in this tissue. To further characterize the subpopulations of B cells in PP's, highly purified B cells were negatively sorted from jejunal PP and fractionated according to co-expression of $CD72^+CD21^+$or $CD72^+CD21^-$ molecules and then stimulated with the TLR9-agonist, CpG ODN. IL-10, IL-12, IFN-γ, and IgM production were then assayed. We observed that only highly purified $CD72^+CD21^+$ B cells spontaneously secreted high levels of IL-10, but they did not produce any IL-12, IFN-γ or IgM suggesting that this cell population contains regulatory B cells. In contrast, $CD72^+CD21^-$ B cells did not secrete IL-10, but secreted IL-12, IFN-γ, and IgM, suggesting they include effector cells. In addition, B cells expressing

surface IgA, IgM and IgG1 all secreted similar levels of IL-10. We further confirmed that only B cells produce IL-10, while other cells in the PP including DCs and T cells do not. Our investigations provide evidence for the existence of two B cell sub-populations in sheep PP; IL-10 secreting regulatory ($CD72^{+}CD21^{+}$) cells, and IL-12/ IFN-γ/ IgM-secreting effector ($CD72^{+}CD21^{-}$) cells.

Key words: Immune regulation; regulatory cells; B_{regs}; effector B cells; Peyer's patches; TLR9; CpG ODN

4.2 INTRODUCTION

B cells are functionally characterized by the ability to produce antibodies [3]. They also have the capacity to produce cytokines and function as antigen presenting cells (APC) [3]. Based on their function, B cells can be divided into two subgroups; effector and regulatory B cells.

Effector B cells are positive regulators of immunity and based on their cytokine secretion patterns have been subdivided into B-effector-1 cells (Be1) and B-effector-2 (Be2) [9, 136]. The Be1 cells secrete IL-12 and IFN-γ, whereas Be2 secrete IL-4, IL-5, IL-6, IL-10 and IL-13 [9]. In addition, effector B cells function as antibody producing cells, present antigens to T cells and modulate T cell-mediated responses [137]. Effector B cells eventually differentiate into plasma cells, able to produce different isotype antibodies [138].

The regulatory role of B cells was first reported by Janeway and colleagues in a murine model of experimental autoimmune encephalomyelitis (EAE) [125]. Using a B cell deficient model, they reported that B cells were not required for the induction of EAE but they contributed to immune regulation resulting in complete recovery from acute EAE. Further evidence for the existence of $B_{regs,}$ was obtained from mouse models of inflammatory bowel disease (IBD) [122, 153], EAE [127], arthritis [131], and lupus [154, 155]. These B_{regs} inhibited the progression of inflammation and/or hastened the recovery from the experimental inflammatory conditions. Notably, B_{regs} cells produce IL-10, the immuno-regulatory cytokine that can suppress harmful immune responses by regulating Th1/Th2 balance and directly dampening innate cell-mediated inflammatory responses [156-158].

Recent investigations in our laboratory revealed a novel B cell population in sheep jejunal Peyer's patches with regulatory function (B_{regs}) that spontaneously produce IL-10 and suppresses TLR9-induced IFN-γ and IL-12 cytokine responses [1]. We have subsequently obtained evidence that these regulatory B cells develop in utero prior to antigen exposure (Jimbo et al. 2014). In these previous studies, $CD21^+$ B cells were isolated using magnetic cells sorting, raising questions as to whether other contaminating cell types may be present. In the present investigation, we sought to determine whether $CD21^+$ B cells are the only source of IL-10 in Peyer's patches. We also investigated whether an effector B cell population co-existed with regulatory B cells in the PPs. Furthermore, we also investigated whether IL-10 secretion was limited to naïve $CD21^+IgM^+$ B cells, or B cells that had undergone isotype switching.

4.3 Materials and methods

4.3.1 Animals and oligonucleotides

Suffolk sheep of either sex were obtained from the Department of Animal and Poultry Science (University of Saskatchewan, Saskatoon, SK, Canada). 2-4 months old lambs were used in these studies. Experiments were conducted according to the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care. All the experimental protocols were approved by the University of Saskatchewan Animal Care Committee.

4.3.2 Oligodeoxynucleotides

Oligodeoxynucleotides of different classes have been shown to be biologically active in sheep both *in vitro* and *in vivo*. We used B-class CpG ODN 2007 to stimulate cells in our experiments. The CpG ODN 2007 and GpC ODN 2007 GC were obtained from Merial Limited (Lyon, France). They have the following sequences and backbone structures; 2007 tcgtcgttgtcgttttgtcgtt and 2007GC tgctgcttgtgcttttgtgctt. The ODN doses were previously optimized and used at a dose of 5ug/ml [1, 139].

4.3.3 Isolation of JPP cells for culture

Lambs were euthanized and jejunal Peyer's patch (JPP) tissues immediately removed and placed in ice-cold DMEM (GibcoBRL) containing antibiotics (100 U/mL), Penicillin, 100 µg/mL Streptomycin sulfate, 0.25 µg/mL Amphotericin B (Sigma-Aldrich). Cells were isolated from JPP as described previously [141]. The number of viable cells isolated from all

tissues was determined by trypan blue dye exclusion and viable cells were counted with a hemocytometer under a light microscope. Cells were re-suspended at the appropriate concentrations in AIM V medium (supplemented with 2% FBS, 100U/mL penicillin, 100 µg/mL streptomycin sulfate, 0.25 µg/mL amphotericin B, 2Mm L-glutamine, 50 µM 2-mercaptoethanol and 10 µg/mL polymyxin B sulfate (Sigma- Aldrich)) and added to round bottomed 96-well plates (Nunc, Naperville, IL, USA).

4.3.4 JPP Labelling for High speed Cell Sorting of negatively selected B cells

For negative sorting of B cells, 3 X 10^8 ovine JPP cells, at a final concentration of 1 X 10^8 cells/mL PBS, were labelled with 18 µg anti-ovine CD25, 18 µg anti-ovine CD11c, 9 µg anti-ovine CD14, 18 µg anti-ovine CD2, 18 µg anti-ovine CD4, 18 µg anti-ovine WC1-N2, 18 µg anti-ovine CD8, 18 µg anti-ovine SWC3 and 15 µg anti-ovine CD16. JPP cells were incubated with mAbs for 20 min at 4 °C, with gentle mixing every 5 min. Cells were then pelleted by centrifuging at 311 x g for 8 min at 4 °C and washed twice with ice-cold PBS. The cell pellet was re-suspended in 3 mL PBS and the following fluorochrome-conjugated secondary antibodies were added: 20 µg goat anti-mouse IgG2a-PE; 20 µg of goat anti-mouse IgG1-PE; and 9 µg goat anti-mouse IgM- allophycocyanin. Cells were incubated in the dark at 4 °C for 15 min and then washed twice with ice-cold PBS.

Table 3. Monoclonal antibodies, isotype controls, and fluorochrome-conjugated secondary antibodies used to detect ovine leukocyte antigens.

Bovine Leukocyte Antigen[1]	**Isotype**	**Clone**	**Supplier**
TcR1	IgG1	86D	VMRD
CD25	IgG2a	CACT108A	VMRD
CD11c	IgM	BAQ153A	VMRD
CD14	IgG1	M-M9	VMRD
CD2	IgG2a	MUC2A	VMRD
WC1-N2	IgG1	BAQ4A	VMRD
CD4	IgG1	17D	VMRD
CD8	IgG1	CACT80C	VMRD
CD172a	IgG2b	74-22-15A	VMRD
CD21	IgG1	MCA1424G	AbD Serotec
CD72	IgM		VIDO
IgM	IgG1		VMRD
IgA	IgG1		VMRD
IgG1	IgM		VMRD
Control	Mouse IgG1	MG100	Invitrogen
Control	Mouse IgG2b	MG2B00	Invitrogen
Control	Mouse IgM	MGM00	Invitrogen
Control	IgM/ Allophycocyanin		BD Biosciences
Control	IgG2b/FITC		Invitrogen[d]
Control	IgG1/PE		Invitrogen

[a]VMRD (Pullman, WA); [b]AbD Serotec (Raleigh, NC); [c]BD Pharmigen (Mississauga, ON); [d]Invitrogen (Burlington, ON)

4.3.5 High-speed Cell Sorting

Labelled JPP cells were adjusted to a final concentration of $1x10^8$ cells/mL in ice-cold PBS, filtered through 35 µm cell strainer capped 12x75 mm polystyrene round bottom tubes (Cat. No. 352235) (BD Falcon ON, CA) to remove cell clumps. High-speed sorting was performed

with a MoFlo XDP (Beckman Coulter Inc. CA, USA) equipped with a 488 argon and 633 HeNe laser. FITC, PE and Allophycocyanin fluorescence was collected through 529/25, 575/25 and 670/30 band-pass filters, respectively. Dot scatter plots of forward light scatter (FSC) and 90^{o} light scatter (SSC) were displayed on a linear scale and used to define the first sort region (R1) which excluded dead cells and debris. Dot plots displaying FSC-Height/FSC-Width were used to define the second sort region (R2) to exclude doublets or larger cell clumps. To negatively sort for B cells, a sort region was set to include all PP B cells and exclude Lin+ cells (R4). Sort conditions included a sheath pressure of 60 psi, a 70 µm nozzle, 0.3 psi differential pressure, and a sort rate of 18-22,000 events/s. Sorted cells were collected in sterile 12x75 mm polypropylene round bottom tubes (VWR international, Mississauga, ON, CA) pre-coated overnight with 4% bovine serum albumen (BSA, Sigma, Ontario CA). Collection tubes were kept on ice throughout the sort and cells were transferred every 30 min to ice-cold AIMV medium (Invitrogen, Burlington, ON) supplemented with plus 10% fetal bovine serum (FBS, Invitrogen, Burlington, ON).

4.3.6 Tissue culture conditions and stimulation with TLR agonists

High-speed sorted cells were re-suspended in AIM V media. Aliquots of $5x10^5$ cells for $CD21^+$ and 3×10^5 for $CD21^-$ cells were cultured in triplicate wells in a final volume of 200 µL. Cells were stimulated with TLR9 agonists B-class CpG 2007 or GpC 2007 as a control at 5 µg/mL and then incubated at 37°C in a 5% CO_2 atmosphere and 95% humidity. For optimal detection of cytokines, cells were stimulated for 48 hours as previously described [141]. Culture supernatants were stored at -20°C until cytokines assays were performed.

4.3.7 Enzyme-linked immunosorbent assay (ELISA) for IgM, IL-10, IL-12 and IFN-γ

ELISAs for quantifying IgM and cytokines in culture supernatants were performed according to previously described procedures as follows; IgM [2], IFN-γ [159], IL-12 [160] and IL-10 [140], with minor modifications. U/ml (units per ml) for IL-10 was defined previously by Kwong et al [140], as the biological activity of IL-10 with one unit being the reciprocal of the IL-10 dilution that inhibited IFN-γ secretion by 50 % of Cos-7 cells.

4.3.8 Flow cytometry

JPP cells were re-suspended at a final concentration of 20 x 10^6 cells/ml in PBSA containing 0.03% sodium azide (EMD Chemicals). 50 µl aliquot of cells was added to each well of a U-bottom 96-well plate (Corning Inc. Life Sciences, Lowell, MA) and 5 µl aliquot of primary monoclonal antibody (mAb) for CD21, CD72, IgM, IgA, IgG1 (Table 3) were each added at a final concentration between 1-5 µg/ml. For each mAb the concentration was titrated to provide maximum labeling intensity without detectable background staining. Cells were incubated on ice for 20 minutes and then washed 3x by adding 200 µl PBSA/0.03% sodium azide. Fluorochrome-conjugated goat anti-mouse immunoglobulin (Ig) isotype-specific secondary antibodies (Life Sciences) were then added at a final concentration of 1 µg/ml and cells were incubated in the dark for 20 minutes on ice. Cells were then washed 3x with 200 µl PBSA/0.03% sodium azide before fixation with 200 µl 2% formaldehyde (Sigma-Aldrich). Cells were stored at 4°C in the dark until analyzed with a FACS Calibur (Becton-Dickinson, Franklin Lakes, NJ) using Cell Quest acquisition and analysis software (Version 3.3). A minimum of 10,000 events were captured for each sample with data collected in list-mode.

4.3.9 Cytospins

Negatively selected B cells were purified by high-speed cell sorting and re-suspended in PBSA containing 0.1% EDTA at a final concentration of 10^6 cells/ml. Glass slides (Superfrost Plus, VWR) were pre-coated in the cytospin (Cytospin 4, Fisher Scientific, Waltham, MA) with 25µlFBS by centrifuging slides at 300 RPM for 3 minutes. A 100 µl aliquot of cell suspension was then added to the cytospin funnel and centrifuged at 300 RPM for 3 minutes. Slides were air dried overnight and then stained with Hemacolor staining kit (EMD Chemicals). Slides were again air-dried overnight before mounting cover slips with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI).

4.3.10 Statistical analysis

Data analysis was performed with the statistical software program, Graph Prism 5 (Graphpad software). Individual group differences were examined by performing one way analysis of variance (ANOVA). Values of $P<0.05$ were considered significant. Means of groups were reported and were compared using the Kruskal-Wallis test and Dunn's test was used to perform the post-hoc tests.

4.4 Results

4.4.1 B cells in Peyer's patches are the only source of IL-10

Previous investigations in our laboratory have shown that an IL-10 secreting regulatory B cell (B_{regs}) are present in jejunal PP of lambs [1]. However, in these previous studies, B cells were enriched using MACS yielding about 95% purity. This raises the question as to whether only $CD21^+$ B cells produce IL-10 in the PP or whether contaminating cells in the remaining 5% were directly or indirectly involved in IL-10 secretion. To address this issue, JPP cells were fractionated using high-speed cell sorting to negatively select B cells (Fig 5).

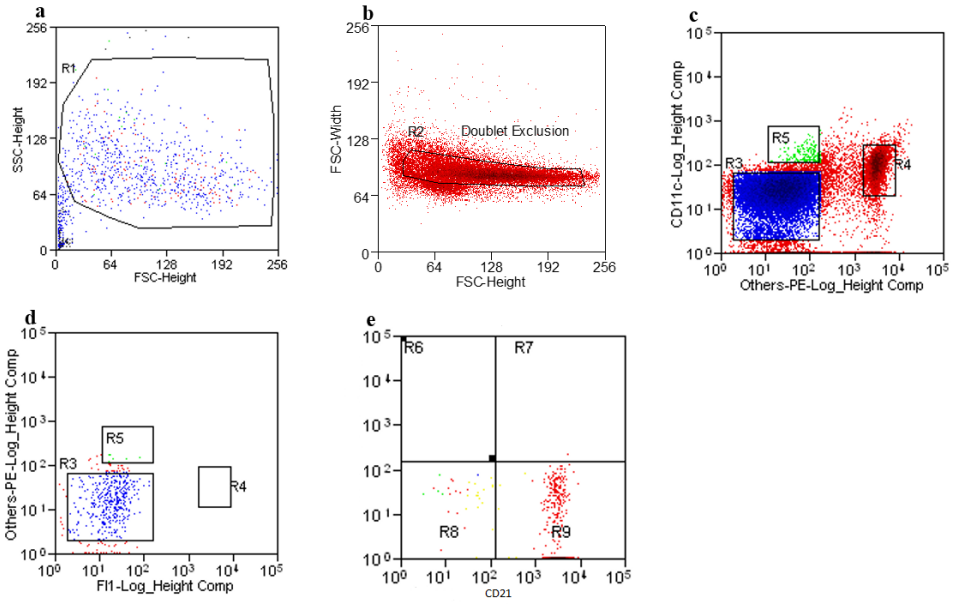
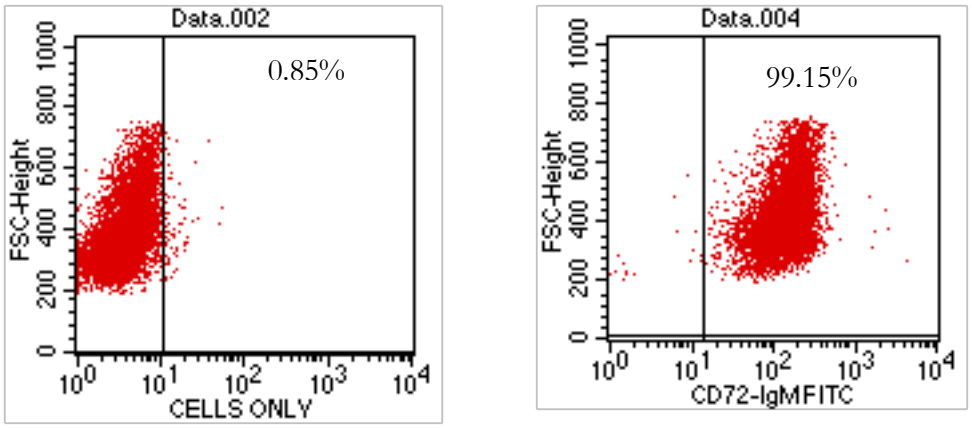


Figure 5. Negative selection of B cells that are negative for other leukocyte lineage markers. Regions for high-speed sorting of negatively selected B cells populations, DCs and other cells (Monocytes, Macrophages, neutrophils, T cells) were first set to exclude cell debris (a) and doublets (b). The B cell sort region (R3) was defined based on the B cells being negative for CD25, CD4, CD8 (T cells), WC-1-N2 (γδT Lymphocytes) CD14 (monocytes), CD172a

(monocytes, granulocytes, dendritic cells and hematopoietic progenitor cells) and CD2 (NK cells) (c). Dendritic cells in R5 region were sorted on the basis of their CD11c expression. The rest of the cells were sorted in the R4 region. Cells within R4 were subsequently sorted into two subpopulations based on CD21 expression into $CD21^-$ (R8) or $CD21^+$ cells (R9). Flow cytometry analysis of Lin^- sorted cell populations' revealed 99% purity for CD72 (d), and 99% purity for other cells (e).This procedure yielded B cell purity of 99% (Fig 6).



CD72

Figure 6. Flow cytometry figures showing sorted B cells purity.

Sorted JPP B cells were stained as described in section 4.3.8 of this thesis. The cells were then analyzed with a FACS Calibur (Becton-Dickinson, Franklin Lakes, NJ) using Cell Quest acquisition and analysis software (Version 3.3). A minimum of 10,000 events were captured for each sample with data collected in list-mode

IL-10 production was then assayed in culture supernatants from the individual sorted JPP cell populations. Unfractionated JPP cells spontaneously secreted IL-10. Stimulation of unfractionated JPP cells with CpG or control GpC ODN did not result in a significant increase

in IL-10 production relative to the medium (Fig 7a). We then assessed IL-10 production in high-speed sorted B cells (JPP B cells). JPP B cells cultured in medium, spontaneously secreted IL-10 and CpG or GpC stimulation did not result in significant increase in B cell production of IL-10 (Fig 7b). Furthermore, the level of IL-10 secretion by purified JPP B cells was similar to unfractionated JPP cells.

In PP, the CD21 marker is expressed on B cells as well as follicular dendritic cells [150]. We therefore sought to determine whether PP DCs might also be a source of IL-10 secretion. No IL-10 was detected in the culture supernatant of sorted $CD11c^+$ (Dendritic cells) cells regardless of whether they were stimulated with CpG or GpC (Fig 7c). Therefore, PP $CD11c^+$ cells did not secrete detectable levels of IL-10.

Previous work in cattle suggested an interaction between B cells and myeloid cells was required for optimal CpG ODN activation of B cells [161, 162]. We therefore investigated whether an interaction between B cells and DCs might enhance IL-10 secretion by B cells. JPP B cells were co-cultured with DCs (B cells + $CD11c^+$). Unstimulated JPP B cells + $CD11c^+$ cells secreted IL-10, and the levels were similar to those observed in purified JPP B cells. Further stimulation of JPP B cells + $CD11c^+$ cells with either CpG or non GpC ODN did not result in significantly increased IL-10 production (Fig 7d). These observations confirm that $CD11c^+$ cells (DC) do not contribute to the IL-10 production by JPP B cells. There was also no IL-10 secretion in the $CD21^-$ cell population regardless of stimulation with CpG or GpC ODN (Fig 7e). These results support the conclusion that B cells are the only source of IL-10 in cultured sheep PP cells.

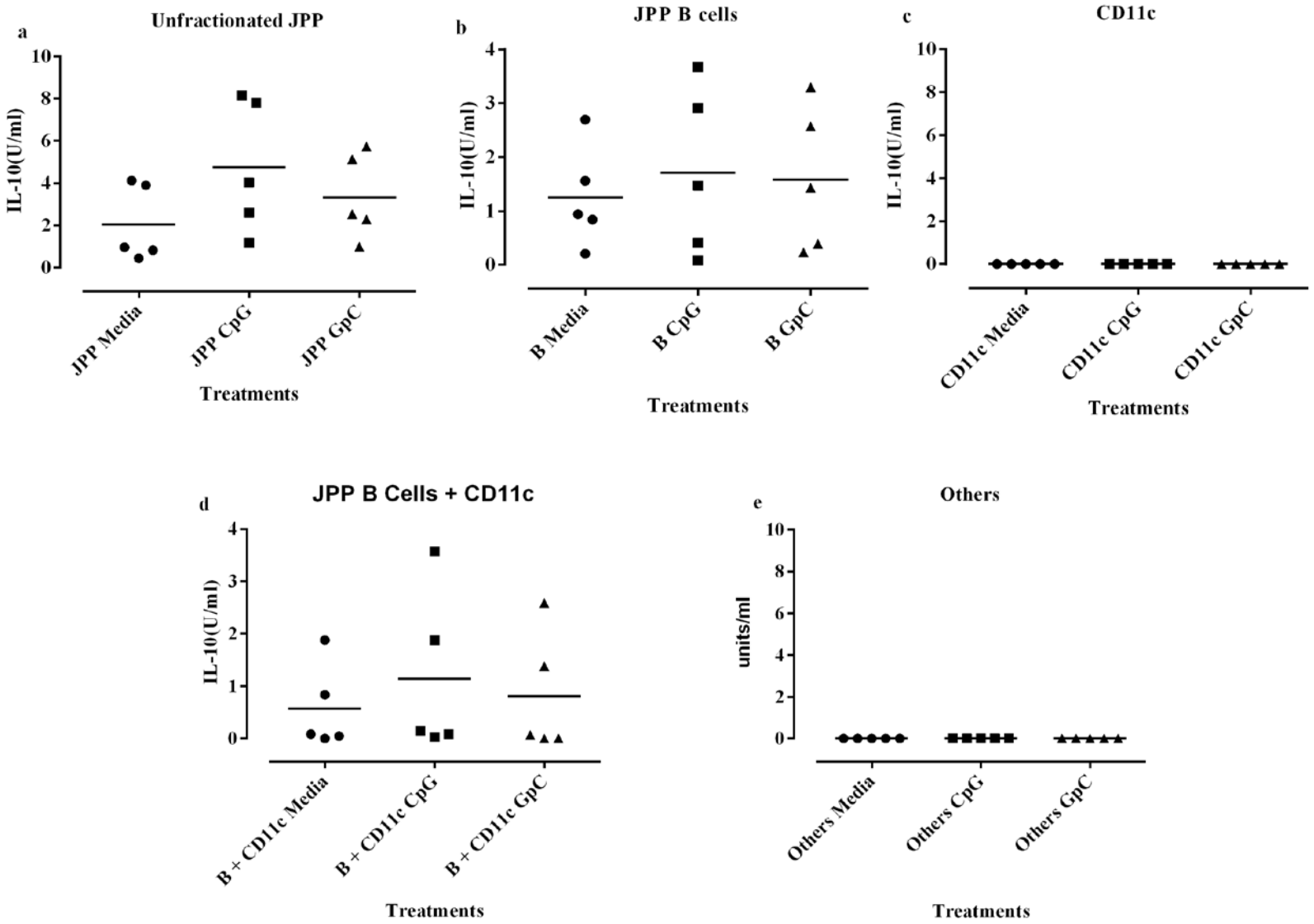


Figure 7. IL-10 production in sorted PP cells. Unfractionated JPP (a), highly purified unfractionated JPP B cells (b), CD11c (DC) (c), B + CD11c (d) and JPP other cells (e), were cultured for 48 hours in medium alone, or in the presence of 5 µg/ml of CpG or GpC ODN. IL-10 production was evaluated in the culture supernatants by IL-10 ELISA. Data for individual animals are presented with mean value indicated by horizontal bar for each group (*n*=5).

4.4.2 $CD72^+CD21^+$ B cells but not $CD72^+CD21^-$ B cells secrete IL-10

All sheep B cells express the CD72 marker except plasma cells, but only a subset of B cells express CD21 [163, 164]. It is not clear whether $CD21^-$ B cells also secrete IL-10. To determine whether $CD21^-$ B cells secrete IL-10, PP B cells were fractionated into $CD72^+CD21^+$ and $CD72^+CD21^-$ cell populations. These cells were then stimulated with CpG and GpC ODN, and IL-10, IL-12, IFN-γ and IgM concentrations were assayed in the supernatants.

Unfractionated JPP cells and $CD72^+CD21^+$ cell populations spontaneously secreted significantly higher levels of IL-10 compared to the $CD72^+CD21^-$ cell population (Fig 8a). Stimulation of unfractionated JPP and $CD72^+CD21^+$ cell populations with CpG or GpC did not result in a significant increase in IL-10 production (Fig 8a). Unstimulated and stimulated $CD72^+CD21^-$ and $CD72^-CD21^-$ cell populations did not secrete any IL-10 (Fig 8a). This observation supports the conclusion that only $CD21^+$ PP B cells produce IL-10, and regulatory B cells are present in this population.

4.4.3 $CD72^+CD21^-$ B cells, but not $CD72^+CD21^+$ B cells secrete IL-12

Effector B cells are characterized by the secretion of a variety of cytokines including *IL-12* [165]. Therefore, we evaluated the production of IL-12 by $CD72^+CD21^+$ and $CD72^+CD21^-$ B cells populations.

Low levels of IL-12 were spontaneously secreted by unfractionated JPP cells (Fig. 8b). There was significantly increased IL-12 production by the unfractionated JPP cell population following CpG ODN stimulation.

$CD72^+CD21^+$ B cells population did not spontaneously secrete a detectable level of IL-12 and stimulation with CpG or GpC ODN did not induce IL-12 secretion (Fig 8b). This is consistent with previous observations [1]. In contrast, $CD72^+CD21^-$ B cells secreted IL-12 following stimulation with CpG ODN (Fig 8b). Therefore $CD21^+$ B cells were not a source of detectable IL-12 production, but B cells in the $CD72^+CD21^-$ population produce this cytokine in response to CpG ODN.

4.4.4 IFN-γ is secreted by $CD72^+CD21^-$ B cells, but not $CD72^+CD21^+$ in PP

Effector B cells are also characterized by the secretion of a variety of cytokines including IFN-γ [165]. There was no detectable IFN-γ secretion by either unstimulated or CpG ODN stimulated unfractionated JPP cells (Fig.8c). Very low levels of IFN-γ were detected in the culture sup of unstimulated $CD72^+CD21^-$ B cells and significantly increased IFN-γ levels were detected following CpG ODN stimulation. Similarly, unstimulated $CD72^-CD21^-$ cells produced very low levels of IFN-γ, but stimulation of these cells with CpG or GpC ODN resulted in no detectable increase in IFN-γ production. These observations support the conclusion that in PP, a major source of IFN-γ is the $CD72^+CD21^-$ B cell population, but TLR agonist stimulation is required (Fig 8c).

4.4.5 $CD72^+CD21^-$ B cells, but not $CD72^+CD21^+$ B cells secrete IgM in the PP

Regulatory B cells in sheep PP do not to secrete IgM [1]. Therefore, we investigated whether there was an effector B cell sub-population in the PP that could secrete IgM. Purified JPP $CD72^+CD21^-$ B cells and $CD72^+CD21^+$B cells were cultured for 48 hours and supernatants were assayed for IgM secretion.

Unfractionated JPP cells spontaneously secreted IgM (Fig. 8d) and stimulation of these cells with CpG or GpC did not result in significantly increased IgM production. $C72^{+}CD21^{+}$ B cells did not spontaneously secrete IgM and CpG or GpC stimulation had no effect on antibody production. In contrast, $C72^{+}CD21^{-}$ B cells spontaneously secreted IgM at levels similar to the unfractionated JPP population. Stimulation of the $C72^{+}CD21^{-}$ cell population with CpG or GpC did not induce a significant increase in IgM secretion (Fig 8d).

There was however no IgM production in the $CD72^{-}CD21^{-}$ population (The rest of the cells that include T cells, macrophages and DCs). These observations suggest that $C72^{+}CD21^{-}$ cells (effector B cells), capable of secreting IgM are present in the PP.

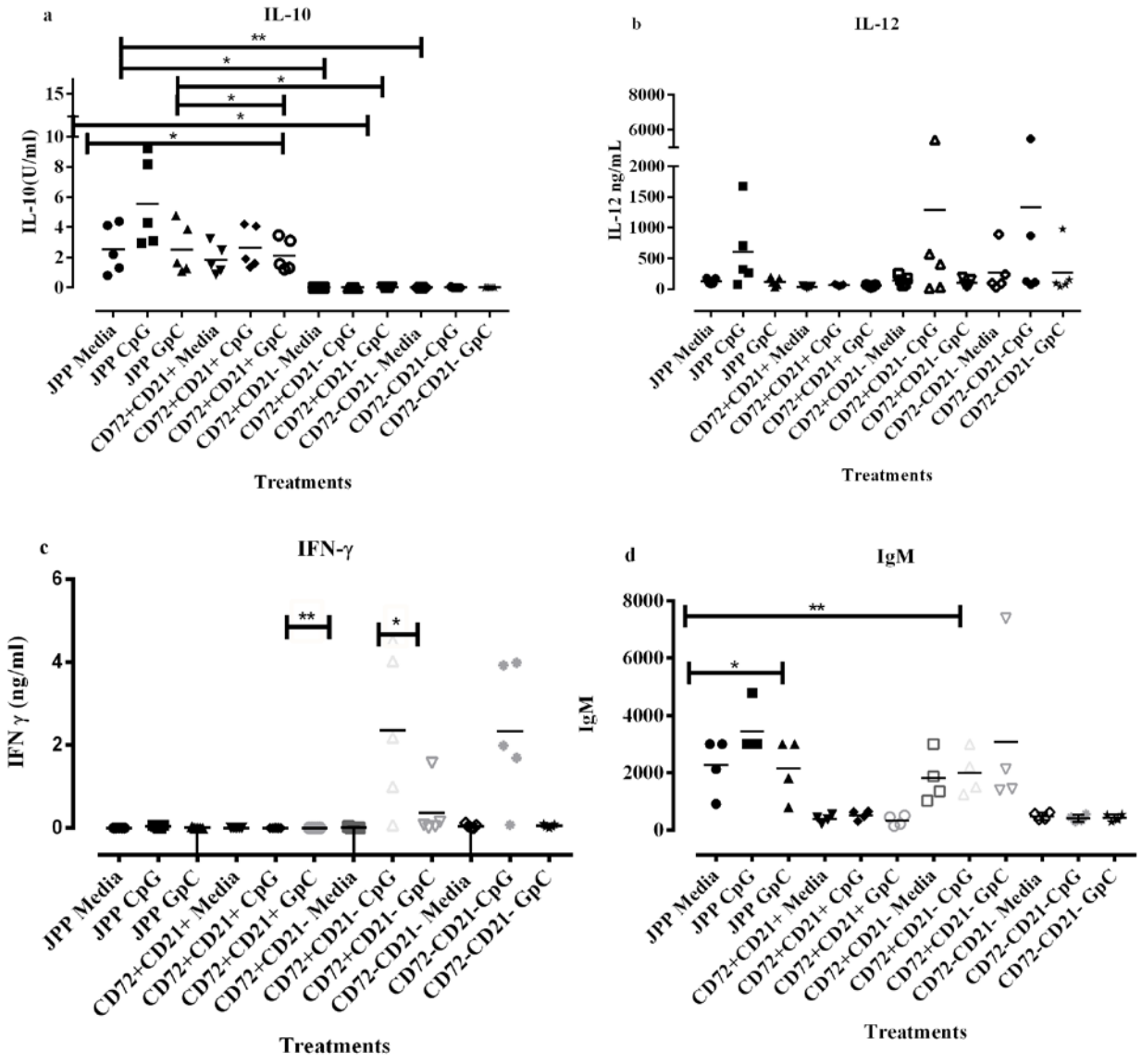


Figure 8. IL-10 (a), IL-12 (b), IFN-γ (c) and IgM (d) production in unfractionated and sorted PP subpopulations. Unfractionated JPP, highly purified JPP $CD72^{+}CD21^{+}$ B cells, JPP $CD72^{+}CD21^{-}$ B cells and JPP $CD72^{-}CD21^{-}$ cells were cultured for 48 hours in medium alone, or in the presence of 5 µg/ml of CpG or GpC ODN. IL-10, IL-12, IFN-γ and IgM production was evaluated in the culture supernatants by IL-10, IL-12, IFN-γ and IgM ELISA. Data for individual animals are presented with the mean value indicated by horizontal bar for each group (*n*=5). JPP; jejunal Peyer's patch cells.

4.4.6 IL-10 secreting CD21$^+$ B cells are sIgM$^+$, sIgA$^+$and sIgG1$^+$

Having confirmed that CD21$^+$ B cells were the sole source of spontaneous IL-10 in PP, we then investigate whether B cells which had undergone isotype switching could still produce IL-10. Therefore, the negatively sorted B cell population was further fractionated based on surface immunoglobulins expression (*sIgM*$^+$, *sIgA*$^+$ or *sIgG1*$^+$ populations) (Fig 9).

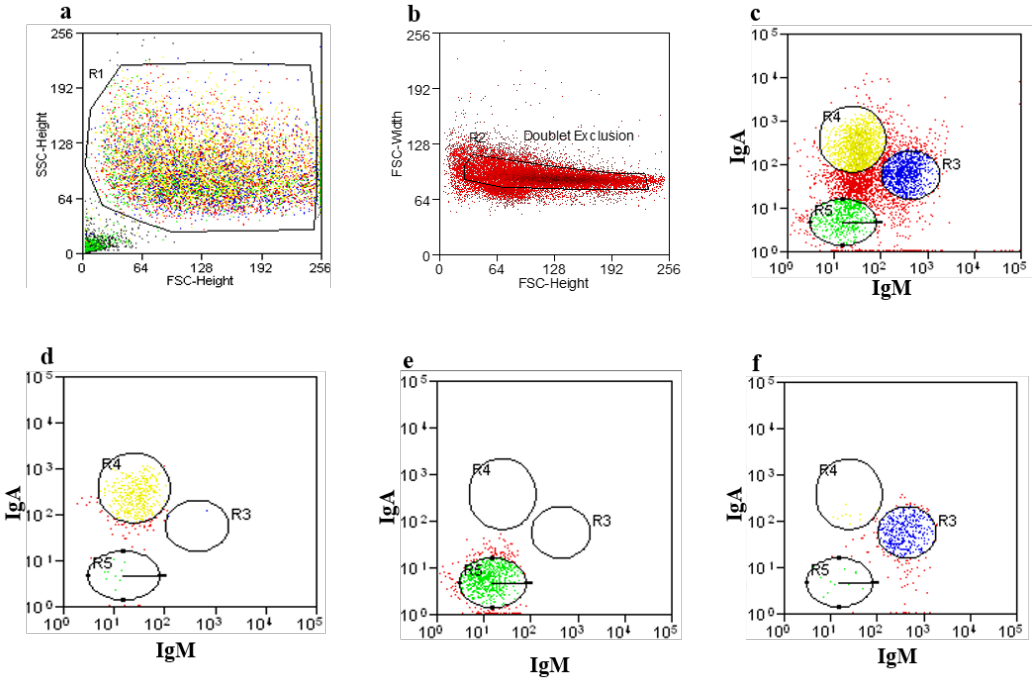


Figure 9. Sorting of B cells based on surface Ig expression.

Regions for high-speed sorting of negatively selected B cells populations were first set to exclude cell debris (a) and doublets (b). B cells were first negatively sorted based on the B cells being negative for CD25, CD4, CD8 (T cells), WC-1-N2 (gamma-delta T Lymphocytes) CD14 (monocytes), CD172a (monocytes, granulocytes, dendritic cells and hematopoietic progenitor cells) and CD2 (NK cells) (Fig 5). Cells were there after sorted into three subpopulations based on expression of IgM (R3), IgA (R4) and IgG1 (R5) (c). Flow

cytometry analysis of sorted cell populations revealed 96% purity for IgG1 (d), 98% purity for IgM (e) and 97% for IgA (f).

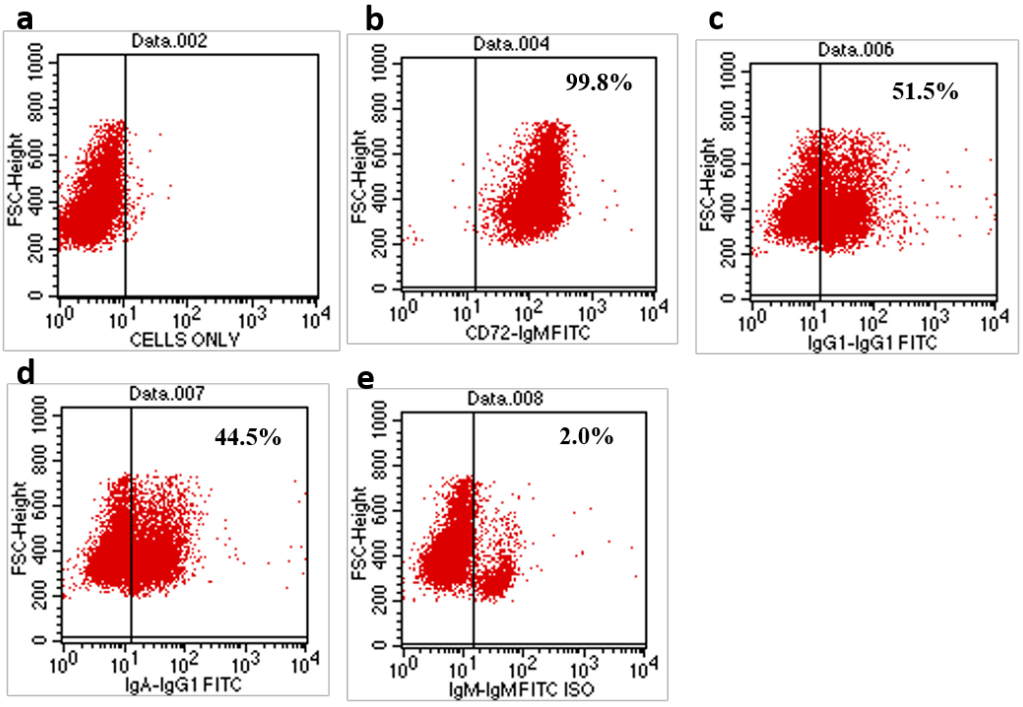


Figure 10. Phenotype analysis of negatively sorted JPP B cells

Sorted JPP B cells were stained as described in section 4.3.8 of this thesis. The cells were then analyzed with a FACS Calibur (Becton-Dickinson, Franklin Lakes, NJ) using Cell Quest acquisition and analysis software (Version 3.3). A minimum of 10,000 events were captured for each sample with data collected in list-mode. Negatively sorted B cells were 99.8% $CD72^+$ (b), 51.5% $sIgG1^+$ (c), 44.4% $sIgA^+$ (d) and 2.0% $sIgM^+$ (e) (Fig 10a). Cytospins were also performed to ascertain the phenotypes of the sorted B cells (Fig. 11).

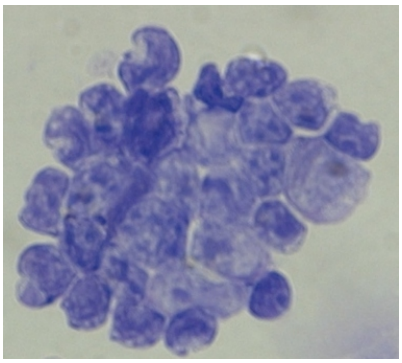


Figure 11. Cytospin of negatively sorted PP B cells

* Cytospin for sorted B cells revealed B cells in 2 sizes. Large and small. Scale bar = 10 μm; magnification = 100X.

The sIgM$^+$, sIgA$^+$ and sIgG1$^+$ B cells all spontaneously secreted similar levels of IL-10 (Fig 12). The fractionated negatively selected B cell population was included a positive control. The levels of spontaneous IL-10 secreted by the unstimulated sIgM$^+$, sIgA$^+$ and sIgG1$^+$ B cells were not significantly different from the levels produced by the unstimulated unfractionated JPP B cells (Fig 12).

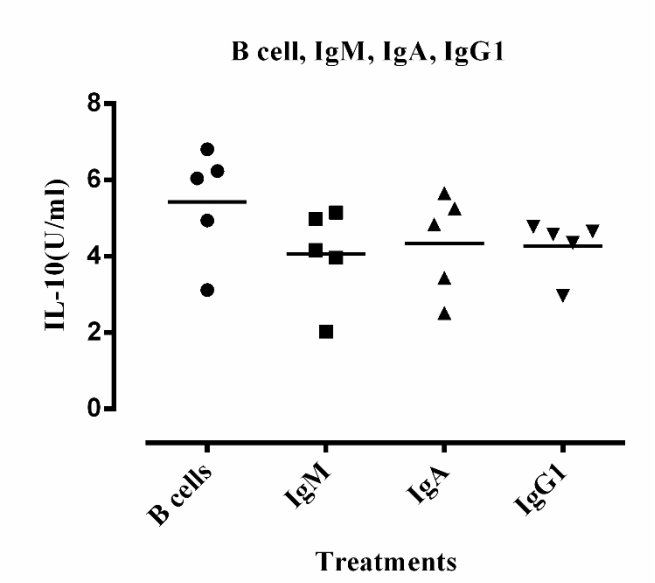


Figure 12. IL-10 production in sorted sIg$^+$ PP B cells. Highly purified JPP B cells, sIgM$^+$ B cells, sIgA$^+$ B cells and sIgG1$^+$ B cells were cultured for 48 hours in medium alone, or in the presence of 5 μg/ml of CpG or GpC ODN. IL-10 production was evaluated in the culture supernatants by IL-10 ELISA. Data for individual animals are presented with mean value indicated by horizontal bar for each group (*n*=5).

4.5 Discussion

In the present investigation, we provide evidence for the existence of two B cell sub-populations present in JPP of sheep; (i) a regulatory B cell ($CD72^{+}CD21^{+}$) and (ii) an effector B cell ($CD72^{+}CD21^{-}$) population. The regulatory B cells secreted IL-10 and the effector B cells secreted IL-12, IFN-γ and IgM. This is consistent with a model proposed by Mizoguchi and Ban [3]. Furthermore, we confirmed that regulatory $CD21^{+}$ B cells were the only source of detectable IL-10 secretion by ovine PP B cells.

Regulatory B cells are now recognized as an important component of the immune system and are characterised by IL-10 secretion [3]. They were first reported by Janeway and colleagues [125] who provided evidence for a direct regulatory role of B cells in experimentally induced autoimmune encephalomyelitis (EAE) [125, 126]. Further evidence of B_{regs} have been shown in other disease models [3]. The mechanisms that regulate B_{reg} development and function are not yet clear, but various molecules, including TLR ligands, CD154 (CD40L), foreign antigens, and IL-21, were shown to promote differentiation of B cells to B_{regs} by signalling through cognate receptors on B cells [127, 166, 167]. Accumulating evidence suggests that the B_{regs} cell population is heterogeneous. Thus, this population can be derived from all B cells when presented with the correct stimulatory context and appropriate duration of stimulus [168].

It is thought that IL-10 is the primary mediator in the immune regulation mediated by B_{regs} [1]. IL-10 mediates suppression of inflammation by several mechanisms, including down regulation of the production of pro-inflammatory cytokines, such as IL-6, IL-12, IL-

17, IL-18, IFN-γ, TNF-α and the expression of MHC class II [143-145]. We have previously reported that $CD21^+$ B cells produce IL-10 [1, 76]. However, the B cells used in these earlier studies were obtained by MACS enrichment and had a purity of 95%. It was possible that contaminating cells, such as FDCs, may have contributed, to the IL-10 production. To address this issue, we negatively sorted B to increase B cell purity to 99%. Using these negatively sorted B cells we have now confirmed that only $CD21^+$ PP B cells secrete IL-10. All the other cells evaluated in the PP including DCs, T cells, monocytes and macrophages did not spontaneously secrete detectable levels IL-10. Thus, using highly purified $CD21^+$ B cells, we have confirmed that B_{regs} within the $CD21^+$ B cell population are the only source of spontaneously secreted IL-10 when cell were isolated from sheep PP. Furthermore, these $CD21^+$ B_{regs} do not secrete detectable levels of IL-12, IFN-γ, or IgM with or without CpG ODN stimulation.

Effector B cells are positive regulators of immune response and can be divided into two subfamilies on the basis of cytokine secretion. B effector 1(Be1) cells are characterised by a Th1-like cytokine secretion pattern and B effector2 (Be2) cells are characterised by a Th2-like cytokine secretion pattern [9, 136]. Effector B cells terminally differentiate into plasma cells that have undergone Ig isotype switching and secrete antibodies [138]. In the present investigation, we provide evidence for the existence of an effector B cell population ($CD72^+CD21^-$) in the PP which produces IL-12, IFN-γ, and IgM. However, further studies are required to determine whether they can be further subdivided into Be1 or Be2 cells. Other cells known to express CD72 including follicular dendritic cells, NK cells, splenic red

pulp macrophages (but not on peripheral blood monocytes), and liver Kupffer cells [109, 169].

Interestingly, we found that $CD72^{+}CD21^{-}$ effector B cells produce IFN-γ upon stimulation with CpG ODN. IFNs are known for their antiviral activity and ability to regulate the functions of cells of innate and adaptive immunity. IFN-γ is a potent activator of the antimicrobial functions of phagocytes, and has an essential role in resistance to many pathogenic bacteria, fungi and intracellular parasites. It is produced primarily by natural killer (NK) cells and by the bystander activation of particular subsets of T cells, for example, NKT cells, $CD8^{+}$ T cells, $CD4^{+}$ T cells and γδ T cells, by dendritic cells (DCs) and B cells [170, 171]. B cells with innate immune functions that produce IFN-γ in huge amounts have recently been discovered [172]. These effector B cells produce IFN-γ in response to challenge with *Listeria monocytogenes, Escherichia coli,* vesicular stomatitis virus and Toll-like receptor ligands. They promote innate responses against intracellular bacterial infection by secreting IFN-γ through a feedback mechanism [172]. The difference between these effector B cells and our effector B cells is that the effector B cells, as described by others, are seen during infection or in disease conditions whereas our investigations reveal that JPP effector B cells exist in healthy animals.

B cells from sheep produce IgM in response to CpG stimulation [2]. It was previously reported that the $CD21^{-}$ population can secrete IgM [2]. We have now confirmed that the $CD21^{-}$ population includes B cells that are $CD72^{+}CD21^{-}$ and $CD72^{-}$CD21. This is consistent with the presence of an effector B cell population in the PP. We were unable to

further fractionate these cells further into the $CD72^{-}CD21^{-}$ population due to very low cell numbers.

Also of interested was the finding that B cells that had undergone isotype switching secrete similar levels of IL-10. It may be that some B cells need to undergo isotype switching to acquire the capacity to secrete IL-10. Alternatively, B_{reg} function may present following isotype switching. The isotype switched B_{regs} may be similar to the recently discovered inducible B_{regs} detected in the blood of sheep infected with bovine leukaemia virus that secrete IL-10 spontaneously and can be induced by CpG ODN to secrete IL-10 (unpublished observation).

The significance of the presence of effector and regulatory B cells in the PP is that they may ensure that effector immune responses generated in intestines can be appropriately regulated to avoid chronic inflammation and pathology. Further investigations are required to provide insight into how these functions are integrated in PP. In conclusion, this study confirms that only $CD21^{+}$ PP B cells spontaneously secrete IL-10. We therefore conclude that B_{regs} are present in this population. Furthermore, we demonstrated that both effector and regulatory B cell populations co-exists in the PP and that isotype-switched B cells may also include B_{regs}.

CHAPTER 5: DISTRIBUTION OF IL-10 SECRETING CD21$^+$ B_{regs} IN OVINE LYMPHOID TISSUES AND THE EXISTENCE OF B_{regs} IN NEONATAL CALVES

Relationship of this study to the dissertation.

In Chapter 4, we confirmed that only CD21$^+$ PP B cells spontaneously secrete IL-10. We also demonstrated that both effector and regulatory B cell populations co-exists in the PP and that isotype-switched B cells may also include B_{regs}. We therefore wondered whether ovine B_{regs} exist in other lymphoid tissues besides PP and whether they are present following isotype switching. We also hypothesized that B_{regs} are present in cattle and are not unique to sheep. Our investigations in this chapter provides evidence that B_{regs} exist at varying frequencies in both mucosal and systemic tissues. In addition, we confirmed the presence of CD21$^+$ B_{regs} in bovine JPP.

5.1 Abstract

We have confirmed that IL-10 secreting CD21$^+$ regulatory B cells (B_{regs}) can be isolated from jejunal Peyer's patches (PP) and this IL-10 production suppresses secretion of IL-12 and IFN-γ. However, it is not known whether these B_{regs} are restricted to JPP or also present in other lymphoid tissues. It is also not known whether B_{regs} are present in other ruminant species. Therefore, CD21$^+$ B cells were purified from the jejunal PP of sheep and cattle as well, and from ovine mesenteric lymph nodes (MLN), tonsils, bronchial lymph nodes (BLN), PBMC, and spleen using magnetic cell sorting. Purified CD21$^+$ B cells were then stimulated with the TLR9-agonist, CpG ODN (CpG oligodeoxynucleotide). The frequency of IL-10-secreting cells in each tissue was evaluated by ELISPOT. We detected IL-10 secreting cells

in $CD21^+$ B cells in both mucosal (jejunal PP, bronchial, MLN and tonsils) and systemic (spleen and blood) lymphoid tissues, and the frequency of $CD21^+$ B cells secreting IL-10 was not changed by CpG ODN stimulation. Mucosal lymphoid tissues (Bronchial and mesenteric LNs and JPP) had high numbers of IL-10 secreting B cells, with JPP having the highest numbers of IL-10 secreting cells while tonsils had the least. The frequency of IL-10 secreting B cells was low in blood and spleen. Our investigations provide evidence that B_{regs} exist at varying frequencies in both mucosal and systemic tissues. In addition, we confirmed the presence of $CD21^+$ B_{regs} in bovine JPP.

Key words: B_{regs}; Bovine; CpG ODN; ELISPOT; MLN**;** regulatory cells; Peyer's patches; sheep; TLR9

5.2 Introduction

The idea that B cells could regulate immune responses originated in 1974, when the ability of B cells to suppress delayed-type hypersensitivity responses in guinea pigs was described [123, 173]. About two decades later, Janeway (1996) and colleagues were the first to provide direct evidence for a direct B cell regulatory role in a mouse model (μMT – a strain genetically deficient in B cells) of experimentally induced autoimmune encephalomyelitis (EAE) [125, 126]. Mizoguchi and colleagues later coined the term regulatory B cells (B_{regs}) to designate B cells with regulatory properties independent of secreted immunoglobulins [122] [3]. B_{regs} exert their regulatory activities through IL-10 production. Regulatory B cells have thus become a focus of increased investigations in recent years, but currently all these studies have used mice.

Ovine $sIgM^+$ $CD21^+$ B cells are representative of naive B cells, are localized in the B cell follicles of spleen, LNs and PP's, and are actively re-circulating [107, 108]. We recently reported that ovine $CD21^+$ B cells isolated from jejunal PP's produced IL-10 with a regulatory function and these B_{regs} were present in the fetus prior to antigen exposure [1, 76]. However, it is not known whether ovine B_{regs} exist in other lymphoid tissues besides PP and whether they are present following isotype switching. It is also not known whether B_{regs} are present in other ruminant species or are unique to sheep. $CD21^+$ B cells are also present in PP of cattle, so we investigated whether B_{regs} may also be present in cattle.

5.3. Materials and methods

5.3.1 Animals

Suffolk sheep of either sex, between 3-4 months old, and male calves of either sex, between 1-2 weeks of age were obtained from the Department of Animal and Poultry Science (University of Saskatchewan, Saskatoon, SK, Canada). Experiments were conducted in accordance with the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care. All experimental protocols were approved by the University of Saskatchewan Animal Care Committee.

5.3.2 CpG Oligodeoxynucleotides

The CpG ODN 2007 and CpG ODN 2007 GC were obtained from Merial limited (Lyon, France). There sequences and backbone structures; were previously discussed [76].

5.3.3 Enzyme-linked immunosorbent SPOT assay (ELISPOT) for IL-10

Jejunal Peyer's patches, bronchial lymph nodes (BLN), mesenteric lymph nodes (MLN), tonsils, spleen and PBMC were isolated from the respective tissues and $CD21^+$ and $CD21^-$ cell populations were fractionated from these populations using magnetic cell sorting. The frequency of IL-10-producing cells within unfractionated, $CD21^+$ and $CD21^-$ JPP, BLN, MLN, tonsils, spleen and PBMC cells was determined by ELISPOT. Flat-bottom, polyvinylidene difluoride-coated, 96-well plates (Millipore, Bedford, MA, USA) were pre-wet with 100 µL 70% ethanol/well (Sigma Chemical Co.), washed with PBS, and coated overnight at 4°C or for 1 h at 37°C with mouse anti-recombinant bovine IL-10 CC318 (Serotec MCA 2110) diluted to 1.0 µg/mL in coating buffer. Plates were then washed four

times with Tris buffered saline /0.05% Tween 20 (Sigma-Aldrich) (TBST) then $5x10^5$ cells were seeded in 100 µl AIMV medium per well in triplicate wells. Plates were incubated at 37°C, 5% CO^2, for 24 h, washed 4 times with PBS, and incubated for 2 h with 0.125 µg/well biotinylated mouse anti-bovine IL-10 antibody (Serotec MCA 2111B). Plates were washed, and 100 µL of streptavidin-alkaline phosphatase (Mabtech, 1:1000 dilution) was added for 1 h. After washing, 100 µL freshly prepared NBT chloride (3 mg/mL) / 5-bromo-4-chloro-3-indolyl phosphate-p-toluidine (1.5 mg/mL) substrate (BioRad, Hercules, CA, USA) in Tris buffer (pH 9.5) was added to each well for 20 min. Plates were washed with distilled water to stop the reaction and air-dried overnight before spot enumeration. The number of IL-10-producing cells per million cells from each tissue were determined by multiplying the number of spots / well by 2 and then averaging values from triplicate wells. U/ml (units per ml) for IL-10 was defined previously by Kwong et al [140], as the biological activity of IL-10 with one unit being the reciprocal of the IL-10 dilution that inhibited IFN-γ secretion by 50 % of Cos-7 cells.

5.3.4 Lymphocyte Proliferative responses

Bovine lymphocyte proliferative responses were assayed according to previously described procedures described [76].

5.3.5 Isolation of Jejunal Peyer's patches, bronchial lymph nodes (BLN), mesenteric lymph nodes (MLN), tonsils, spleen and PBMC cells

Lambs were euthanised and JPP, BLN, MLN, tonsils, spleen tissues immediately removed and placed in ice-cold DMEM (GibcoBRL) containing antibiotics (100 U/mL), Penicillin, 100 µg/mL Streptomycin sulfate, 0.25 µg/mL Amphotericin B (Sigma-Aldrich).

PBMC were isolated as previously described [1]. Single cell suspensions were isolated from JPP, BLN, MLN, tonsils, spleen and blood as described previously [141]. The number of viable cells isolated from all tissues was determined by trypan blue dye exclusion and viable cells were counted with a hemocytometer under a light microscope. Cells were re-suspended at the appropriate concentrations in AIM V medium, (supplemented with 2% FBS, 100U/mL penicillin, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B, 2Mm L-glutamine, 50 μM 2-mercaptoethanol and 10 μg/mL polymyxin B sulfate (Sigma- Aldrich) and added in 100 μ/well in round bottomed 96-well plates (Nunc, Naperville, IL, USA).

5.3.6 Magnetic activated cell sorting (MACS)

JPP, BLN, MLN, tonsil and spleen cells suspensions and PBMC were stained with mouse anti-bovine CD21 (IgG1 isotype; AbD Serotec, UK) for 15 min at 4^0C. The JPP, BLN, MLN, tonsils, spleen and PBMC cells were then washed twice with MACS buffer (PBSA, 0.5M EDTA, 10% BSA). Cells were pelleted by centrifugation for 8 min at 440 x g. The JPP, BLN, MLN, tonsil and spleen cells suspensions and PBMC were then stained with goat anti-mouse IgG1 phycoerythrin (PE) conjugate (ABD Serotec, UK) for 15 min at 4^0C before washing as above. The JPP, BLN, MLN, tonsils, spleen and PBMC cells were then incubated with anti-PE magnetic beads for 15 min at 4^0C and passed through the LC MACS column (Miltenyi Biotec, Bergish Gladbach, Germany) according to manufacturer's instruction. The $CD21^+$ B cell fraction was eluted, washed in PBSA and re-suspended in AIM V media

5.3.7 Tissue culture conditions and stimulation with TLR agonists

Isolated $CD21^+$ cells were re-suspended in AIM V media. Aliquots of $5x10^5$ cells for unfractionated JPP, BLN, MLN, tonsils, spleen and PBMC cells, $5x10^5$ for $CD21^+$ cells and $3x10^5$ cells for $CD21^-$ cells were cultured in triplicate wells in a final volume of 200 μL. Cells were stimulated with B-class CpG 2007 or GpC 2007 at 5 μg/mL and then incubated at 37°C for 48 hours in a 5% CO_2 atmosphere and 95% humidity. For optimal detection of cytokines, cells were stimulated for 48 hrs as previously described [141]. Culture supernatants were stored at -20ºC until assayed for secreted cytokines.

5.3.8 Enzyme-linked immunosorbent assay (ELISA) for IFN-γ, IFN-α, IL-12 and IL-10

The IFN-γ, IFN-α, IL-12 and IL-10 enzyme-linked immunosorbent assays used in these studies were shown previously to detect ovine and bovine IFN-γ, IFN-α, IL-12 and IL-10 cytokines as described elsewhere [141, 160, 174].

5.3.9 Statistical analysis

Data analysis was performed with the statistical software program, Graph Prism 5 (Graphpad software). Data that were not normally distributed were transformed by ranking. Individual group differences were examined by performing one way analysis of variance (ANOVA). Values of $p<0.05$ and $p<0.01$, were considered significant and very significant respectively. Kruskal-Wallis test and Dunn's test was used to perform the post-hoc tests.

5.4. RESULTS

We have previously shown that IL-10 secreting $CD21^+$ B_{regs} exist in jejunal PPs of lambs of different ages [76]. However, it is not known whether these B_{regs} are also present in other lymphoid tissues. We therefore performed experiments to determine the frequency of B_{regs} in the spleen, mesenteric lymph node (MLN) PBMC, tonsils and bronchial lymph nodes (BLN) and compared to JPP.

5.4.1 Frequency of IL-10 secreting cells in JPP

The frequency per million cells of IL-10 secreting cells present in JPP of 3-4 month old lambs was determined with an ELISpot essay. There was on average 207 (90 - 372 cells per million) IL-10 secreting cells per million cells in the unfractionated JPP population. There was, however, no change in the frequency of IL-10 producing B cells following stimulation with CpG or non CpG ODN (Fig 13a). Similarly, enriched JPP $CD21^+$ B cells had a similar frequency of IL-10 secreting cells (124 - 500 cells per million). The number of $CD21^+$ B cells secreting IL-10 did not change significantly even after stimulation with CpG or non GpC ODN (Fig 13a). There were however, no or few (0 - <100 cells per million) IL-10 secreting cells in the unstimulated $CD21^-$ cell population and this did not change with CpG stimulation. These results are consistent with our previous observations in PP cells isolated from this age group of lambs [1, 76].

5.4.2 *Frequency of IL-10 secreting cells in bronchial lymph nodes (BLN)*

There were on average 80 (6 -142 cells per million) IL-10 secreting cells per million cells in the unstimulated BLN cell unfractionated population. This frequency did not change following stimulation with CpG or GpC ODN (Fig 13b). Similarly, enriched unstimulated

BLN $CD21^+$ B cells contained IL-10 secreting cells with an average frequency of 70 IL-10 secreting cell per million somatic cells and this frequency did not change significantly following CpG or non-CpG ODN stimulation (Fig 13b). There were relatively (8 to <38 cells per million) fewer IL-10 secreting cells in the unstimulated $CD21^-$ cell population, and this frequency did not change with CpG or non-CpG ODN stimulation. These results confirm that $CD21^+$ B cells spontaneously secreting IL-10 are present in BLN.

5.4.3 Frequency of IL-10 secreting cells in Mesenteric Lymph Node (MLN)

There were on average 37 (10 - 80 cells per million) IL-10 secreting cells per million unfractionated and unstimulated MLN cells. The frequency of spontaneous IL-10 secreting cells in the MLN cell population was not significantly increased following stimulation with CpG or non CpG ODN (Fig 13c). Enriched MLN $CD21^+$ B cells had spontaneous IL-10 secreting cells, with on average 32 IL-10 secreting cells per million cells and this did not significantly change following CpG or non CpG ODN stimulation (Fig 13c). There were very few IL-10 secreting cells in the $CD21^-$ cell population following CpG or non CpG stimulation. The average frequency of IL-10 secreting cells in the $CD21^-$ MLN cell population was 19 cells per million leukocytes.

5.4.4 Frequency of IL10 secreting cells in the Tonsils

The IL-10 ELISpot analysis of IL-10 secreting cells in the tonsils revealed an average of 16 spontaneous IL-10 secreting cells per million leukocytes (8 – 22 cells per million) in the tonsils. The frequency of IL-10 secreting cells did not increase following stimulation with CpG or non CpG ODN (Fig 13d). Similarly, enriched $CD21^+$ B cells had an average

frequency of 17 spontaneous IL-10 secreting cells per million leukocytes and IL-10 secreting cell frequency was not significantly increased following CpG or non CpG ODN stimulation (Fig 13d). In the $CD21^-$ population, no IL-10 secreting cells were detected either before or after CpG or non CpG stimulation (Fig 13d).

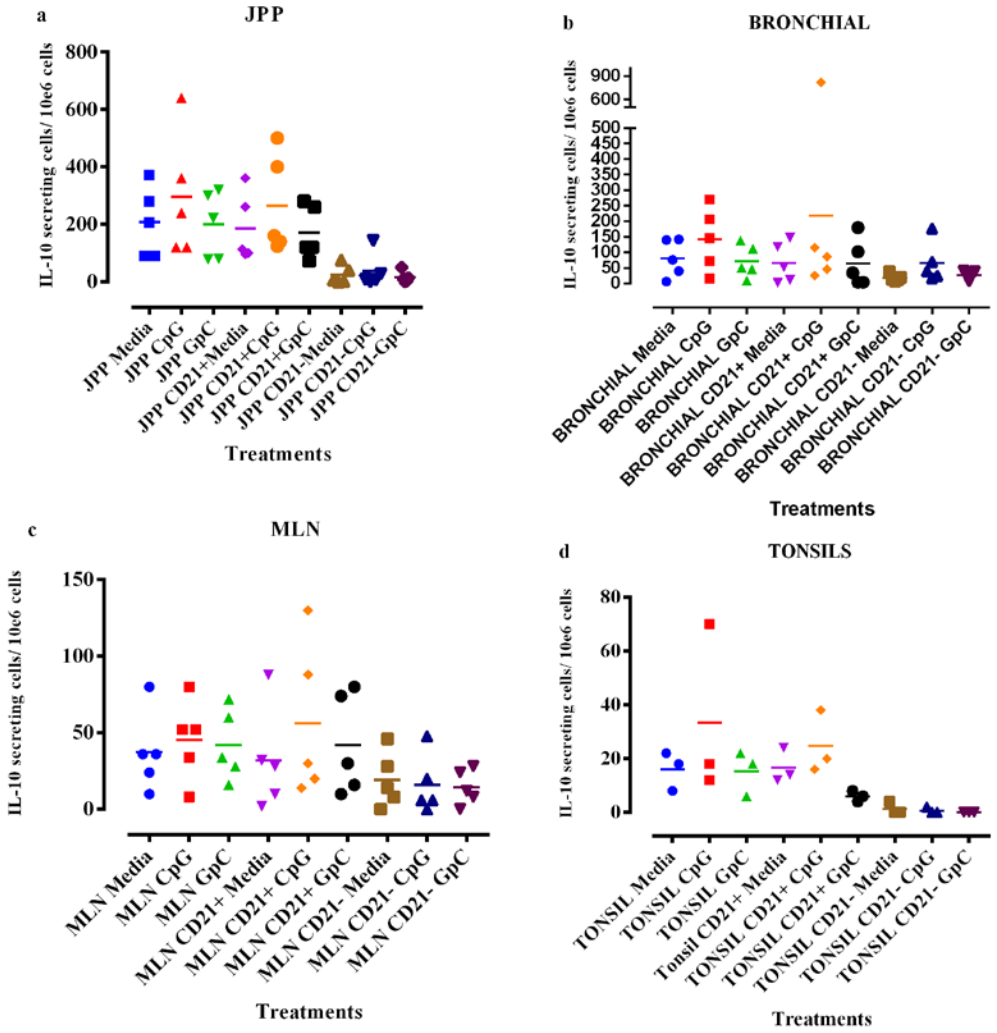


Figure 13. IL-10 secreting cells in JPP (a), BLN (b), MLN (c) and tonsils (d). Unfractionated, $CD21^+$ B cells and $CD21^-$ JPP, BLN, MLN and tonsil cells were cultured for 48 hours in medium alone, or in the presence of 5 µg/ml of CpG or GpC ODN. The frequency of IL-10 secreting cells was enumerated using ELISpot. Data for individual animals are presented with mean value indicated by horizontal bar for each group.

5.4.5 Frequency of IL-10 secreting cells in the systemic lymphoid tissues (spleen and blood)

5.4.5.1 Frequency of IL-10 secreting cells in PBMC

The frequency of spontaneous IL-10 secreting cells in the unfractionated PBMCs was low with on average 30 cells per million leukocytes (6 – 52 cells per million) and this frequency did not change significantly following stimulation with CpG ODN (Fig 14a). Enriched $CD21^+$ PBMC had a similar low frequency of IL-10 secreting cells (50 IL-10 secreting cells per million cells) and the frequency of IL-10 secreting cells did not increase significantly following CpG ODN stimulation (Fig 14a). The $CD21^-$ PBMC fraction had very few IL-10 secreting cells (0 to 62 cells per million) and this frequency, (15 cells per million) did not change significantly after stimulation with CpG or non CpG ODN.

5.4.5.2 Frequency of IL-10 secreting cells in the spleen

There were on average 51 spontaneous IL-10 secreting cells (0 – 180 cells per million) in unfractionated splenocytes. This frequency did not change significantly following stimulation with CpG or non CpG ODN (Fig 14b). Similarly, enriched $CD21^+$ splenocytes spontaneously secreted IL-10 with an average of 130 IL-10 secreting cells per million (26 – 280) and their frequency was not significantly changed following CpG or non CpG ODN stimulation (Fig 14b). There were, however, very few IL-10 secreting $CD21^-$ cells (0 – 11 cells per million), with or without CpG or non CpG ODN stimulation.

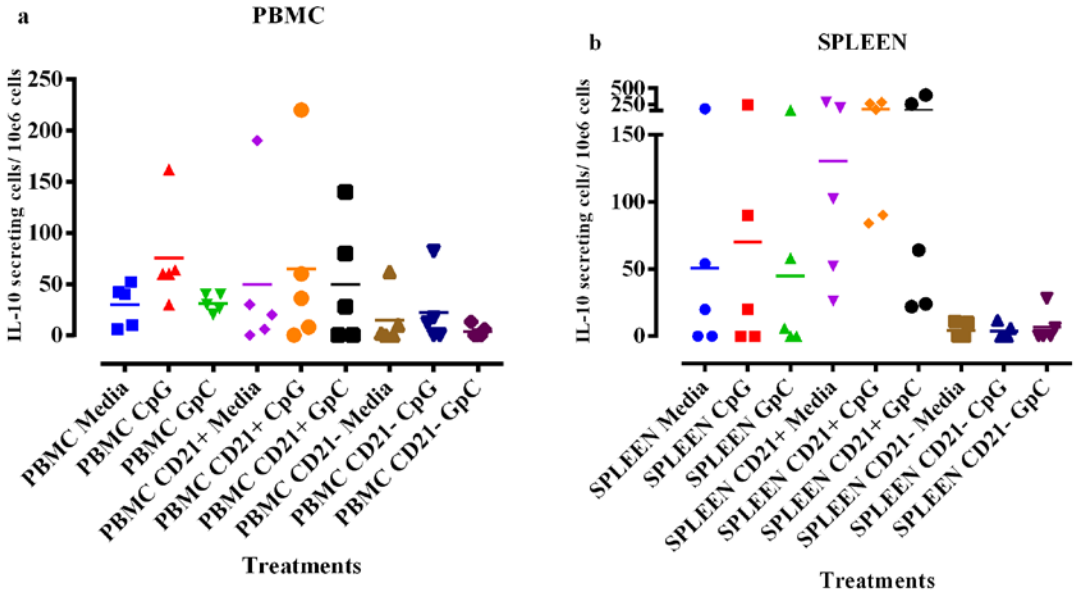


Figure 14. IL-10 secreting cells in PBMC (a) and spleen (b). Unfractionated, $CD21^+$ and $CD21^-$ PBMCs and splenocytes were cultured for 48 hours in medium alone, or in the presence of 5 µg/ml of CpG or GpC ODN. The frequency of IL-10 secreting cells was enumerated using ELISPOT. Data for individual animals are presented with mean value indicated by horizontal bar for each group (*n*=5).

5.4.7 IL-10 production by neonatal bovine $CD21^+$ B cells

We previously reported that PP $CD21^+$ B cells from sheep spontaneously secrete IL-10 and we called these cells B_{regs} [76]. These cells dampen other pro-inflammatory responses in the PP [76]. We therefore performed experiments to determine whether B_{regs} were also present in the JPP of newborn (1-8 days old) calves.

Unfractionated JPP cell population spontaneously secreted significant ($P<0.05$) levels of IL-10 when compared to the $CD21^-$ cell population. Stimulation of these cells with CpG

or GpC did not result in a significant change in the level of IL-10 secretion (Fig 15a). Similarly, unstimulated $CD21^+$ B cells secreted IL-10 and stimulation with CpG or GpC did not alter in IL-10 secretion (Fig 15a). There was no detectable IL-10 secretion by the stimulated or unstimulated $CD21^-$ cell populations. These results confirm that bovine $CD21^+$ B cells are the only source of IL-10 in JPP of bovine neonates and that these cells spontaneously secrete IL-10 spontaneously.

5.4.8 IL-12 production by bovine neonatal JPP cells

Previous work in our laboratory has shown that B_{regs} from ovine PP from sheep spontaneously secreted IL-10 but not IL-12 or IFN-γ [1]. We therefore evaluated IL-12 production by bovine $CD21^+$ B cells.

There was very low spontaneous IL-12 secretion by unfractionated JPP cells (Fig 15b). Stimulation of JPP cells with CpG or non CpG ODN (GpC) did not significantly change IL-12 secretion. Furthermore, IL-12 secretion was not detected in unstimulated $CD21^+$ B cells or following CpG or GpC ODN stimulation. These results are consistent with previous observations [76]. Interestingly, $CD21^-$ JPP cells spontaneously secreted IL-12 and IL-12 secretion increased significantly following CpG ODN stimulation (Fig 15b). Thus, stimulation of JPP $CD21^+$ does not induce IL-12 production, but CpG stimulated JPP $CD21^-$ secrete detectable levels of IL-12.

5.4.9 IFN-γ production by bovine neonates PP B cells

Low levels of spontaneous IFN-γ secretion were observed with unfractionated

cells (Fig 15c). There was however no significant change in IFN-γ levels following stimulation with CpG or GpC ODN. There was no detectable IFN-γ secretion in the $CD21^+$ JPP B cell population following stimulation with CpG or GpC ODN which was consistent with our earlier observation [76]. There was however, a low level of spontaneous IFN-γ secretion by the $CD21^-$ JPP cells and stimulation with CpG or GpC ODN significantly increased IFN-γ production (Fig 15c). $CD21^-$ JPP cells were the only source of IFN-γ in the bovine JPP. Therefore, bovine PP $CD21^+$ did not secrete IFN-γ, but PP $CD21^-$ cells did secrete this cytokine following CpG ODN stimulation.

5.4.10 IFN-α production by neonatal bovine PP B cells

Low levels of IFN-α were spontaneously secreted by unfractionated JPP cells (Fig 15d) and there was no significant change in IFN-α secretion following stimulation with CpG or GpC ODN. Interestingly, there was no detectable spontaneous IFN-α secretion by $CD21^+$ JPP B cells. Furthermore no IFN-α secretion was detected following CpG or GpC ODN stimulation. These results are consistent with our previous observations with ovine JPP cells [1]. Moreover, there was a low level of spontaneous IFN-α secretion by $CD21^-$ JPP cells. The levels of IFN-α increased significantly compared to the $CD21^+$ JPP cells following stimulation with CpG. This result indicates that the $CD21^-$ cell population was the major source of IFN-α secreting cells in the PP (Fig 15d). Therefore, bovine PP $CD21^+$ do not secrete IFN-α, but bovine PP $CD21^-$ secrete this cytokine following CpG stimulation.

5.4.11 Lymphocyte proliferation responses of bovine neonatal PP B cells

We have previously reported that ovine JPP $CD21^+$ B cells (B_{regs}) do not proliferate [76]. Therefore, we investigated whether bovine $CD21^+$ JPP B cells could also proliferate. Unfractionated bovine JPP cells displayed a significant proliferative response when stimulated with CpG ODN (Fig 15e). Similarly, $CD21^-$ JPP cells significantly proliferated following CpG ODN stimulation. In contrast, bovine $CD21^+$ B cells did not proliferate following CpG ODN stimulation (Fig 15e). These data indicate that bovine $CD21^+$ PP B cells do not proliferate in direct response to CpG ODN stimulation. They may require other cells such as myeloid cells to respond to CpG stimulation [175].

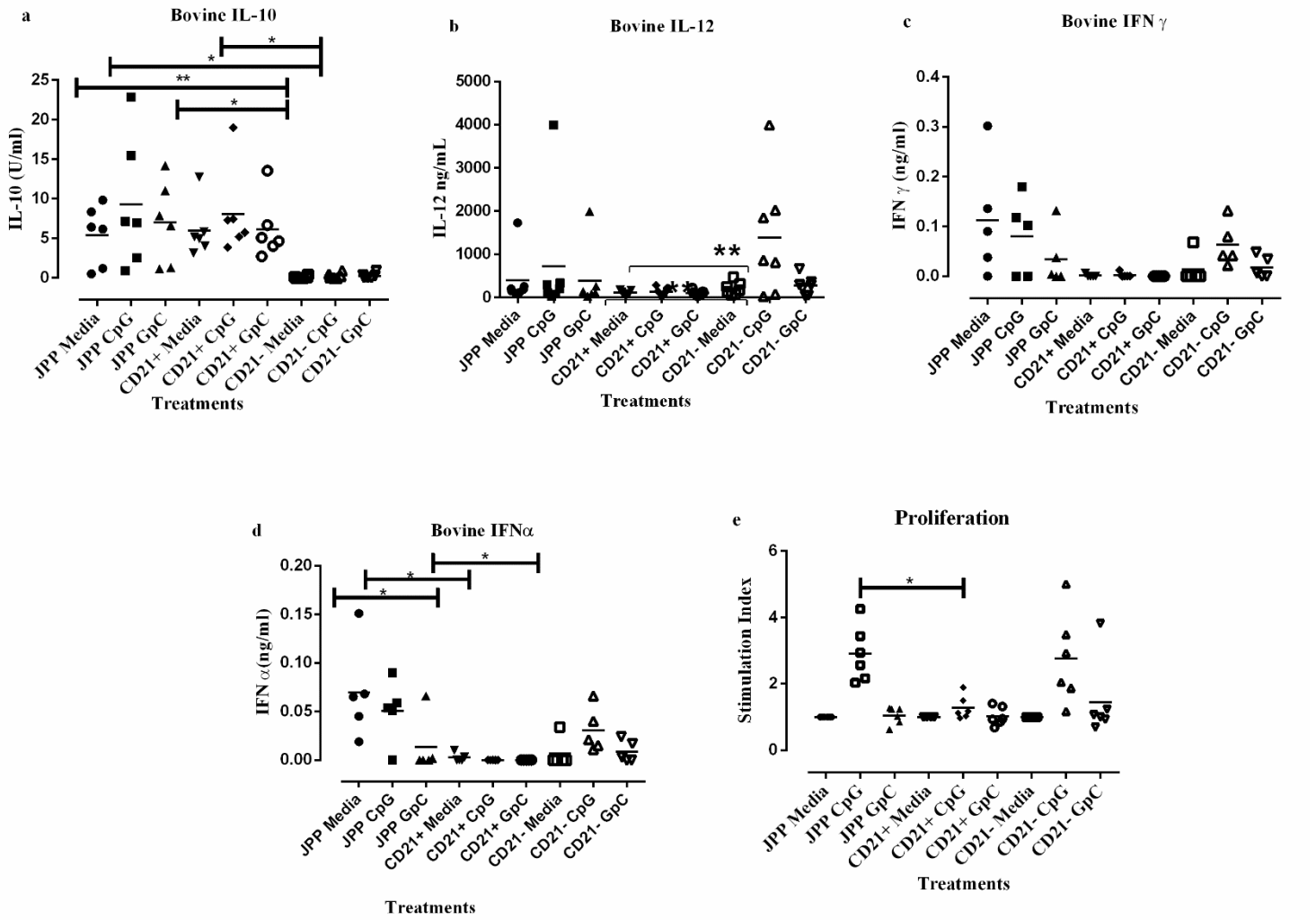


Figure 15. Interleukin (IL)-10 (a), IL-12 (b), IFN-γ (c) and IFN-α (d) Secretion and proliferative responses (e) of neonatal bovine JPP cells. Unfractionated JPP (JPP), $CD21^+$ JPP B cells ($CD21^+$) and $CD21^-$ JPP cells ($CD21^-$) were cultured for 48 hours in medium, or in the presence of 5 µg/ml CpG or GpC ODN. IL-10, IL-12, IFN-γ and IFN-α production was evaluated in the culture supernatants by ELISA. For proliferative responses, cells were cultured for 66 hours in medium alone or in the presence of 5 µg/ml CpG or GpC ODN. Radioactive thymidine was then added in the cultures and cells incubated for an extra 6 hours to quantify proliferative responses. Data for individual animals are presented with mean value indicated by horizontal bar for each group (*n*=5).

5.5 Discussion

In this paper, we surveyed the frequency of IL-10 producing B_{regs} cells, in mucosal and systemic lymphoid tissues of young sheep. We further investigated whether IL-10 producing B_{regs} were unique to sheep or also present in another ruminant species. We observed that IL-10 secreting $CD21^+$ B cells were present in both mucosal and systemic lymphoid tissues. These investigations provide evidence that IL-10 secreting B_{regs} are distributed in a variety of musocal and systemic tissues, but their frequencies vary. We also confirmed that IL-10 secreting B_{regs} were not unique to sheep but are also present in cattle.

Our first aim was to survey the existence of $CD21^+$ B_{regs} in the various tissues of sheep. Two subpopulations of B cells have been described in sheep. These two B cell sub-populations have distinct recirculation characteristics and tissue distribution [108]. Phenotypically, the two subpopulations are distinguished by their surface expression of complement receptors, CD21 (CR2) and CD11b/ CD18 (CR3) [108]. The $CD11b^+$ B cells are non-circulating B cells. Unlike these cells, the $CD21^+$ B cells are sIgM low and co-express L- selectin. They populate the splenic and PP follicles, are absent in the splenic marginal zones and are the only B cells found in afferent and efferent lymph and all lymph nodes [108]. In this experiment we show that the frequency of IL-10 secreting $CD21^+$ B cells varies significantly in different lymphoid tissues. These $CD21^+$ B cells are similar to the cells Gupta et al, described in sheep since they are also variedly distributed among lymphoid tissues [108].

The reason for this variation in B_{reg} frequency is presently unknown but should be subject of future investigations. One reason may be due to lymphocyte re-circulation that ensures B_{regs} are distributed throughout the body, similar to T_{regs} recirculation [176]. It may also be that some tissues have requirements for a higher frequency of B_{regs}. For example, mucosal tissue may require a high frequency of B_{regs} due to constant exposure to microbial products at these sites. B_{regs} may be required to control the level of immune activation and avoid chronic inflammation. Some IL-10 secreting cells were also present among the $CD21^-$ population isolated from different tissues. This may be due to contamination of IL-10 secreting B cells with the $CD21^-$ cell fraction as a result of using the MACs for fractionating JPP cells into $CD21^+$ and $CD21^-$ subpopulations.

In our second investigation of B_{regs} in neonatal calves, we observed that IL-10 secreting $CD21^+$ B cells could be isolated from the JPP of newborn calves. These results are similar to our previous observations with ovine JPP [1, 76]. Furthermore, we report that bovine $CD21^+$ PP B cells do not secrete IL-12 and IFN-γ, either with or without CpG or GpC ODN stimulation. IL-12 is a pro-inflammatory cytokine that influences development of Th-1 type responses and is primarily produced by monocytes, macrophages and dendritic cells following microbial stimulation. T cells and NK cells up-regulate IFN-γ production, proliferate and carry out cytotoxic activity following stimulation with IL-12 [152]. In this study, IL-12 was secreted by the bovine $CD21^-$ JPP subpopulation but not by $CD21^+$ B cells population (B_{regs}). Similar results were seen when PP cells were assayed for IFN-γ secretion. As had been shown before for IL-12, the B_{regs} did not produce IFN-γ. Therefore, bovine $CD21^+$ B cell population do not secrete the pro-inflammatory cytokines, IL-12 and IFN-γ but

produce the anti-inflammatory cytokine IL-10. This observation provides evidence that $CD21^+$ B cells, with a B_{reg} phenotype also exists in cattle. Also, these purified $CD21^+$ B cells did not proliferate in response to CpG ODN stimulation [175]. In contrast, bovine PP $CD21^-$ cells produced detectable levels of IL-12, IFN-γ and did proliferate after stimulation with CpG. Therefore consistent with our results in Chapter 4, bovine PP B cells may also be subdivided into a regulatory ($CD21^+$) and an effector ($CD21^-$) B cell population.

In conclusion, we confirmed that IL-10 secreting $CD21^+$ B cells could be isolated from both mucosal and systemic lymphoid tissues in the sheep and do exist in PP of neonatal calves.

6.0 GENERAL DISCUSSION

The intestinal mucosa is constantly exposed to a diverse array of microbes, some of which are pathogenic. Although the real magnitude of the host exposure to pathogens and commensal microbes is difficult to assess, it appears that these encounters rarely result in inflammation and disease. Mucosal immune defense mechanisms are responsible for host protection, however the control of inflammatory responses is important in preserving the integrity of the mucosal barriers.

Peyer's patches are the primary sites for induction of antigen-specific immune responses in the intestinal tract. The intestine also expresses PRR, including TLR receptors which detects PAMPs from microbes [177]. Understanding how TLR responses are regulated in PP will provide valuable information on how innate immune responses are regulated in the intestines. MLNs are the key site for induction of tolerance to food proteins, but here they act as a firewall to preserve systemic ignorance of commensal organisms, rather than an obligatory site for induction of the adaptive response itself [178].

In this thesis, we started by studying the development of regulatory B cells in sheep PP. We clearly show that B_{regs} develop in utero prior to antigen exposure and are present in young sheep. This was achieved by demonstrating the presence of IL-10 secreting $CD21^{+}$ B in the JPP of fetal, neonatal and young lambs.

Like their T cell counterparts (T_{regs}), B_{regs} are capable of inhibiting inflammatory responses and inducing immune tolerance through the production of IL-10 and TGF [3,

142]. IL-10 is an immune regulatory cytokine with pleiotropic functions, including suppression of inflammation [143]. IL-10 is produced by different cells including B cells, monocytes, macrophage, neutrophils and DCs [122, 127, 131]. We show that in addition to the 3-4 month old lambs, neonatal and fetal lamb $CD21^+$ B cells also secrete IL-10 and presumably have a regulatory function. This information supports the conclusion that there is a competent mucosal immune system present at birth, capable of protecting the newborn against unnecessary inflammatory responses induced by enteric flora. The $CD21^+$ B cells isolated from fetal PPs would not have been exposed to exogenous antigens and yet they produced detectable levels of IL-10 meaning that PP B_{regs} production of IL-10 is antigen-independent and B_{reg} development occurs spontaneously. We therefore propose that PP may function as a site for the production of natural B_{regs} analogous to the thymus function as a site for natural T_{reg} generation [151]. Presumably the suppressive effects of IL-10 produced by PP $CD21^+$ B cells dampens potentially harmful inflammatory responses (e.g. IL-12 and IFN-γ) in response to CpG-DNA (and other PAMPs) following exposure to commensal microflora at the time of birth.

In the foetus, this suppressive effect may develop in preparation for colonization of the gut lumen with different microbiota which occurs during birth. We consistently observed that $CD21^+$ B cells isolated from the jejunal PP of older lambs do not proliferate in response to CpG ODN, results that were consistent with earlier observations [1]. However $CD21^+$ B cells isolated from the JPPs of fetal and neonatal lambs do proliferate following CpG ODN stimulation which may imply that at these early stages of development the B cells do not have a defect in the TLR9 signal transduction pathway as reported for older

lambs [2]. Alternatively, there may be insufficient B_{reg} activity (due to low numbers of B_{regs} in fetus) to block CpG ODN stimulation. The other possibility may be technical; perhaps a subpopulation of fetal and neonatal $CD21^-$ B cells capable of proliferating, co-purify with $CD21^+$B cells.

We confirmed that regulatory $CD72^+CD21^+$ B cells are the only detectable source of IL-10 in PP of sheep when using an ELISA. This is important given that in our previous studies, we used MACS to enrich the $CD21^+$ B cells population. This procedure could only achieve 95% purity, suggesting that the other 5% cell population may contribute to the IL-10 production within the $CD21^+$ B cells population. Additionally, DCs also express CD21 and may contribute to IL-10 production. By using a procedure that negatively sorts B cells, we were able to achieve a B cell purity in excess of 99%. Using this highly purified B cell population, we have definitively confirmed that B cells are the only source of IL-10 in PP. DC and other cell types tested did not contribute to IL-10 production.

In Chapter 3, we provide evidence for the existence of regulatory ($CD72^+CD21^+$) and effector ($CD72^+CD21^-$) B cell subpopulations in the JPP of sheep. To our knowledge, this is the first time effector B cells have been described in ovine PP. The effector B cell population ($CD72^+CD21^-$) in the PP produced IL-12, IFN-γ, and IgM following stimulation with CpG ODN. We confirmed that the $CD21^-$ population consists primarily of B cells that are $CD72^+CD21^-$, though it was impossible to completely eliminate the $CD72^-CD21^-$ population due to very low cell numbers. The presence of effector and regulatory B cells in the PP may

ensure that effector immune responses generated in the intestine can be appropriately regulated.

The frequency of IL-10 secreting B_{regs} is very low (<0.02%) within the $CD21^+$ population. The significance of this remains to be studied. T_{regs} exists in different subpopulations either as natural or inducible T_{regs} [179]. The sheep B_{regs} have not been studied under disease conditions and therefore it may be that their low numbers increase during disease, similar to T_{regs}. We propose that in disease conditions, some naturally occurring B cells may be induced to become B_{regs} as we have just discovered in an experiment with bovine leukaemia virus infected sheep. We observed that inducible B cells that secrete high levels of IL-10 were present in blood (data not included in thesis).

In chapter 4, we investigated the presence of IL-10 producing regulatory B cells in a second ruminant species. We confirmed that IL-10 secreting B_{regs} could be isolated from cattle and are not unique to sheep. We observed that among bovine $CD21^+$ B cells, there are cells that secrete IL10 but fail to secrete IL-12 or IFN-γ following stimulation with CpG or GpC ODN. These B cells also do not directly proliferate in response to CpG stimulation. These results are consistent with our earlier observations with ovine JPP cells [1, 76]. However, bovine B cells may be different from sheep B cells since they require co-stimulatory signals to proliferate in response to CpG ODN stimulation.

We also surveyed the distribution of IL-10 producing regulatory B cells, in ovine mucosal and systemic lymphoid tissues. These investigations provide evidence that IL-10

secreting B_{regs} are present at different frequencies within the $CD21^+$ B cell population isolated from different lymphoid tissues. As had been shown in our previous reports, the JPP contained the highest frequency of IL-10 secreting B_{regs} when compared to other mucosal tissues evaluated (Mesenteric Lymph nodes, Tonsils and Bronchial lymph nodes) and peripheral lymphoid tissues (PBMC, and Spleen). The reason for the high frequency of B_{regs} in the JPP's is not known but may be due to the fact that these PP are located in direct contact with the intestinal lumen and are continuously exposed to microbial molecules. It may be necessary to continually dampen inflammatory responses to maintain homeostasis. It is interesting to note that there were more IL-10 secreting cells in the mucosal associated lymphoid tissues (PP's, MLN and BLN), compared to the peripheral lymphoid tissues (PBMC and spleen). There also seems to be a difference in the secretion of IL-10 by JPP $CD21^-$ cells by ELISA and ELISpot. This may have resulted from technical issues emanating from the fractionation of the PP cells using MACS which may have resulted in some $CD21^+$ B cells co-purifying with the $CD21^-$ population. ELISpot is also a more sensitive assay than ELISA [180].

7.0 CONCLUSION

In conclusion, we demonstrate that regulatory B cells could be isolated from fetal lambs prior to antigen exposure, are present in newborns and 3-4 month old sheep and are widely distributed throughout mucosal and systemic lymphoid tissues. Furthermore, functionally similar $CD21^+$ B_{regs} were isolated from the JPP of neonatal calves. Moreover, we demonstrated that ovine JPP B cells can be functionally divided into two distinct subpopulations; a regulatory and an effector population. Further investigations are required to provide detailed information on how these two populations function to regulate immune responses in the intestine

References

1. Booth, J.S., et al., *A novel regulatory B-cell population in sheep Peyer's patches spontaneously secretes IL-10 and downregulates TLR9-induced IFNalpha responses.* Mucosal Immunol, 2009. **2**(3): p. 265-75.
2. Booth, J.S., et al., *TLR9 signaling failure renders Peyer's patch regulatory B cells unresponsive to stimulation with CpG oligodeoxynucleotides.* J Innate Immun, 2010. **2**(5): p. 483-94.
3. Mizoguchi, A. and A.K. Bhan, *A case for regulatory B cells.* J Immunol, 2006. **176**(2): p. 705-10.
4. Kumar, H., T. Kawai, and S. Akira, *Pathogen recognition in the innate immune response.* Biochem J, 2009. **420**(1): p. 1-16.
5. Kumar, H., T. Kawai, and S. Akira, *Pathogen recognition by the innate immune system.* Int Rev Immunol, 2011. **30**(1): p. 16-34.
6. Akira, S., K. Takeda, and T. Kaisho, *Toll-like receptors: critical proteins linking innate and acquired immunity.* Nat Immunol, 2001. **2**(8): p. 675-80.
7. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. **11**(5): p. 373-84.
8. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-20.
9. Medzhitov, R., *Toll-like receptors and innate immunity.* Nat Rev Immunol, 2001. **1**(2): p. 135-145.
10. Blasius, A.L. and B. Beutler, *Intracellular toll-like receptors.* Immunity, 2010. **32**(3): p. 305-15.
11. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, Jr., *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity.* Nature, 1997. **388**(6640): p. 394-7.

12. Beutler, B., *Innate immunity: an overview.* Mol Immunol, 2004. **40**(12): p. 845-59.

13. Barton, G.M. and J.C. Kagan, *A cell biological view of Toll-like receptor function: regulation through compartmentalization.* Nat Rev Immunol, 2009. **9**(8): p. 535-42.

14. Takeuchi, O., et al., *Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins.* J Immunol, 2002. **169**(1): p. 10-4.

15. Schroder, N.W., et al., *Lipoteichoic acid (LTA) of Streptococcus pneumoniae and Staphylococcus aureus activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved.* J Biol Chem, 2003. **278**(18): p. 15587-94.

16. Sen, G.C. and S.N. Sarkar, *Transcriptional signaling by double-stranded RNA: role of TLR3.* Cytokine Growth Factor Rev, 2005. **16**(1): p. 1-14.

17. Tapping, R.I., et al., *Toll-like receptor 4, but not toll-like receptor 2, is a signaling receptor for Escherichia and Salmonella lipopolysaccharides.* J Immunol, 2000. **165**(10): p. 5780-7.

18. Hayashi, F., et al., *The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5.* Nature, 2001. **410**(6832): p. 1099-103.

19. Henneke, P., et al., *Role of lipoteichoic acid in the phagocyte response to group B streptococcus.* J Immunol, 2005. **174**(10): p. 6449-55.

20. Crozat, K. and B. Beutler, *TLR7: A new sensor of viral infection.* Proc Natl Acad Sci U S A, 2004. **101**(18): p. 6835-6.

21. Triantafilou, K., et al., *TLR8 and TLR7 are involved in the host's immune response to human parechovirus 1.* Eur J Immunol, 2005. **35**(8): p. 2416-23.

22. Bauer, S., et al., *Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition.* Proc Natl Acad Sci U S A, 2001. **98**(16): p. 9237-42.

23. Zhang, X., et al., *IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25+CD4+ regulatory T cells.* Int Immunol, 2004. **16**(2): p. 249-56.

24. Zhang, D., et al., *A toll-like receptor that prevents infection by uropathogenic bacteria*. Science, 2004. **303**(5663): p. 1522-6.

25. Mishra, B.B., U.M. Gundra, and J.M. Teale, *Expression and distribution of Toll-like receptors 11-13 in the brain during murine neurocysticercosis.* J Neuroinflammation, 2008. **5**: p. 53.

26. Koblansky, A.A., et al., *Recognition of Profilin by Toll-like Receptor 12 Is Critical for Host Resistance to Toxoplasma gondii.* Immunity. **38**(1): p. 119-130.

27. Shi, Z., et al., *A novel Toll-like receptor that recognizes vesicular stomatitis virus.* J Biol Chem, 2011. **286**(6): p. 4517-24.

28. Oldenburg, M., et al., *TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification.* Science, 2012. **337**(6098): p. 1111-5.

29. Utaisincharoen, P., et al., *CpG ODN enhances uptake of bacteria by mouse macrophages.* Clin Exp Immunol, 2003. **132**(1): p. 70-5.

30. Klinman, D.M., *Use of CpG oligodeoxynucleotides as immunoprotective agents.* Expert Opin Biol Ther, 2004. **4**(6): p. 937-46.

31. Lim, W.H., et al., *Human plasmacytoid dendritic cells regulate immune responses to Epstein-Barr virus (EBV) infection and delay EBV-related mortality in humanized NOD-SCID mice.* Blood, 2007. **109**(3): p. 1043-50.

32. Fiola, S., et al., *TLR9 contributes to the recognition of EBV by primary monocytes and plasmacytoid dendritic cells.* J Immunol, 2010. **185**(6): p. 3620-31.

33. Krug, A., et al., *TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function.* Immunity, 2004. **21**(1): p. 107-19.

34. Coban, C., et al., *Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin.* J Exp Med, 2005. **201**(1): p. 19-25.

35. Krieg, A.M., et al., *CpG motifs in bacterial DNA trigger direct B-cell activation.* Nature, 1995. **374**(6522): p. 546-9.

36. Rankin, R., et al., *CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved.* Antisense Nucleic Acid Drug Dev, 2001. **11**(5): p. 333-40.

37. Brown, W.C., et al., *DNA and a CpG oligonucleotide derived from Babesia bovis are mitogenic for bovine B cells.* Infect Immun, 1998. **66**(11): p. 5423-32.

38. Kamstrup, S., D. Verthelyi, and D.M. Klinman, *Response of porcine peripheral blood mononuclear cells to CpG-containing oligodeoxynucleotides.* Vet Microbiol, 2001. **78**(4): p. 353-62.

39. Jorgensen, J.B., et al., *CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (Salmo salar L.) leucocytes to produce supernatants with antiviral activity.* Dev Comp Immunol, 2001. **25**(4): p. 313-21.

40. Pontarollo, R.A., et al., *Augmentation of cellular immune responses to bovine herpesvirus-1 glycoprotein D by vaccination with CpG-enhanced plasmid vectors.* J Gen Virol, 2002. **83**(Pt 12): p. 2973-81.

41. Verthelyi, D. and D.M. Klinman, *Immunoregulatory activity of CpG oligonucleotides in humans and nonhuman primates.* Clin Immunol, 2003. **109**(1): p. 64-71.

42. Wernette, C.M., et al., *CpG oligodeoxynucleotides stimulate canine and feline immune cell proliferation.* Vet Immunol Immunopathol, 2002. **84**(3-4): p. 223-36.

43. Zhang, Y., et al., *Induction of interleukin-6 and interleukin-12 in bovine B lymphocytes, monocytes, and macrophages by a CpG oligodeoxynucleotide (ODN 2059) containing the GTCGTT motif.* J Interferon Cytokine Res, 2001. **21**(10): p. 871-81.

44. Harandi, A.M., K. Eriksson, and J. Holmgren, *A protective role of locally administered immunostimulatory CpG oligodeoxynucleotide in a mouse model of genital herpes infection.* J Virol, 2003. **77**(2): p. 953-62.

45. Gomis, S., et al., *Protection of chickens against Escherichia coli infections by DNA containing CpG motifs.* Infect Immun, 2003. **71**(2): p. 857-63.

46. Gramzinski, R.A., et al., *Interleukin-12- and gamma interferon-dependent protection against malaria conferred by CpG oligodeoxynucleotide in mice.* Infect Immun, 2001. **69**(3): p. 1643-9.

47. Shi, T., et al., *Intranasal CpG-oligodeoxynucleotide is a potent adjuvant of vaccine against Helicobacter pylori, and T helper 1 type response and interferon-gamma correlate with the protection.* Helicobacter, 2005. **10**(1): p. 71-9.

48. Tewary, P., et al., *Immunostimulatory oligodeoxynucleotides are potent enhancers of protective immunity in mice immunized with recombinant ORFF leishmanial antigen.* Vaccine, 2004. **22**(23-24): p. 3053-60.

49. Zhu, F.G. and J.S. Marshall, *CpG-containing oligodeoxynucleotides induce TNF-alpha and IL-6 production but not degranulation from murine bone marrow-derived mast cells.* J Leukoc Biol, 2001. **69**(2): p. 253-62.

50. Sun, S., et al., *Type I interferon-mediated stimulation of T cells by CpG DNA.* J Exp Med, 1998. **188**(12): p. 2335-42.

51. Krieg, A.M., *CpG motifs in bacterial DNA and their immune effects.* Annu Rev Immunol, 2002. **20**: p. 709-60.

52. Klinman, D.M., et al., *CpG DNA: recognition by and activation of monocytes.* Microbes Infect, 2002. **4**(9): p. 897-901.

53. Assaf, A., et al., *A threshold level of TLR9 mRNA predicts cellular responsiveness to CpG-ODN in haematological and non-haematological tumour cell lines.* Cell Immunol, 2009. **259**(1): p. 90-9.

54. Vollmer, J., et al., *Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities.* Eur J Immunol, 2004. **34**(1): p. 251-62.

55. Hornung, V., et al., *Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides.* J Immunol, 2002. **168**(9): p. 4531-7.

56. Meffre, E., *The establishment of early B cell tolerance in humans: lessons from primary immunodeficiency diseases.* Ann N Y Acad Sci, 2011. **1246**: p. 1-10.

57. Rawlings, D.J., et al., *Integration of B cell responses through Toll-like receptors and antigen receptors.* Nat Rev Immunol, 2012. **12**(4): p. 282-94.

58. Kraehenbuhl, J.P. and M.R. Neutra, *Transepithelial transport and mucosal defence II: secretion of IgA.* Trends Cell Biol, 1992. **2**(6): p. 170-4.

59. McGhee, J.R. and K. Fujihashi, *Inside the Mucosal Immune System.* PLoS Biol, 2012. **10**(9): p. e1001397.

60. Honda, K. and K. Takeda, *Regulatory mechanisms of immune responses to intestinal bacteria.* Mucosal Immunology, 2009. **2**(3): p. 187-196.

61. Faria, A.M. and H.L. Weiner, *Oral tolerance.* Immunol Rev, 2005. **206**: p. 232-59.

62. Weiner, H.L., *Oral tolerance, an active immunologic process mediated by multiple mechanisms.* J Clin Invest, 2000. **106**(8): p. 935-7.

63. Schmidt, J.E., *Medical discoveries : who and when*. 1959, Springfield, Ill.: Charles C. Thomas.

64. Barr, W.G., et al., *The accessory cell function of murine Peyer's patches.* Cell Immunol, 1985. **92**(1): p. 41-52.

65. Neutra, M.R., N.J. Mantis, and J.P. Kraehenbuhl, *Collaboration of epithelial cells with organized mucosal lymphoid tissues.* Nat Immunol, 2001. **2**(11): p. 1004-9.

66. Lowden, S. and T. Heath, *Lymph pathways associated with Peyer's patches in sheep.* J Anat, 1992. **181 (Pt 2)**: p. 209-17.

67. Heel, K.A., et al., *Review: Peyer's patches.* J Gastroenterol Hepatol, 1997. **12**(2): p. 122-36.

68. Griebel, P.J. and W.R. Hein, *Expanding the role of Peyer's patches in B-cell ontogeny.* Immunol Today, 1996. **17**(1): p. 30-9.

69. Mutwiri, G., et al., *Ileal and jejunal Peyer's patches play distinct roles in mucosal immunity of sheep.* Immunology, 1999. **97**(3): p. 455-61.

70. Reynolds, J.D. and D. Kirk, *Two types of sheep Peyer's patches: location along gut does not influence involution.* Immunology, 1989. **66**(2): p. 308-11.

71. Yasuda, M., et al., *The sheep and cattle Peyer's patch as a site of B-cell development.* Vet. Res., 2006. **37**(3): p. 401-415.

72. Pabst, R. and J.D. Reynolds, *Peyer's patches export lymphocytes throughout the lymphoid system in sheep.* J Immunol, 1987. **139**(12): p. 3981-5.

73. Reynolds, J.D., *Evidence of extensive lymphocyte death in sheep Peyer's patches. I. A comparison of lymphocyte production and export.* J Immunol, 1986. **136**(6): p. 2005-10.

74. Reynolds, J.D. and B. Morris, *The evolution and involution of Peyer's patches in fetal and postnatal sheep.* Eur J Immunol, 1983. **13**(8): p. 627-35.

75. Miyasaka, M., et al., *Studies on the differentiation of T lymphocytes in sheep. I. Recognition of a sheep T-lymphocyte differentiation antigen by a monoclonal antibody T-80.* Immunology, 1983. **49**(3): p. 545-53.

76. Jimbo, S., et al., *IL-10 secreting CD21 B cells are present in jejunal Peyer's patches of sheep during fetal development.* Cell Tissue Res, 2014.

77. Belkaid, Y. and S. Naik, *Compartmentalized and systemic control of tissue immunity by commensals.* Nat Immunol, 2013. **14**(7): p. 646-53.

78. Colotta, F., et al., *The type II 'decoy' receptor: a novel regulatory pathway for interleukin 1.* Immunol Today, 1994. **15**(12): p. 562-6.

79. LeBouder, E., et al., *Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk.* J Immunol, 2003. **171**(12): p. 6680-9.

80. Janssens, S., et al., *Regulation of interleukin-1- and lipopolysaccharide-induced NF-kappaB activation by alternative splicing of MyD88.* Curr Biol, 2002. **12**(6): p. 467-71.

81. Janssens, S. and R. Beyaert, *Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members.* Mol Cell, 2003. **11**(2): p. 293-302.

82. Kobayashi, K., et al., *IRAK-M is a negative regulator of Toll-like receptor signaling.* Cell, 2002. **110**(2): p. 191-202.

83. Watanabe, T., et al., *NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses.* Nat Immunol, 2004. **5**(8): p. 800-808.

84. Brint, E.K., et al., *ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance.* Nat Immunol, 2004. **5**(4): p. 373-379.

85. Garlanda, C., et al., *Intestinal inflammation in mice deficient in Tir8, an inhibitory member of the IL-1 receptor family.* Proc Natl Acad Sci U S A, 2004. **101**(10): p. 3522-6.

86. Polentarutti, N., et al., *Unique pattern of expression and inhibition of IL-1 signaling by the IL-1 receptor family member TIR8/SIGIRR.* Eur Cytokine Netw, 2003. **14**(4): p. 211-8.

87. Diehl, G.E., et al., *TRAIL-R as a negative regulator of innate immune cell responses.* Immunity, 2004. **21**(6): p. 877-89.

88. Chuang, T.-H. and R.J. Ulevitch, *Triad3A, an E3 ubiquitin-protein ligase regulating Toll-like receptors.* Nat Immunol, 2004. **5**(5): p. 495-502.

89. McCartney-Francis, N., W. Jin, and S.M. Wahl, *Aberrant Toll receptor expression and endotoxin hypersensitivity in mice lacking a functional TGF-beta 1 signaling pathway.* J Immunol, 2004. **172**(6): p. 3814-21.

90. Aliprantis, A.O., et al., *Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2.* Science, 1999. **285**(5428): p. 736-9.

91. Aliprantis, A.O., et al., *The apoptotic signaling pathway activated by Toll-like receptor-2.* Embo j, 2000. **19**(13): p. 3325-36.

92. Geuking, M.B., et al., *Intestinal bacterial colonization induces mutualistic regulatory T cell responses.* Immunity, 2011. **34**(5): p. 794-806.

93. Park, S.G., et al., *T regulatory cells maintain intestinal homeostasis by suppressing gammadelta T cells.* Immunity, 2010. **33**(5): p. 791-803.

94. Hardy, R.R., P.W. Kincade, and K. Dorshkind, *The protean nature of cells in the B lymphocyte lineage.* Immunity, 2007. **26**(6): p. 703-14.

95. Chevallier, et al., *B-1-like cells exist in sheep. Characterization of their phenotype and behaviour.* Immunology, 1998. **95**(2): p. 178-184.

96. Cariappa, A., et al., *The recirculating B cell pool contains two functionally distinct, long-lived, posttransitional, follicular B cell populations.* J Immunol, 2007. **179**(4): p. 2270-81.

97. Vaughan, A.T., A. Roghanian, and M.S. Cragg, *B cells—Masters of the immunoverse.* The International Journal of Biochemistry & Cell Biology, 2011. **43**(3): p. 280-285.

98. Wardemann, H., et al., *Predominant autoantibody production by early human B cell precursors.* Science, 2003. **301**(5638): p. 1374-7.

99. Zhang, X., *Regulatory functions of innate-like B cells.* Cell Mol Immunol, 2013. **10**(2): p. 113-21.

100. Kurt-Jones, E.A., et al., *Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus.* Nat Immunol, 2000. **1**(5): p. 398-401.

101. Bretscher, P.A., *A two-step, two-signal model for the primary activation of precursor helper T cells.* Proc Natl Acad Sci U S A, 1999. **96**(1): p. 185-90.

102. Baumgarth, N., *A two-phase model of B-cell activation.* Immunol Rev, 2000. **176**: p. 171-80.

103. Janeway, C.A., Jr., *Pillars article: approaching the asymptote? Evolution and revolution in immunology. Cold spring harb symp quant biol. 1989. 54: 1-13.* J Immunol, 2013. **191**(9): p. 4475-87.

104. Harwood, N.E. and F.D. Batista, *Early events in B cell activation.* Annu Rev Immunol, 2010. **28**: p. 185-210.

105. Lanzavecchia, A., *Antigen-specific interaction between T and B cells.* Nature, 1985. **314**(6011): p. 537-9.

106. Rock, K.L., B. Benacerraf, and A.K. Abbas, *Antigen presentation by hapten-specific B lymphocytes. I. Role of surface immunoglobulin receptors.* J Exp Med, 1984. **160**(4): p. 1102-13.

107. Young, A.J., et al., *Structure and expression of ovine complement receptor type 2.* Vet Immunol Immunopathol, 1999. **72**(1-2): p. 67-72.

108. Gupta, V.K., et al., *Two B cell subpopulations have distinct recirculation characteristics.* Eur J Immunol, 1998. **28**(5): p. 1597-603.

109. Adachi, T., et al., *CD72 negatively regulates signaling through the antigen receptor of B cells.* J Immunol, 2000. **164**(3): p. 1223-9.

110. Ying, H., et al., *Regulation of mouse CD72 gene expression during B lymphocyte development.* J Immunol, 1998. **161**(9): p. 4760-7.

111. Iwasaki, A. and R. Medzhitov, *Regulation of adaptive immunity by the innate immune system.* Science, 2010. **327**(5963): p. 291-5.

112. Hua, Z. and B. Hou, *TLR signaling in B-cell development and activation.* Cell Mol Immunol, 2013. **10**(2): p. 103-106.

113. Loder, F., et al., *B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals.* J Exp Med, 1999. **190**(1): p. 75-89.

114. Ueda, Y., et al., *T-independent activation-induced cytidine deaminase expression, class-switch recombination, and antibody production by immature/transitional 1 B cells.* J Immunol, 2007. **178**(6): p. 3593-601.

115. Hasan, M., et al., *Defective Toll-like receptor 9-mediated cytokine production in B cells from Bruton's tyrosine kinase-deficient mice.* Immunology, 2008. **123**(2): p. 239-49.

116. Capolunghi, F., et al., *CpG drives human transitional B cells to terminal differentiation and production of natural antibodies.* J Immunol, 2008. **180**(2): p. 800-8.

117. Lund, F.E., et al., *Regulatory roles for cytokine-producing B cells in infection and autoimmune disease.* Curr Dir Autoimmun, 2005. **8**: p. 25-54.

118. Pistoia, V., *Production of cytokines by human B cells in health and disease.* Immunol Today, 1997. **18**(7): p. 343-50.

119. Harris, D.P., et al., *Cutting edge: the development of IL-4-producing B cells (B effector 2 cells) is controlled by IL-4, IL-4 receptor alpha, and Th2 cells.* J Immunol, 2005. **175**(11): p. 7103-7.

120. Wedlock, D.N., et al., *Vaccination of cattle with Mycobacterium bovis culture filtrate proteins and CpG oligodeoxynucleotides induces protection against bovine tuberculosis.* Vet Immunol Immunopathol, 2005. **106**(1-2): p. 53-63.

121. Di Sabatino, A., et al., *Impairment of splenic IgM-memory but not switched-memory B cells in a patient with celiac disease and splenic atrophy.* J Allergy Clin Immunol, 2007. **120**(6): p. 1461 - 1463.

122. Mizoguchi, A., et al., *Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation.* Immunity, 2002. **16**(2): p. 219-30.

123. Katz, S.I., D. Parker, and J.L. Turk, *B-cell suppression of delayed hypersensitivity reactions.* Nature, 1974. **251**(5475): p. 550-1.

124. Marino, E., et al., *CD4(+)CD25(+) T-cells control autoimmunity in the absence of B-cells.* Diabetes, 2009. **58**(7): p. 1568-77.

125. Wolf, S.D., et al., *Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice.* J Exp Med, 1996. **184**(6): p. 2271-8.

126. Alsalami, M.T. and L.J. Filippich, *Haematology of foetal sheep.* Aust Vet J, 1999. **77**(9): p. 588-94.

127. Fillatreau, S., et al., *B cells regulate autoimmunity by provision of IL-10.* Nat Immunol, 2002. **3**(10): p. 944-50.

128. Adkins, B., et al., *Neonatal tolerance revisited again: specific CTL priming in mouse neonates exposed to small numbers of semi- or fully allogeneic spleen cells.* Eur J Immunol, 2004. **34**(7): p. 1901-9.

129. Fujimoto, M., *Regulatory B cells in skin and connective tissue diseases.* J Dermatol Sci, 2010. **60**(1): p. 1-7.

130. Mann, M.K., et al., *Pathogenic and regulatory roles for B cells in experimental autoimmune encephalomyelitis.* Autoimmunity, 2012. **45**(5): p. 388-99.

131. Mauri, C., et al., *Prevention of arthritis by interleukin 10-producing B cells.* J Exp Med, 2003. **197**(4): p. 489-501.

132. Nishimura, S., et al., *Adipose Natural Regulatory B Cells Negatively Control Adipose Tissue Inflammation.* Cell Metab, 2013.

133. Iwata, Y., et al., *Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells.* Blood, 2011. **117**(2): p. 530 - 541.

134. Yanaba, K., et al., *A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses.* Immunity, 2008. **28**(5): p. 639 - 650.

135. Matsushita, T., et al., *Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression.* J Clin Invest, 2008. **118**(10): p. 3420-30.

136. Airoldi, I., et al., *Expression and function of IL-12 and IL-18 receptors on human tonsillar B cells.* J Immunol, 2000. **165**(12): p. 6880-8.

137. Graham, S.P., et al., *Immunostimulatory CpG oligodeoxynucleotides enhance the induction of bovine CD4+ cytotoxic T-lymphocyte responses against the polymorphic immunodominant molecule of the protozoan parasite Theileria parva.* Vet Immunol Immunopathol, 2007. **115**(3-4): p. 383-9.

138. Shapiro-Shelef, M. and K. Calame, *Regulation of plasma-cell development.* Nat Rev Immunol, 2005. **5**(3): p. 230-42.

139. Booth, J.S., et al., *Innate immune responses induced by classes of CpG oligodeoxynucleotides in ovine lymph node and blood mononuclear cells.* Vet Immunol Immunopathol, 2007. **115**(1-2): p. 24-34.

140. Kwong, L.S., et al., *Development of an ELISA for bovine IL-10.* Vet Immunol Immunopathol, 2002. **85**(3–4): p. 213-223.

141. Griebel, P.J., *Isolation of lymphoid follicles from Peyer's patches.* Immunology Methods Manual. The Comprehensive Sourcebook of Techniques, 1996. **4**: p. 2079.

142. Mauri, C. and P.A. Blair, *Regulatory B cells in autoimmunity: developments and controversies.* Nat Rev Rheumatol, 2010. **6**(11): p. 636-643.

143. Saraiva, M. and A. O'Garra, *The regulation of IL-10 production by immune cells.* Nat Rev Immunol, 2010. **10**(3): p. 170-81.

144. D'Andrea, A., et al., *Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells.* J Exp Med, 1993. **178**(3): p. 1041-8.

145. de Waal Malefyt, R., et al., *Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes.* J Exp Med, 1991. **174**(5): p. 1209-20.

146. Jung, C., J.P. Hugot, and F. Barreau, *Peyer's Patches: The Immune Sensors of the Intestine.* Int J Inflam, 2010. **2010**: p. 823710.

147. van Nierop, K. and C. de Groot, *Human follicular dendritic cells: function, origin and development.* Semin Immunol, 2002. **14**(4): p. 251-7.

148. Mueller, S.N. and R.N. Germain, *Stromal cell contributions to the homeostasis and functionality of the immune system.* Nat Rev Immunol, 2009. **9**(9): p. 618-629.

149. Press, C.M., et al., *Development of accessory cells in B-cell compartments is retarded in B-cell-depleted fetal sheep.* Dev Immunol, 1998. **6**(3-4): p. 223-31.

150. Herrmann, L.M., et al., *CD21-positive follicular dendritic cells: A possible source of PrPSc in lymph node macrophages of scrapie-infected sheep.* Am J Pathol, 2003. **162**(4): p. 1075-81.

151. Cupedo, T., et al., *Development and activation of regulatory T cells in the human fetus.* Eur J Immunol, 2005. **35**(2): p. 383-90.

152. Trinchieri, G., *Interleukin-12 and the regulation of innate resistance and adaptive immunity.* Nat Rev Immunol, 2003. **3**(2): p. 133-46.

153. Dalwadi, H., et al., *B cell developmental requirement for the G alpha i2 gene.* J Immunol, 2003. **170**(4): p. 1707-15.

154. Brummel, R. and P. Lenert, *Activation of Marginal Zone B Cells from Lupus Mice with Type A(D) CpG-Oligodeoxynucleotides.* The Journal of Immunology, 2005. **174**(4): p. 2429-2434.

155. Lenert, P., et al., *TLR-9 Activation of Marginal Zone B Cells in Lupus Mice Regulates Immunity Through Increased IL-10 Production.* Journal of Clinical Immunology, 2005. **25**(1): p. 29-40.

156. Fiorentino, D.F., et al., *IL-10 inhibits cytokine production by activated macrophages.* The Journal of Immunology, 1991. **147**(11): p. 3815-22.

157. O'Farrell, A.-M., et al., *IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat3-dependent and -independent pathways.* EMBO J, 1998. **17**(4): p. 1006-1018.

158. Grünig, G., et al., *Interleukin-10 Is a Natural Suppressor of Cytokine Production and Inflammation in a Murine Model of Allergic Bronchopulmonary Aspergillosis.* The Journal of Experimental Medicine, 1997. **185**(6): p. 1089-1100.

159. Mutwiri, G., et al., *Induction of immune responses in newborn lambs following enteric immunization with a human adenovirus vaccine vector.* Vaccine, 2000. **19**(9-10): p. 1284-93.

160. Hope, J.C., et al., *Development of detection methods for ruminant interleukin (IL)-12.* Journal of Immunological Methods, 2002. **266**(1–2): p. 117-126.

161. Ruprecht, C.R. and A. Lanzavecchia, *Toll-like receptor stimulation as a third signal required for activation of human naive B cells.* European Journal of Immunology, 2006. **36**(4): p. 810-816.

162. Bajer, A.A., et al., *Peripheral blood-derived bovine dendritic cells promote IgG1-restricted B cell responses in vitro.* J Leukoc Biol, 2003. **73**(1): p. 100-6.

163. Nakayama, E., I. von Hoegen, and J.R. Parnes, *Sequence of the Lyb-2 B-cell differentiation antigen defines a gene superfamily of receptors with inverted membrane orientation.* Proc Natl Acad Sci U S A, 1989. **86**(4): p. 1352-6.

164. Naessens, J., et al., *Characterization of a bovine leucocyte differentiation antigen of 145,000 MW restricted to B lymphocytes.* Immunology, 1990. **69**(4): p. 525-30.

165. Lund, F.E. and T.D. Randall, *Effector and regulatory B cells: modulators of CD4+ T cell immunity*. Nat Rev Immunol, 2010. **10**(4): p. 236-247.

166. Yoshizaki, A., et al., *Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions.* Nature, 2012. **491**(7423): p. 264-8.

167. Yanaba, K., et al., *The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals.* J Immunol, 2009. **182**(12): p. 7459-72.

168. Lu, L., *Frontiers in B-cell immunology.* Cell Mol Immunol, 2013. **10**(2): p. 95-96.

169. Alcon, V.L., et al., *B-cell co-receptor CD72 is expressed on NK cells and inhibits IFN-gamma production but not cytotoxicity.* Eur J Immunol, 2009. **39**(3): p. 826-32.

170. Lertmemongkolchai, G., et al., *Bystander activation of CD8+ T cells contributes to the rapid production of IFN-gamma in response to bacterial pathogens.* J Immunol, 2001. **166**(2): p. 1097-105.

171. Cui, J., et al., *Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors.* Science, 1997. **278**(5343): p. 1623-6.

172. Bao, Y., et al., *Identification of IFN-gamma-producing innate B cells.* Cell Res, 2013.

173. Neta, R. and S.B. Salvin, *Specific suppression of delayed hypersensitivity: the possible presence of a suppressor B cell in the regulation of delayed hypersensitivity.* J Immunol, 1974. **113**(6): p. 1716-25.

174. Mena, A., et al., *Innate immune responses induced by CpG oligodeoxyribonucleotide stimulation of ovine blood mononuclear cells.* Immunology, 2003. **110**(2): p. 250-7.

175. Buchanan, R.M., et al., *B-cell activating factor (BAFF) promotes CpG ODN-induced B cell activation and proliferation.* Cellular Immunology, 2011. **271**(1): p. 16-28.

176. Yang, E., et al., *Both retention and recirculation contribute to long-lived regulatory T-cell accumulation in the thymus.* Eur J Immunol, 2014.

177. Fukata, M. and M. Arditi, *The role of pattern recognition receptors in intestinal inflammation.* Mucosal Immunol, 2013. **6**(3): p. 451-463.

178. Macpherson, A.J. and K. Smith, *Mesenteric lymph nodes at the center of immune anatomy.* The Journal of Experimental Medicine, 2006. **203**(3): p. 497-500.

179. Cebula, A., et al., *Thymus-derived regulatory T cells contribute to tolerance to commensal microbiota.* Nature, 2013. **497**(7448): p. 258-262.

180. Tanguay, S. and J.J. Killion, *Direct comparison of ELISPOT and ELISA-based assays for detection of individual cytokine-secreting cells.* Lymphokine Cytokine Res, 1994. **13**(4): p. 259-63.