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Functional Characterization of IL-40 a novel B cell cytokine and Physiological implications of CCL28 ablation

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Functional Characterization of IL-40
a novel B cell cytokine
and
Physiological Implications of CCL28 ablation

DISSERTATION

Submitted in Partial Satisfaction of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

In Biomedical Sciences

By

Monica Ivonne Vazquez

Dissertation Committee:
Professor Albert Zlotnik, Chair
Professor Kenneth J. Longmuir
Professor Craig M. Walsh

2015

DEDICATION

TO

My husband Brian Vazquez
And
son Isaiah Vazquez

They have been part of my entire life as a student from my undergraduate years
to my graduate career.
They both provided a much needed sense of balance
and always
brought me back to what matters the most:
Family.

My little girl, Penelope, who always brings a smile to face
and has given me the strength to continue.
Getsemani, expected April 7, 2015, who I cannot wait to meet.

Thank you for your unending love and support.

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Dean's List 2008

LIST OF ABBREVIATIONS

(in order of appearance in text)

Chapter 1

LRR – leucine rich repeat
TCR – T cell receptor
BCR – B cell receptor
AID – Activation induced deaminase
CLP – common lymphoid progenitor
IL – interleukin
IFN – interferon
TNF – Tumor Necrosis Factor
TGF – Transforming Growth Factor
BSF-1 – B cell stimulating factor-1
SCID – Severe Combined Immunodeficiency
LIF – Leukemia inhibitory factor
PBMC – Peripheral blood mononuclear cells
EBV – Epstein Barr Virus
LPS – lipopolysaccharide
MHC – Major Histocompatibility Complex
Breg – B Regulatory
IFN-I – Type I IFN
TNFR – TNF Receptor
TRADD – TNFR associated death domain protein
NF-κB – Nuclear Factor κ-B
RANKL – Receptor activator of NF-κB ligand
RA – Rheumatoid Arthritis
IBD – Irritable Bowel Disease
EAE – Experimental Autoimmune Encephalomyelitis
BLyS – B Lymphocyte stimulating factor
FasL – fas ligand
BAFF – B cell activating factor
SLE – Systemic Lupus Erythematosus
BMP – Bone Morphogenic Proteins
GDF – Growth and Differentiation Factors
hESC – human embryonic stem cells
Ag – antigen
Treg – T regulatory
mIg – membrane bound immunoglobulin
TβR – TGF-β receptor
PP – Peyer's patches
GPCR – G-protein coupled receptor
FO B cell – Follicular B cell
MZ B cell – Marginal Zone B cell
GC – Germinal Center

CSR – Class Switch Recombination
SHM – Somatic Hypermutation
mAb – monoclonal antibody
T-I – T independent
T-D – T dependent
Blimp-1 – B lymphocyte induced maturation protein-1
Pax5 – paired box 5
XBP-1 – X-box binding protein-1
Bcl-6 – B cell lymphoma 6
IRF-4 – Interferon regulatory factor-4
FDC – Follicular dendritic cells
APRIL – a proliferation inducing ligand
BAFFR – BAFF Receptor
TACI – transmembrane activator and calcium modulator and cytophilin ligand interactor
BCMA – B cell maturation antigen
ASC – Antibody secreting cell
APC – antigen presenting cell
MOG – Myelin oligodendrocyte glycoprotein
Be – B effector cell
MS – Multiple Sclerosis
MALT – Mucosal associated lymphoid tissue
ILF – Isolated lymphoid follicle
MLN – mesenteric lymph node
M cell – microfold cell
NALT – Nasal associated lymphoid tissue
BALT – Bronchus associated lymphoid tissue
GALT – Gut associated lymphoid tissue
LTic – Lymphoid tissue inducer cell
FAE – follicle associated epithelium
WT – Wild Type
MG – Mammary Gland
TEB – terminal end branches
BTK – Burton Tyrosine Kinase
XLA – X-linked globunemia
CVID – common variable immunodeficiency
ICOS – inducible T cell co-stimulator
ISAG – Immune system associated gene
BIGE – Body Index of Gene Expression
IL-40 – Interleukin 40
Il-40$^{-/-}$ - IL-40 deficient mouse

Chapter 2

KLK3 – Kallikren3
CD – Cluster of Differentiation
NK – natural killer cell
NKT – natural killer T cell
TSPAN33 – Tetraspanin33
UPL – Universal Probe Library
qPCR – Quantitative real-time PCR
IACUC – Institutional Animal Care and Use Committee
VVWR – Vaccinia Virus Western Reserve
FCS – Fetal Calf Serum
PEC – peritoneal exudate cells
HI-VACV – Heat-Inactivated Vaccinia Virus
RS – Reed-Sternberg cells
B-ALL – B-acute lymphoblastic leukemia

Chapter 3

HEV – high endothelial venules
EST – expressed tag sequence
Ccl28$^{-/-}$ - CCL28 deficient mouse
HD – Hodgkin 's disease
GF – germ free
SPF – specific pathogen free
F1 – first filial
IVF – *in vitro* fertilization
VNO – vomeronasal organ

ABSTRACT OF THE DISSERTATION

Functional Characterization of IL-40 a novel B cell cytokine

And

Physiological implications of CCL28 ablation

By

Monica Ivonne Vazquez

Doctor of Philosophy in Biomedical Sciences

University of California, Irvine, 2015

Professor Albert Zlotnik, Chair

Immune responses are regulated by an intricate balance between cytokine production and actions of various immune cell types. Cytokines are key regulators of the immune system and represent molecular messengers that provide extrinsic cues that direct the genesis of an immune response and its ultimate down-regulation. Here, I aim to characterize two cytokines that play distinct roles in the development of humoral immune responses.

First, I describe a novel B cell cytokine, Interleukin 40 (IL-40) that is expressed in hematopoetic tissues (fetal liver and bone marrow) and by a lymphocyte population not regularly associated with cytokine secretion (activated B cells). Interestingly, the *IL-40* gene is only present in mammalian genomes, indicating that it may participate in a mammalian-specific immune function. One such mammalian specific function which is also immune related is the process of lactation. The mammary gland undergoes extensive remodeling upon the onset of lactation. Milk ducts become defined to transport milk and the immune microenvironment undergoes significant

changes that lead to the production of IgA. Importantly, IL-40 is up-regulated immediate after lactation. An IL-40 deficient mouse (*Il-40*$^{-/-}$ mouse), exhibits an altered B cell phenotype, reduced serum and mucosal IgA, and a blunted response to HI-VACV (heat-inactivated vaccinia virus). I next focus on CCL28, a mucosal chemotactic cytokine. CCL28 is responsible for the recruitment of IgA secreting cells to mucosal sites via its receptor CCR10. Additionally, CCL28 is a potent antimicrobial peptide that can inhibit gram positive/gram negative bacteria, and *C. albicans*. Here, I functionally characterize the first mouse with a targeted deletion of the Ccl28 gene, (*Ccl28*$^{-/-}$ mouse). The *Ccl28*$^{-/-}$ mouse exhibits altered levels of IgA in mucosal secretions, and high susceptibility to salmonella infection.

Overall, I have functionally characterized two distinct cytokine knock-out mouse models, IL-40 and CCL28. I predict that through my doctoral work I have laid the foundation for a better understanding of the role that these two cytokines play in the fields of B cell biology, cytokine discovery, mucosal immunology and the pathogenesis of mucosal infections.

CHAPTER 1

Introduction and Significance

The Evolution of the Immune System

The immune system is a highly evolved mechanism designed to protect us from pathogens present in our environment. In the event that a pathogen is able to breach our primary defense, represented by barrier tissues such as skin and mucosal epithelia, we are equipped with an arsenal of molecular and cellular weaponry that has continually adapted over the millions of years of host-pathogen interactions. In its earliest stages, the immune system consisted of a group of generic receptors capable of recognizing conserved pathogen patterns that could elicit a response by the host [1-3]. The ability to recognize conserved patterns present on pathogens is the fundamental basis of the innate immune system. Phagocytic cells and other cell types associated with innate immunity appear in early invertebrates (Figure 1.1).

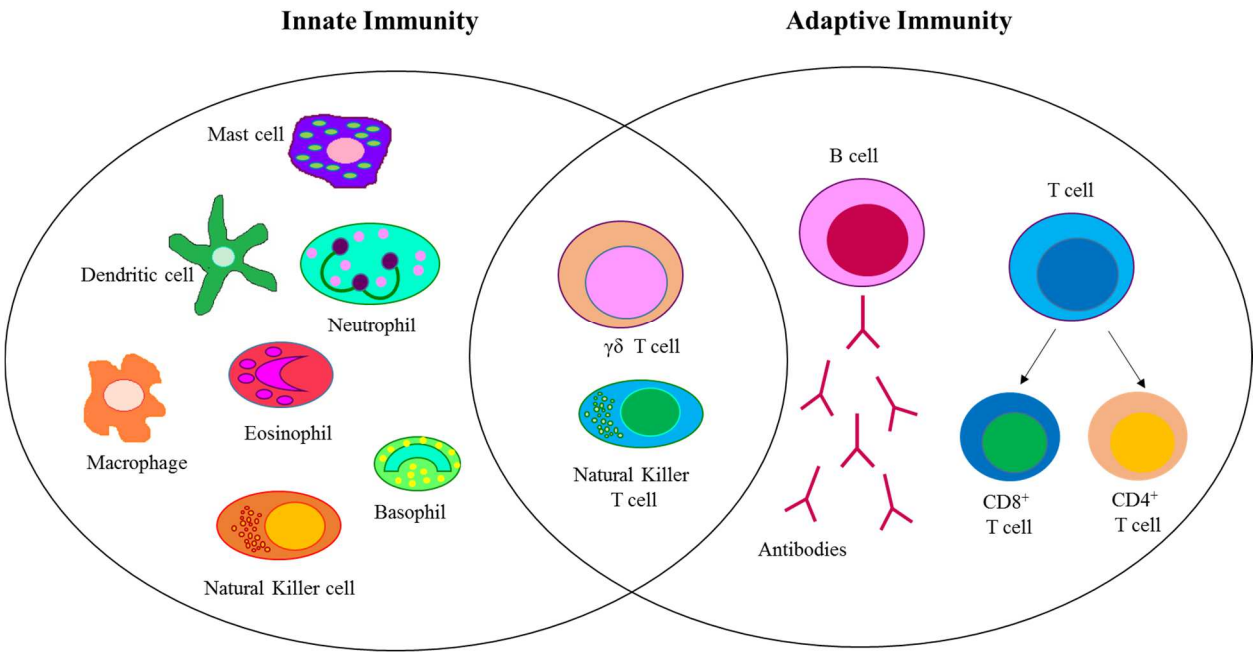

Figure 1.1. Cells of the immune system. Cells from the innate immunity branch provide rapid responses. Some these cells: natural killer, dendritic cell, mast cell, macrophage, and neutrophil, can phagocytose pathogens. Cells from the adaptive immunity branch are slow to respond, but provide a more efficient response.

Despite the capacity to recognize conserved patterns on pathogens the innate system lacks the ability to remember a previous assailant and further the ability to generate a response specific to that same assailant.

Lymphocyte progenitors, defined as cells capable of generating diverse receptors with the ability to migrate and be long-lived, are not seen until the appearance of basal vertebrates [4-7]. The ability to generate diverse antigen receptors, an element associated with the adaptive immune system, is first seen in agnathans, jawless vertebrates like the lamprey and hagfish. Activated lymphocytes in agnathans produced leucine-rich-repeat (LRR) proteins with a high degree of variability [8]; albeit a response not as elegant as that found in gnathostomes, jawed vertebrates. The generation of variable proteins is a crucial step towards specificity in immune responses. Gnathostomes are the first species to express the crucial gene in the recombinatorial process that is responsible for generating a variable T cell receptor or B cell receptor (TCR/BCR): activation-induced deaminase, *AID* [8, 9]. The adaptive immune system in vertebrates developed two main types of lymphocytes: T and B cells (Figure 1.1).

Despite a common precursor, common lymphoid progenitor (CLP) (Figure 1.2), they develop in distinct anatomical regions and this leads to a fundamental functional difference that allows for a division of labor within the immune system. Before discussing B cells I will cover cytokines, because a brief introduction on these important mediators is required in order to appreciate the B cell journey.

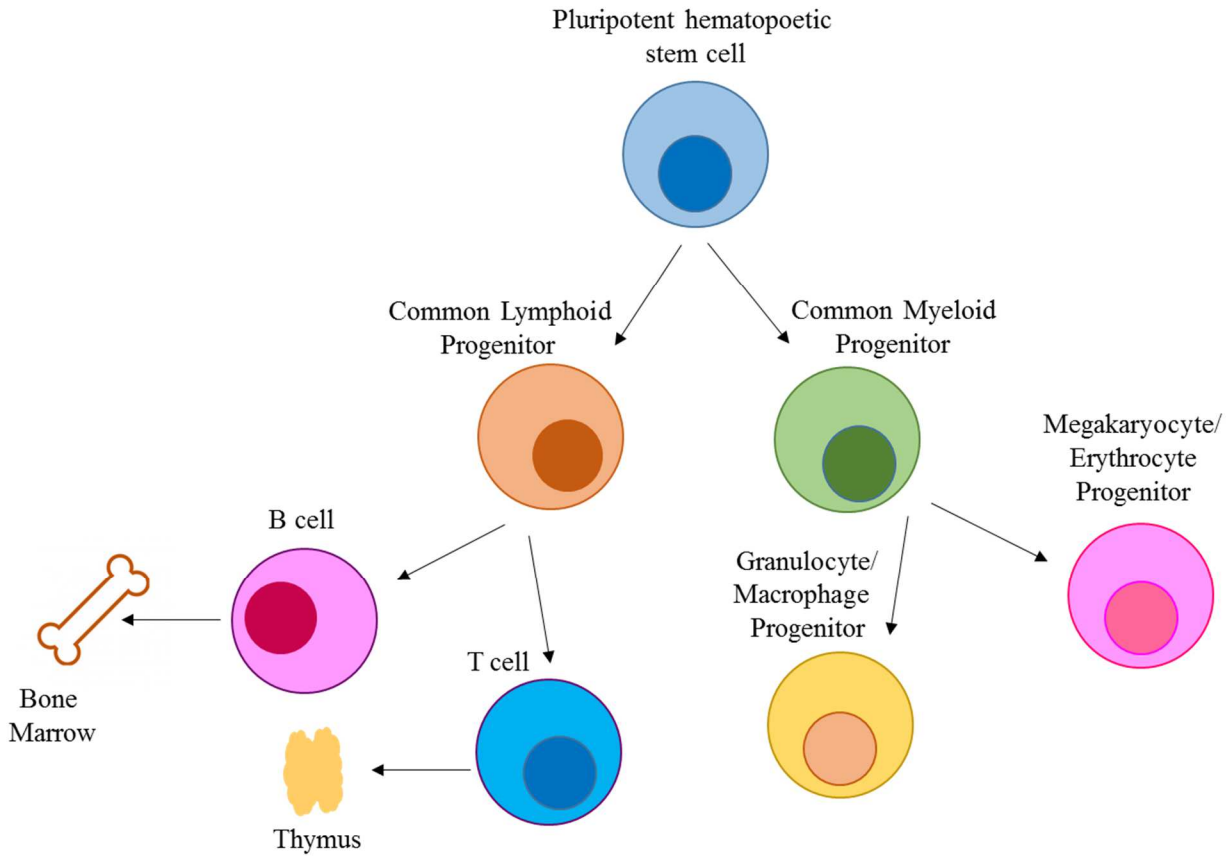

Figure 1.2. Condensed schematic of hematopoiesis. Shows where B and T cells development diverges from a common lymphoid progenitor and anatomic sites of continued development.

Cytokines

Cytokines are secreted proteins produced by leukocytes that can act on other leukocytes or tissues. They have pleiotropic effects and are involved in diverse biological processes including embryonic development, cell growth regulation, proliferation, differentiation, apoptosis, homeostasis, and modulation of inflammation [10]. Due to their multiple biological functions the term cytokine has expanded over the years. Currently the accepted definition of a cytokine is a small secreted protein made by a given cell, that acts on another similar cell which may be of the same or a different cell type [11].

Within the immune system, cytokines are produced by activated cells or are induced by other cytokines upon cellular activation. Hence, they represent molecular messengers through which cells of the immune system communicate to develop and modulate different immune responses. In addition to their biological properties, cytokines serve as biomarkers of subpopulations of lymphocytes. Due to the large number of cytokines they have been divided into super-families based on sequence homology which in turn reflects a shared ancestral precursor gene; some examples include the Interleukins (IL), Interferons (IFN), Tumor Necrosis Factor (TNF), Transforming Growth Factor (TGF), and the chemokines.

The Interleukin Superfamily

Interleukins, as their name implies, are proteins produced and secreted by leukocytes that can act on other leukocytes or tissues [12]. The term interleukin was first used in 1979 to describe two different molecules secreted by leukocytes with a similar molecular weight. These two early interleukins are what we now know as IL-1 and IL-2 [13]. Since the introduction of the term, and

concurrent identification of the first two interleukins, 37 more interleukins have been described [14, 15]. In the same way that cytokines are divided into super-families the Interleukin super-family is further classified into families based on similar biological properties and to a lesser extent sequence homology [16].

The IL-1 Family

A report published in 1948 was the first to describe the temperature elevating effects mediated by a soluble factor present in polymorphic nuclear cells from a rabbit. This report was the first to identify a cytokine, which we now know as IL-1 [13]. Since the initial report of IL-1 there have been 38 more interleukins that have been identified and characterized. The IL-1 family is composed of pro-inflammatory interleukins which include: IL-1α, IL-1β, IL-1Rα, IL-18, IL-33, and IL-37 [16]

The IL-1 family members induce their pro-inflammatory effects indirectly and these effects are reminiscent of the effects induced by the binding of toll like receptors (TLR) because of this similarity the IL-1 family is associated with the innate response [17]. For example IL-1β signaling results in increased production of cyclooxygenase type 2, nitric oxide, and expression of adhesion molecules. In addition to chemokine gradients, which serve to recruit lymphocytes, the above effects ultimately lead to the infiltration of cells that lead to global inflammation.

The Common γ-chain Family

The interleukins that belong to the common γ-chain cytokine family are recognized for their importance in the development and maintenance of lymphocytes. The interleukins that comprise this family include IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. These interleukins are grouped together because they share a common signaling component in their receptor: the γ-chain also known as CD132 [18].

IL-2 is best known for its effects on T cells. It is upregulated shortly after activation and can sustain T cell proliferation *in vitro* [18]. IL-4 is a product of activated $CD4^+$ T cells and functions as a B cell growth factor [19]. Initially described as B cells stimulating factor 1, BSF-1, (because its ability to co-stimulate B cell proliferation) it also acts synergistically with CD40L, a TNF family member discussed below, that enables cell division, survival and differentiation [20, 21]. IL-4 stimulates the differentiation towards the secretion of specific immunoglobulin isotypes: IgG1 and IgE [19]. This polarizes the immune response towards a Th2 type response while at the same time inhibiting Th1 responses; therefore IL-4 works as a key cross-regulator of the immune system.

IL-7 is important to both T and B cell development. Stromal cell derived IL-7 drives B cell development in the bone marrow [22] and has a role in immunoglobulin gene rearrangement [23-26]. In humans, mutations in the IL-7Rα gene result in Severe Combined Immune Deficiency (SCID) making IL-7 indispensable for T cell development. However, SCID patients maintain normal B cell populations [27, 28]. IL-9 was first characterized as a T and mast cell growth factor [29]. The main producers of IL-9 are T cells secreting it as an autocrine growth factor. A subpopulation of T cells has been identified based on their production of IL-9 known as Th9

cells [30]. IL-9 also affects B cells. In mice, IL-9 was found to increase the number of peritoneal B-1 cells [31, 32]. It is also involved in augmenting IL-4 induced IgE and IgG production from human B cells [33, 34]. These briefly reviewed functions of a few members of the common-γ chain family confirms that these cytokines are primarily involved in maintaining the balance of specific immune cell types.

The IL-6 family

The IL-6 family, like the common-γ chain family, is grouped based on a shared component of their signaling receptor. In the case of the IL-6 family the common molecules is gp130. However, gp130 is ubiquitously expressed, reflecting the wide range of functions exhibited by members of this family, which include IL-6, Oncostatin M, Leukemia inhibitory factor (LIF) and ciliary neurotrophic factor [35]. For example, Oncostatin M was first reported as an inhibitor of melanoma cell line and lung carcinoma cells [36]. It has since then been linked to a variety of other biological processes including: bone metabolism, liver regeneration and inflammatory conditions [37]. LIF is crucial for embryogenesis and can induce pluripotent stem cells [38]. Ciliary neurotropic factor is produced by Schwann cells and astrocytes [39]. It can also sustain the differentiation of neurons in vitro and can rescue neurons from axomoty-induced death *in vivo* [40].

IL-6 is produced by a variety of cell types such as T cell, fibroblasts, and peripheral blood mononuclear cells (PBMC) [41]. T cell-derived IL-6 can exert its effects on various immune cell populations including B cells. IL-6 induces immunoglobulin secretion in both naïve B cells that have been activated and Epstein Barr virus (EBV) immortalized cells [42]. Because of this observation it was initially named BSF-2. Its production is stimulated by inflammatory

conditions including viral infections, lipopolysaccharide (LPS) stimulation, and the cytokines produced as a result of the inflammation like IL-1, TNF-α, and IFN-γ [43-46].

The IL-10 family

This cytokine family is known for its anti-inflammatory effects and is made up of IL-10, IL-19, IL-20, IL-24, and IL-26. Their main function is to protect epithelial tissues from damage caused by inflammation and infection [47]. Yet, their ability to down regulate the immune system is a double edge sword. While preventing overt immune responses and resolving these is imperative, uncontrolled tissue repair directed by IL-20 can lead to psoriasis [48]. Hence, it is evident that even putative anti-inflammatory interleukins need to be regulated.

IL-10 is the founding member of this family, and was first identified as the product of a B cell lymphoma [49]. Its anti-inflammatory properties were readily apparent as it was able to inhibit type 1 cytokines such as IL-1, TNF-α, and IL-12 and could down regulate major histocompatibility complex (MHC) class II expression on activated monocytes [50-53]. IL-10 is produced by a variety of immune cells including B cells. This recently identified B cell subset known as B regulatory (Breg) or B10 cells makes up about 1-2% of splenic B cells. Furthermore, adoptive transfer of this population to mice lacking $CD20^{+}$ B cells or $CD19^{-/-}$ mice reversed inflammation via IL-10 production [54].

Interferons

In 1957, a protein that could interfere with viral replication was identified by Isaacs and Lindenmann; hence their subsequent name interferons [55]. There are three types of interferons, classified by the type of receptor to which they bind, including: type I, type II, and type III [56].

Type I interferons include IFN-α and IFN-β, type II is represented by IFN-γ [57], and type III consists of IFN-λ. Type I interferons are responsible for promoting antiviral states in response to viral infection; however a sustained antiviral state is dependent on type II, IFN-γ, production [58-60].

Type I IFN's (IFN-I) are constitutively produced in the bone marrow and promote the generation and selection of normal B cell populations [61]. Treatment with IFN-α/β on mature B cells results in partial activation that is associated with increased sensitivity to BCR engagement [62]. This suggests a possible link between innate and acquired immune responses in the sense that IFN-I (typically associated with innate responses) treatment results in the amplification of B cell responses, which are associated with acquired responses.

IFN-II, or IFN-γ, is associated with type1 immune responses which are driven by Th1 polarized cells. Under certain conditions it functions as an inhibitor of B cell responses and can inhibit production of certain immunoglobulin isotypes (Table 1.1) [63, 64]. However its inhibitory effects are limited to pre-activated B cells and do not have an effect on resting B cells. Depending on the activation conditions used, IFN-γ can induce B cell proliferation; this suggests that the manner in which B cells are activated, LPS or CD40 ligation, is the critical factor in the fate of the B cell [65]. More importantly, it suggests a delicately evolved, highly fine-tuned adaptation of the humoral immune response to IFN-γ.

Immunoglobulin secretion by terminally differentiated B cells is a direct consequence of the cytokines shaping the response (Table 1.1). IFN-γ, a Th1 cytokine, is involved in the induction/repression of various immunoglobulin classes. In humans, IFN-γ reduces total IgG production, and it specifically inhibits IgG1, a major component of total IgG, while increasing

IgG2, with no notable effects on either IgG3 or IgG4 production levels [66]. This phenotype is also observed in the mouse. LPS-activated B cells treated with IFN-γ produced increased levels of IgG2a and IgG3 while IgG1, IgM, and IgE were inhibited [64]. These data indicate that IFN-γ has similar functions in both humans and mice and that it is involved in the humoral response by directly controlling the immunoglobulin isotypes produced by plasma cells, terminally differentiated B cells.

Tumor Necrosis Factor Family

The TNF family is made up 18 different genes that encode 19 different transmembrane proteins [67]. These cytokines exhibit limited homology other than their conserved extracellular domain that is rich in cysteine residues. This feature has been maintained among the members of the family because it is necessary for proper signaling. Signaling in this family requires various adaptor proteins such as Tumor necrosis factor receptor (TNFR)-associated death domain protein (TRADD) [68].

Members of this family are involved in various biological processes including: differentiation/development, proliferation, inflammation, apoptosis, and survival. For example, the signaling provided by TNF, Lymphotoxin-α (LT-α), LT-β, and Receptor activator of Nuclear Factor κ-B (NF-κB) (RANKL) during development is essential in the genesis of secondary lymphoid organs [69, 70]. TNF, the most extensively studied member of this family, has been linked to various inflammatory autoimmune disorders like Rheumatoid Arthritis (RA), Irritable Bowel Disease (IBD), and Experimental Autoimmune Encephalomyelitis (EAE) [71, 72].

Various lymphocyte populations depend on TNF members to fully mature and be able to exert an effect during an immune response. CD40 and B lymphocyte stimulating factor (BLyS), for example, act as survival factors for B cells. Upon activation B cells upregulate CD40 [73] which

binds to CD40L, CD154, that is expressed on mature $CD4^+$ T cells. Ligation of CD40 also induces up-regulation of fas ligand (FasL) by the B cells. If the B cells was appropriately activated, by cognate antigen, then it is not susceptible to fas-mediated cell death; however, if the B cells was inappropriately activated, then it succumbs to fas-mediated apoptosis [74]. This ingenious interplay between two members of the TNF family serves as a check point that ensures that only necessary responses are promoted, while at the same time inhibiting overt inflammation that would result from generating a response to an innocuous antigen. B cell Activating Factor (BAFF) functions parallel those of CD40. It promotes B cell proliferation *in vitro* and its overexpression leads to various manifestations of autoimmunity. For example, in a murine model overexpression of BAFF resulted in the formation of auto-antibodies, proteinuria, Ig deposits in the kidneys, and increased formation of germinal centers (GC) [71]. Likewise, disruption of CD40/CD40L in mice leads to inflammatory disorders like RA and Systemic Lupus Erythematosus (SLE) [71]

Transforming Growth Factor

There are 33 genes in the TGF superfamily. Similar to other cytokine families, members of this family are involved in diverse cellular processes that span the entire life of the cell: development, homeostasis, proliferation, survival/apoptosis, and differentiation [75]. Ligation results in the formation of a heterotetracomplex made up of two type II receptors and two type I receptors. The activated type I receptors then recruit Smad proteins, which are required for appropriate signal transduction in the TGF family [75].

The TGF superfamily has been grouped into three groups: Bone Morphogenic proteins (BMP's), Growth and Differentiation factors (GDF) [75], and Activins/nodal family. The BMP's function

as regulators of bone development, maintenance, and repair and in regulation of embryological development [76]. GDFs are involved in the regulation of cell differentiation during embryogenesis and adult life [76]. Activins/nodal group has been associated in the maintenance of pluripotency and undifferentiated state of human embryonic stem cells (hESC's) [77]. Due to the pleiotropic effects of these cytokine their deregulation leads to various disorders that affect the various systems including skeletal-muscle, pulmonary, connective tissue, and neurodegenerative diseases [78].

Within the immune system TGF-β exerts its effects on various lymphocyte populations. In regards to cells of myeloid lineage, monocytes and macrophages, it acts as a suppressor by inhibiting proliferation and down-regulating production of reactive oxygen species and nitrogen intermediates [79]. TGF-β also regulates the maturation of T cells by directly inhibiting antigen (Ag) specific proliferation of naïve $CD4^+$ T cells a result of T regulatory (Treg) secreted TGF-β [80] and inhibits aberrant T cell expansion by controlling intracellular calcium concentrations [81]. With regards to B cells, TGF-β regulates the expression of cell surface markers, inhibits membrane bound IgD (mIgD), mIgM and CD23 while inducing MHC class II and the preferential secretion of IgA by activated B cells (Table 1.1) [82].

As discussed above TGF-β functions as pleiotropic cytokine within the immune system. To identify the specific effects of TGF-β on B cells a conditional knock-out of TGF-β receptor (TβR) was generated using Cre/loxP technology that targeted B cells only, which resulted in a mouse deficient in TβR B cells, a TβR-$B^{-/-}$ mouse [83]. Interestingly, the TβR-$B^{-/-}$ mice exhibited an increase in both B-1 cells in the peritoneum and B cells in the Peyer's patches (PP); however B-2 cells had a reduced life-span. Furthermore, the TβR-$B^{-/-}$ mouse exhibits a dramatic increase in levels of serum immunoglobulins, but IgA is nearly absent. Taken together, this indicates that

TGF-β is necessary for the induction of IgA *in vivo* and that it is required for the proper homeostasis of B cell subsets [83].

	IgM	IgG1	IgG2a	IgG2b	IgG3	IgA	IgE
IL-4	Inhibits	Induces			Inhibits		Induces
IL-6	Induces						
IFN-γ	Inhibits	Inhibits	Induces		Induces		Inhibits
TGF-β	Inhibits		Induces		Inhibits	Induces	

Table 1.1. Role of cytokines in murine isotype class switching.

Chemokines

Chemokines are small secreted proteins that control both the innate and acquired branches of the immune system. Their primary function is to direct lymphocytes to effector sites; hence they are analogous to 'traffic directors' in the sense that they guide different lymphocyte subsets to a given destination. Chemokines are therefore known as chemotactic cytokines. The chemokine family is categorized based on structural characteristics such as the position of the cysteine residues in their N-terminus. There are four subfamilies of chemokines: 1.) CXC chemokines, or α-chemokines, have two cysteine residues separated by one amino acid, 2.) CC chemokines, or β-chemokines, have two consecutive cysteine residues, 3.) C chemokines, or γ-chemokines, have one cysteine residue, and 4.) CX_3C chemokines, or δ-chemokines, have two cysteine residues separated by three amino acids [84].

In order for chemokines to attract cells to given site two events must occur: 1) the chemokine ligand must be secreted by a cell and 2) it, the chemokine ligand, must form a gradient for the

chemokine receptor expressing cell(s) to follow. This implies that chemokines must be secreted in large amounts in order to achieve their function. This is in stark contrast to cytokine levels that are secreted to induce a response upon target cells; a direct consequence of the distance between cells that are being acted upon cytokines versus cells being recruited to specific sites by chemokines. Upon successful recruitment of a given cell type chemokines bind their receptors and signaling is accomplished via G-protein coupled receptors (GPCR's).

The hallmark characteristics of chemokine receptors include: a seven transmembrane domain, that contains a 'DRY box' required for G protein coupling [85], ligand binding domain in the extracellular NH2 terminus, and a COOH-terminal region rich in serine/threonine residues in the intracellular cytoplasmic tail [86]. Signaling is initiated in the target cell via the Gαi subunit, which leads to a signaling cascade that results in a chemotactic response [87].

B cell development

Lymphocytes initiate their development during gestation in the fetal liver. In the mouse, pluripotent stem cells, from the yolk sac, can be visualized as early as day 7-9 of gestation, then these migrate to the murine fetal liver between days 10-11 of gestation [88]. These new arrivals will eventually give rise to B cells. Cells taken from the fetal liver at day 15 of gestation form progenitor B cells in the presence of IL-7 and stromal cell contact [88]. After birth the process of hematopoiesis takes place in the bone marrow. In the bone marrow CLP's can give rise to either a T cell or B cell. Progenitor T cells migrate out of the bone marrow and continue their development in the thymus. B cells remain within the bone marrow to continue their development. The developmental status of B cells can be tracked by the expression of specific cell surface markers that identify the developing lymphocyte as a B cell (Figure 1.3).

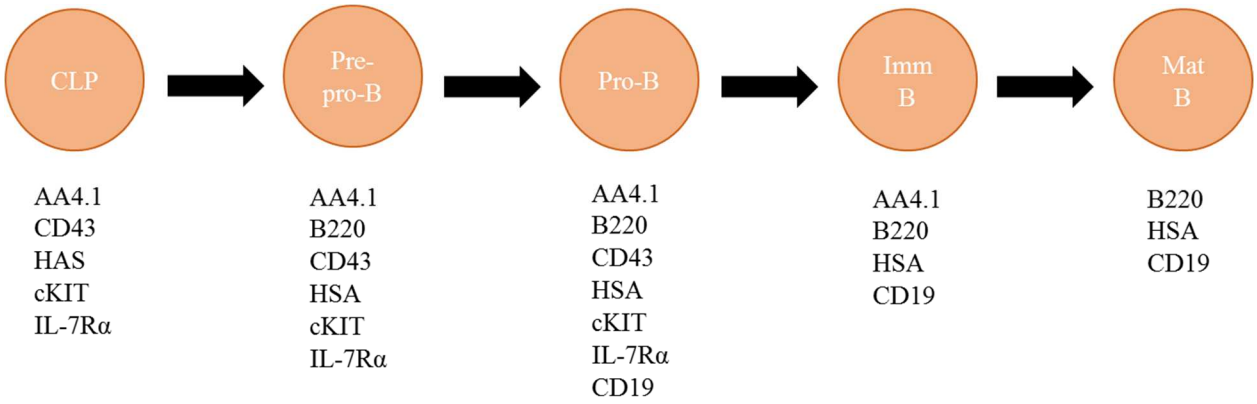

Figure 1.3. Diagram of B cell development from CLP to B cell commitment. Shows cell surface phenotype and expression of B cell related genes. Imm B represents immature B cell. Mat B represents mature B cell.

As the surface topology of the developing B cell is under continual gradual change so are the genes within the nucleus. B cells undergo an extensive process to rearrange the heavy and light chains of the immunoglobulin genes, known as V-D-J and V-J recombination [89]. Upon completion of this gene rearrangement the B cell expresses a unique BCR. The BCR serves as a check point for B cells. B cells are presented with self-antigen to test the affinity of the nascent BCR which results in two different outcomes: 1) high affinity interactions result in clonal deletion and the B cell will be eliminated, 2) low affinity interactions have two potential outcomes: anergy in which the B cell becomes unresponsive and short-lived or editing of the BCR to eliminate self-reactivity [90] (Figure 1.4).

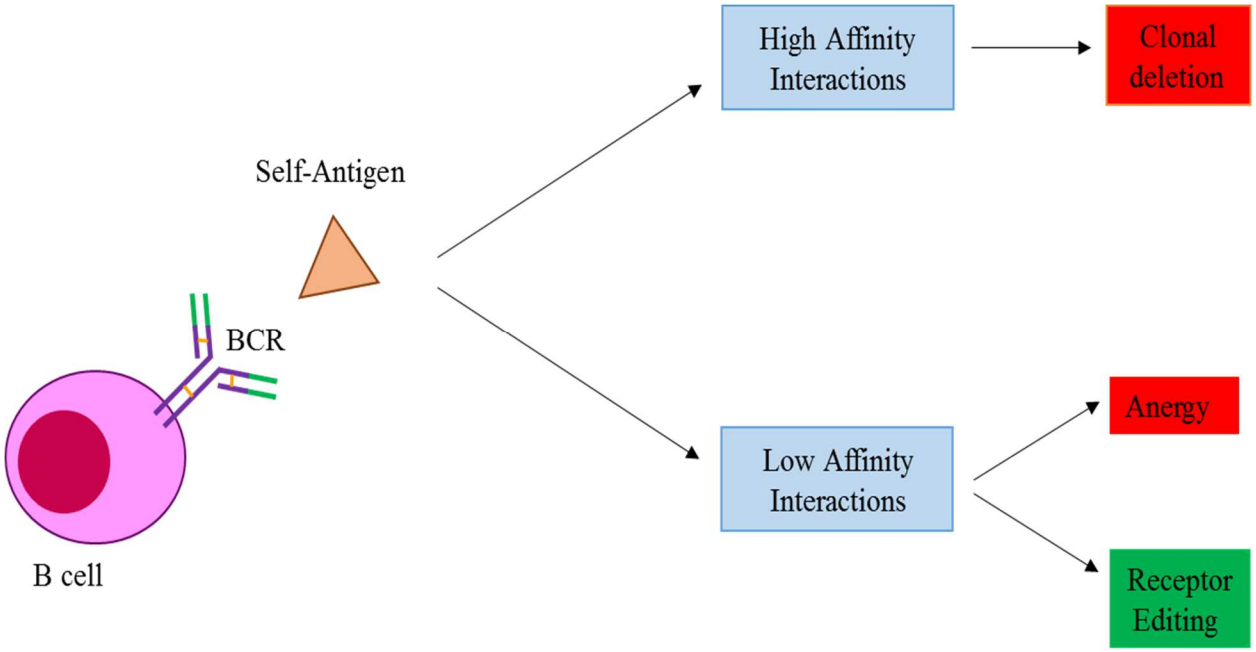

Figure 1.4. BCR functions as a checkpoint in the bone marrow. Developing B cells are exposed to self-antigens in the bone marrow. High affinity interactions result in the elimination of the B cell via clonal deletion. Low affinity interactions can lead to elimination via anergy or receptor editing. B cells that undergo receptor editing are not eliminated.

The BCR is required for further B cell development and survival [91]. At this point the B cells may exit the bone marrow and migrate throughout the periphery, but they are still considered "immature" or "transitional" B cells. This nomenclature is based on the cell surface markers which at this stage include mIgM and mIgD. Despite being immature B cells, these cells can respond to certain type-I antigens like LPS which will induce a rapid antibody response.

B cell subtypes

There are two main types of B cells: B-1 cells and B-2 cells. The difference between the two includes the expression of CD5, anatomical localization, self-renewing capacity, and their origin. B-1 B cells are $CD5^+$ and can be further divided into B1a and B1b cells [92]. These cells are generally thought of as fetal B cells because they can be readily generated from fetal precursors, but not from adult precursor cells [90, 92]. BCR specificity is imperative to the genesis of this subtype of B cells. Transgenic mice have revealed that either non-specific antigens such as anti-H-2, MHC H-2, or highly specific antigens such as those in immune-privileged sites lead to a substantial decrease in B-1 cells, but do not affect B-2 cells [90]. Therefore BCR specificity plays a significant role in the development of B-1 cells.

B-2 cells migrate to secondary lymphoid tissues in the periphery including the spleen, lymph nodes and gut. This subtype is further divided into either Follicular B cells (FO B cells) or Marginal Zone B cells (MZ B cells). FO B cells are free to circulate the periphery and can enter and exit secondary lymphoid tissues. However, upon recognizing their cognate antigen they will either become short-lived plasma cells or enter a GC reaction [93]. Within the GC, the B cell will undergo clonal expansion, class switch recombination (CSR), and somatic hypermutation (SHM). These processes culminate in the production of high affinity antibody producing plasma cells and memory B cells (Figure 1.5) [94].

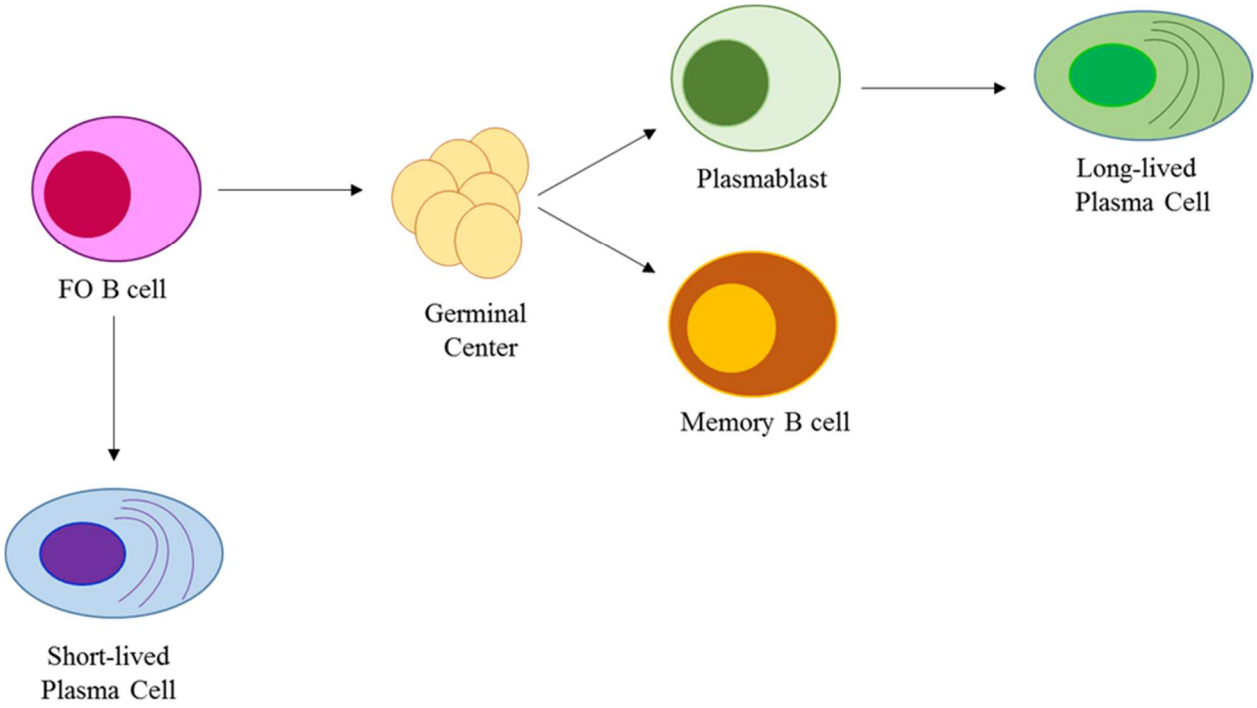

Figure 1.5. Follicular (FO) B cells differentiate into plasma cells via two routes. FO B cell that enter a GC can become a memory B cell or a long lived plasma cell. FO B cells can also become short lived plasma cells.

Memory B cells, generated during the GC reaction, persist in the bone marrow and are able to differentiate into plasma cells upon secondary exposure [95]. MZ B cells are sessile cells that reside in the marginal zone of the spleen. Their location enables a rapid antibody response to blood borne antigens (Figure 1.6) [95]. MZ B cells are similar to B-1 B cells in various aspects. For instance, MZ B cells are a self-renewing population that can respond with antibodies without T cell help. All the B cell subtypes ultimately contribute to the production of immunoglobulin, by differentiating into plasma cells.

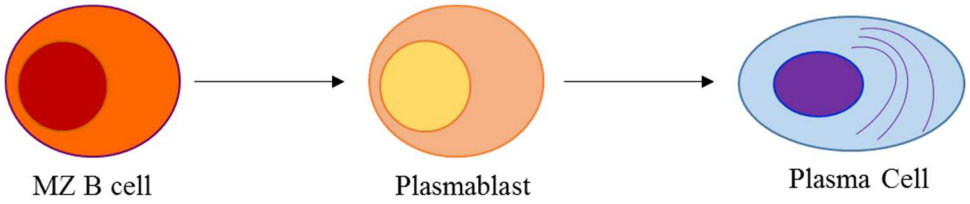

Figure 1.6. Marginal Zone (MZ) B cells differentiation into plasma cells. MZ B cells can differentiate into plasma cells without entering a GC.

Discovering the Functions of B cells: A Historical Perspective

B cell developmental stages and their associated functions within the immune system was discovered in reverse chronological order. For example serum gamma globulin was discovered in 1948 [96]. This was recognized as antibody production, a function associated with the terminal differentiation stage of B cells, the plasma cell. However, plasma cells were not identified until later [97]. Along with the discovery of antibodies came two different views that attempted to explain antibody formation: the "Natural Selection" view from the Jerne laboratory [98] and the "Clonal Expansion" view from the Burnet laboratory [99]. The "Clonal Expansion" theory was later proven through experimental data [100]. Hence, the initial function ascribed to B cells was antibody production, yet this function is attained upon fully differentiating into plasma cells [99].

Upon the concurrent discovery of both T and B lymphocytes, a division of labor was proposed [99]. This separation was between cells derived from the thymus, T cells, which required an intact thymus and are responsible for delayed hypersensitivity reactions [101, 102] and cells from the bone marrow, B cells, which are responsible for antibody formation [103, 104].

Other differentiated states and functions of B cells began to be discovered through the use of animal models and clinical studies with patients that suffered from immune deficiencies. The advent of monoclonal antibodies (mAb's) has facilitated the mapping of B cell surface topology. Through the use of mAb most of the cell surface markers restricted to B cells have been identified. Moreover, mAb technology represents an example of B cell biology contributing a novel experimental tool that is useful in all areas of biology [105].

CD20 was the first B cell restricted cell surface markers to be identified with mAb; as such it was initially named B1 [106]. The rapid identification of many cell surface markers made it apparent that a unified naming nomenclature was needed; hence the introduction of CD, cluster of differentiation, classification system. Remarkable progress in B cell biology came about after the identification of B cell restricted markers. For example, this facilitated the generation of model systems designed to characterize these B cell restricted molecules. Accordingly, mice deficient in each of these genes have been developed and used to describe each of the molecules and their role on B cell development and function.

B cell Functions: Antibody production, Antigen presentation, and Cytokine production

Antibody Production by terminally differentiated B cells, plasma cells

Antibody production is a function attributed to plasma cells, the final differentiated B cells stage. There are two main routes to becoming a plasma cells, but prior to differentiating into antibody producing plasma cells, B cells must encounter their cognate antigen and become activated. Naïve B cells that exit the bone marrow have undergone several check points to ensure they are

not self-reactive and have recombined the heavy and light chains of their immunoglobulin genes. At this point, the B cell expresses mIgM and mIgD, both of which serve as a receptor and are specific for the same antigen.

The route a given B cell takes to becoming a plasma cells depends on the nature of its cognate Ag [107, 108]. If a B cell recognizes a T-independent (T-I) Ag, then it does not require T cell help to produce Ab. However, T-I activation generally will not result in the secretion of class switched high-affinity antibodies nor will the B cell become a long-lived plasma cell [95]. On the other hand, if a B cell recognizes a T-dependent (T-D) antigen then it will receive T cell help in the form of CD40 ligation. CD40 ligation leads to two possible outcomes: generation of a short-lived plasma cell that produces IgM and participates in the initial phase of the response or migration and formation of a GC in which the B cell undergoes SHM, CSR, and affinity maturation to ultimately become either a long-lived plasma cells or a memory B cell [109, 110].

The transition from B cell to plasma cell is regulated by various transcription factors. B lymphocyte-induced maturation protein-1 (Blimp-1) is a transcription factor required for plasma cell differentiation and immunoglobulin secretion hence it is widely used as a marker of plasma cells [111]. Its activation leads to the repression of paired box 5 (Pax5) another transcription factor necessary for B cell development, which in turn allows the activation of other genes necessary for plasma cell commitment including: X-box binding protein-1 (XBP-1) and J-chain [22, 112]. The formation of GC, the site of plasma cell differentiation, is also critical to the plasma cell lineage commitment. B-cell lymphoma 6 (Bcl-6) is required for the formation of GC and functions in the quality control of the reactions underway in the GC by preventing B cells from prematurely differentiating within the GC [113]. Interferon regulatory factor-4 (IRF-4), is

yet another factor important in the GC, is required for CSR and plasma cell differentiation. It positively regulates Blimp-1 and its ablation results in plasma cell differentiation is arrest [114]. However its role in the formation of GC is yet to be fully defined [108].

The transcriptional factors that drive plasma cell differentiation are regulated by cytokines produced by other immune cells within the GC [115]. Activated T cells in the GC produce IL-2, which is required for GC formation and plasma cell differentiation [116]. Furthermore, *in vitro* data demonstrated that lack of IL-2, achieved using an anti-IL-2 antibody, leads to a reduction of immunoglobulin production by B cells that were co-cultured with activated T cells [116]. IL-4 is not required for GC formation, but as a B cell differentiation factor it participates in the induction of *AID* which is a prerequisite for SHM and CSR [117]. IL-6, like IL-2, is a product of follicular dendritic cells (FDC's) as such it participates in GC formation [118]. In the process of plasma cell differentiation, IL-10 is thought of as a master regulator that plays a role in the ultimate fate decision for B cells either plasma cell or memory B cell. In its presence, IL-10 induces B cells in the GC to become long-lived plasma cells, on the other hand its absence leads differentiating B cells to become memory B cells instead [119, 120]. IL-21 is the key orchestrator of GC formation and ultimately plasma cell differentiation [121].

Chemokines are also linked to GC and plasma cell differentiation. The up-regulation of CCR7 by B cells induces a chemotactic response towards CCL19 and CCL21, both of which are expressed on T cells that reside in the T cell zone of the lymph nodes [122]. CXCR4 and CXCR5 are required for the proper architecture of the GC itself [123]. CXCR4 is also responsible for the recruitment of long-lived plasma cell to the bone marrow where its ligand CXCL12 is expressed.

In the bone marrow long lived plasma cells are sustained by the microimmune environment and maintain their ability to produce high-affinity antibodies [124].

Members of the TNF family: APRIL, a proliferation inducing ligand, and BAFF, are also involved in the maintenance of plasma cells. BAFF binds three different receptors: BAFFR (BAFF Receptor), TACI (transmembrane activator and calcium-modulator and cytophilin ligand interactor), and BCMA (B cell maturation antigen). The latter two can also be bound by APRIL [125, 126]. Blocking of either shared receptor, via gene ablation or using of monoclonal antibodies, results in a reduction of antibody secreting cells (ASC) [127]. Of the two cytokines, APRIL seems to be more important in the maintenance of plasma cells. This is supported by the APRIL deficient mouse that exhibits decreases in both immunoglobulin titers and plasma cells [128].

B cells also function as Antigen Presenting Cells (APC)

In addition to differentiating into plasma cells, B cells also function as antigen presenters and cytokine producers. The latter two functions are not specific to B cells and are also attributed to different cells of the immune system. Other immune cells that function as antigen presenting cells (APC's), are DC's and macrophages. As APC's, B cells are able to induce tolerance to a specific antigen or can initiate an immune response. However, a B cell is not immediately able to present Ag. To gain this ability the B cell must undergo a specific activation process that confers antigen presentation capacity.

Upon encountering Ag, APC's will ingest it in one of three ways: phagocytosis, pinocytosis, or receptor-mediated endocytosis [129]. B cells rely on the BCR to recognize Ag and ingest it via

receptor-mediated endocytosis. In the context of B cells as APC's the BCR is a critical determinant of how well Ag is presented to $CD4^+$ T cells. For example, high affinity binding between the BCR and the antigen requires less Ag uptake by the B cells and results in efficient antigen presentation [130, 131]. Furthermore, high affinity binding interactions affect the processing of the antigen itself because some of the actual Ag is occluded. This results in certain epitopes being favored over others thus directly affecting the Ag repertoire and diversity of Ag presented to T cells [132-134]. In this regard the BCR modulates the quality control of the immune response.

B cells seem to be excellent APC's that can initiate an immune response at the mere sighting of Ag. However, since the frequency of a B cell bearing a receptor for a given Ag is incredibly low, 1 in $10^4 – 10^5$, this usually does not occur [129]. Given the sophistication of the processing of the Ag and the BCR built in control mechanisms it may seem wasteful to underutilize B cells as an APC. However, this outcome is favorable to the organism, otherwise this mechanism could lead to non-stop inflammation.

In the rare event that a B cell encounters an Ag that it bears a receptor for, ingests and processes it two events must occur in order for proper activation of the B cell that leads clonal expansion. The first is BCR engagement with the antigen, which provides the first activation signal. Next, the CD40 molecule, on the B cell, must bind to CD154, on the T cell, which provides a secondary co-stimulatory signaling that is required for B cell activation. The first signal promotes growth, proliferation and survival and induces expression of CD86, a co-stimulatory molecule that aids in T cell activation [135, 136]. Hence, this interaction between B and T cell results in mutual benefits to both lymphocytes. The secondary signal sustains B cell activation

and promotes the increased expression of MHC class II and CD80, another co-stimulatory molecule important in T cell activation [137]. However, the secondary signal required by B cells to become APC's, CD40 ligation, implies that the $CD4^+$ T cell that engages the B cell has already been activated, and as a result now expresses CD154 [129]. This reveals that B cells are not the preferred APC's because of their activation requirements depend on the previous activation of another lymphocyte.

B cells as cytokine producers

The hallmark function of B cells, antibody production, relies on external signaling provided by T cell derived cytokines. This reflects the important role of T cells as the initiators and modulators of the immune response, while B cells are regarded as mere responders to cytokines provided by T cells [82]. Indeed, naïve B cells do not secrete many cytokines upon activation. In contrast, T cells initiate strong cytokine production almost immediately following activation. This inherent difference between T and B cells reflects the B cells need for further differentiation and specific activation conditions in order to become cytokine producers.

Additional signaling is provided by the immune microenvironment and specific differentiation stage of the B cell. For example, the main cytokines produced by naïve B cells upon activation are the chemokines CCL22 and CCLl7 [141-143]. These two chemokines share the same receptor (CCR4) which is strongly expressed in $CD4^+$ Th2 type T cells. This reflects the ability of activated B cells to recruit Th2 cells. In turn the recruited Th2 cells produce cytokines, such as IL-4, that shape the B cell response and induce the differentiation of B cells towards cytokine producing B cells (Figure 1.7).

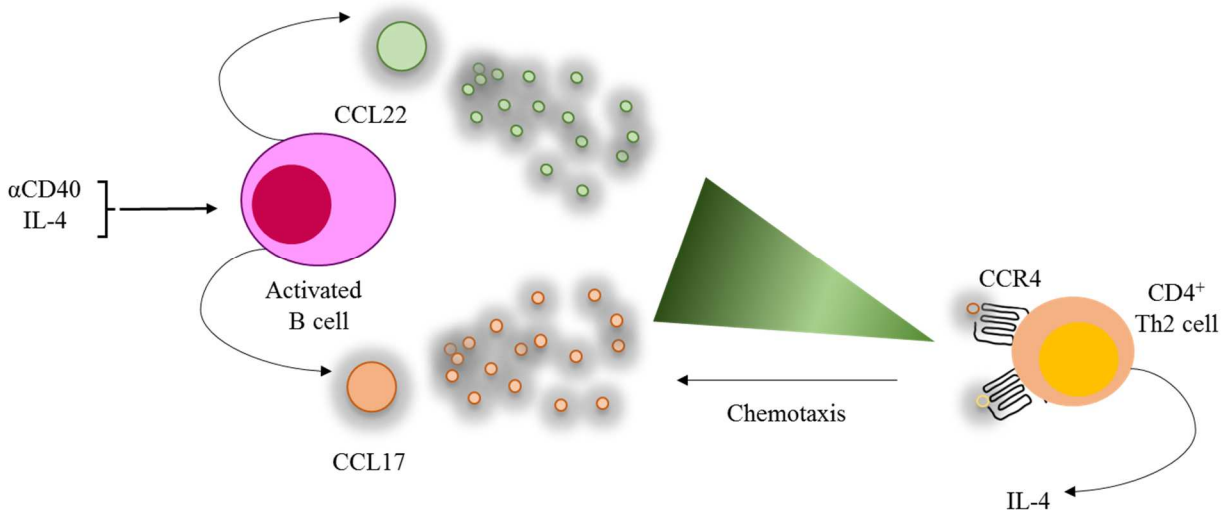

Figure 1.7. Activated B cells produce CCL17 and CCL22 to recruit CD4$^+$ T cells. Unlike T cells that initiate cytokine production immediately upon activation, B cells required additional cues provided by the immune microenvironment to produce cytokines. Here an activated B cell produces chemokines that will recruit a Th2 cell that will provide these molecular signals to the B cell.

Therefore, cytokine secretion by B cells is regulated by extrinsic signaling provided by other immune cell types. Once B cells acquire the capacity to produce cytokines, they become capable of cross-regulating responses via polarization/inhibition and can even negatively regulate the entire immune system.

One example of B cell derived cytokine modulating the immune response is B cell derived IL-6. IL-6 production by B cells leads to inflammation which exacerbates inflammatory conditions and autoimmune pathologies. Conversely, lack of IL-6 can lead to immune defects. For example, an IL-6 deficient mouse is resistant to myelin oligodendrocyte glycoprotein (MOG)-induced EAE. This phenotype is a consequence of poor lymphocyte proliferation that leads to reduced inflammation and demyelination in the central nervous system [144-147]. Furthermore, mouse studies have demonstrated that IL-6-deficient B cells do not support the polarization of T cells to

Th17, a cell type linked to various inflammatory conditions [147]. In humans, IL-6 is elevated in both SLE and Castleman's disease patients [147-149]. However, the exact origin of IL-6 producing B cells remains unknown. Overall, IL-6 over-production by B cells promotes inflammation through the generation of pathogenic antibodies and increased proliferation of pathogenic T cells.

B cell derived cytokines can also promote tolerance and reduce inflammation as in the case of the recently identified Breg/B10 cell. This subset was identified based on its ability to secrete IL-10 [54]. However it will not be discussed in this section because it has a major role in maintaining the homeostasis of the gut mucosa and will be discussed then.

B cells can be polarized to be effector cells

The first report of B cells as cytokine producers described ROHA-9, an EBV transformed human B cell line that constitutively produced IL-1 which led to enhanced T cell proliferation [150, 151]. These findings have been replicated using murine B cells. For example, EBV transformed murine B cells secrete IL-5 which promotes proliferation of eosinophil precursors and B cell proliferation and antibody production [152]. This set the stage for B cells as effectors of the immune response and suggested that B cells are able to modulate the magnitude of the immune response by both antigen-presentation and cytokine production.

B cells are classified into effector subtypes depending on the cytokines they secrete. There are two main B effector (Be) populations: Be1 and Be2, which drive either Th1/Th2 responses and cross-regulate the other [153, 154]. In a recent study, it was demonstrated that cytokine secretion by human B cells is depends on the manner of the stimulation received [155]. For example, B

cells stimulated with CD40L and BCR signaling proliferated and produced pro-inflammatory cytokines including TNF-α, lymphotoxin, and IL-6; however, stimulation with CD40 alone led to a significant induction of IL-10 which down-regulates unnecessary responses [155]. In the context of autoimmunity this 'cytokine network' is deregulated; cells collected from multiple sclerosis (MS) patients had a reduced capacity to secrete IL-10 [156]. Naïve B cells from patients with MS retained the ability to be polarized in vitro to Be1 cells which secrete pro-inflammatory cytokines, or Be2 cells which secrete anti-inflammatory cytokines. Treatment with Mitoxantrone, a therapy used for MS, recapitulated these results *in vivo* suggesting an *in vivo* 'cytokine network' switch [156]. Taken together these studies demonstrate that in addition to receiving activation signaling B cells require further cues from their microimmune environment to produce cytokines and that the cytokines produced are dictated by the environmental cues provided.

B cells are important in the maintenance of the immune system

In addition to providing molecular cues necessary to the evolution of the immune response, B cells ensure the proper genesis of the immune system and maintain its integrity. For example, mice that develop without B cells exhibit a dramatic decrease in thymocyte number and diversity, defects in the spleen dendritic cell (DC and T cell compartments), lack of PP organogenesis and FDC networks, absence of MZ macrophages, and reduced chemokine expression [99, 138, 139]. Furthermore, B cells are involved in wound healing, tissue rejection, and can also influence tumor immunity [99, 140].

Mucosal Associated Immune System

The Mucosal Associated Immune System or (MALT) represents a scattered collection of secondary immune system structures such as: PP, Isolated Lymphoid follicles (ILF's), and mesenteric lymph nodes (MLN). These microstructures are important in the induction of immune responses and have a significant role in "educating" the immune system. The structures protected by the MALT are: the gastrointestinal, respiratory, and urogenital tracts along with the following exocrine glands: conjunctivae, lachrymal, salivary glands and lactating breast [157]. These sites are very important for the organism as they represent the primary sites of gas exchange, food absorption, sensory activity, and reproduction.

Given their important function there are additional mechanisms that protect these structures. A monolayer of epithelial cells acts as a physical/mechanical barrier [158]. Since the barrier is only one cell in width it is permeable and therefore permits the physiological processes to take place. However, this ingenuity is also a detriment because it cannot discriminate between potential pathogens and innocuous substances. Another layer of protection built into the system is a chemical barrier. This barrier is achieved by the production of mucins, heavily glycosylated proteins that form a mucus-like substance that prevents bacteria and/or other antigens from coming into direct contact with the epithelial cells [159].

The mechanical and chemical barriers described above are designed to prevent/inhibit the entry of potential infectious agents. Yet, despite their presence many pathogens are still able to gain entry into a host via mucosal portals. Moreover, these sites are confronted with a variety of antigens some of which are not harmful including food antigens in the gut and pollen in the respiratory tract. These innocuous antigens represent a unique opportunity to "educate" the immune system to tolerate that substance. This formidable task is achieved by the various

immune cells, including B cells and T cells that migrate to the scattered secondary immune structures and cells like the microfold cell (M cell) and DC's present below the epithelial monolayer that protects the structures of the MALT.

The MALT is subdivided into distinct anatomical regions

The MALT can be subdivided into specific anatomical regions. For example, the NALT, Nasal Associated Lymphoid Tissue, represents the mucosal epithelia of the nasal cavity. The BALT, Bronchus Associated Lymphoid Tissue, is comprised of respiratory tract including the lungs and airway. The GALT, Gut Associated Lymphoid Tissue, constitutes the gastrointestinal tract. These compartments are recognized as members of the mucosal immune system by the presence of organized lymphoid tissue.

The NALT

In rodents the NALT contains an aggregate of lymphoid tissue, similar to tonsils, that is analogous to the Waldeyer's ring found in humans [160]. The Waldeyer's ring, or lingual tonsils, are situated at the interface between the upper gastrointestinal tract, GALT, and the terminal end of the NALT. This unique location enables this immune effector site to come into contact with both airborne antigens and foodborne antigens [161]. Hence the NALT represents a unique inductive site for B cell responses. This site is populated with recirculating naïve B cells and functions as an important site of B cell induction [162]. This secondary immune tissue participates both in mucosal immunity and maintenance of homeostasis in the MALT and has an active role in initiating local systemic immune responses [162].

Morphologically the lingual tonsils are divided into 4 distinct regions including the reticular crypt epithelium, extrafollicular area, mantle zones of the follicle, and follicular GC [162]. Follicular GC are a hallmark characteristic of this tissue and are where activated B cells undergo

SHM, CSR, and affinity maturation in order to produce high affinity antibodies. Similar to the salivary and lacrimal glands, the nasal and bronchial mucosal secretions also contain IgA antibodies [163, 164]. This observation supports the concept that the effector sites of the NALT are seeded by B cells that were activated in the lingual tonsils.

The GALT

The GALT comes into contact with many different antigens some are pathological like Salmonella and others are innocuous like food antigens such as wheat. To discriminate between harmful and benign antigens the GALT relies on two main immune microstructures: ILF's and PP. ILF's were recently identified in the mouse small intestine [165]. These lymphoid structures require LT-β signaling during their formation and can develop de novo in response to local stimuli [166]. PP represent a unique interface between the intestinal lumen and the lamina propria and are exquisitely situated to function as immune sensors of the gut. They begin to appear during gestation in both human and mice. By 30 weeks gestation the human fetal small intestine already contains an impressive 60 PP's, upon birth and subsequent colonization by commensal organisms the PP count increases and reaches an all-time high during puberty with an average count of 240 PP's [167]. However, given their anatomical location human data is difficult to gather; hence the development of these microstructures is much better defined in the mouse.

The development of PP in mice occurs in three steps during embryogenesis. The first step is marked by VCAM-1 expression in the stromal cells of the small intestine. Next, the VCAM-1^{+} cells recruit lymphoid tissue inducer cells, LTic, that will become the PP. Finally, circulating lymphocytes are attracted and they fill their corresponding niche [167]. Interestingly, while the

genesis of PP has been fully characterized during embryogenesis, the post-natal development of these structures is not completely understood.

The PP contains three distinct areas: follicular area, interfollicular area, and follicle-associated epithelium, FAE. The first two contain lymphoid follicles, germinal centers and immune cells such as B cells, DC's, and macrophages. The FAE also contains immune cell infiltrates, but the hallmark cell contained here is the M-cell [168]. This is a specialized cells that can transcytose intact antigens and present these to the immune cells below. This is the distinguishing feature of the PP that allows it to function as an immune sensor of the gut. The nature of the antigen that is taken up determines whether the immune response that is induced will be a tolerizing response, a response to food antigens, or an inflammatory response, a response to viral antigen.

Immune cells and their functions in the mucosa

A variety of immune cells participate in the induction/repression of immune responses in the MALT. The most prevalent population of immune cells found are B cells, T cells, and DC's. The lymphocytes present exhibit an activated phenotype even in the absence of inflammation or disease [169]. This is only seen in the mucosa and represents a constant state of readiness. Within the GALT, the PP contain immune cells that have a Th1 phenotype. This was confirmed in humans by various laboratories that note production of inflammatory cytokines associated with Th1 polarization like IFN-γ, TNF-α, and IL-12 whereas Th2 type cytokines including IL-4, IL-6, and IL-10 are secreted are relatively lower levels [170, 171]. The cellular composition of the human PP is poorly defined due to the difficulty in obtaining this tissue from patients. However, in mice PP contain mostly B cells, about 60% defined as $B220^+$ cells, T cells, represent 25% of the immune cells present defined as $CD3^+$ cells, followed 10% DC's, $CD11c^+$ cells, and

the rest are macrophages, defined as F4/80$^+$ cells. Interestingly, of the T cells present most are CD4$^+$ T cells that bear memory cell surface markers [167]. The presence of these cells reinforces the idea that immune cells situated at this location are primed to initiate an immune response.

CD4$^+$ T cells are able to produce either inflammatory or anti-inflammatory cytokines depending on their polarization to Th1 or Th2. The cytokines produced shape the local immune microenvironment and have a direct effect on B cells and the immunoglobulin type that is produced. As described above, T cells in the PP of the GALT have a propensity to produce Th1 type cytokines; yet one of the first observed differences between the systemic immune system and the mucosal immune system is that IgA is the predominant immunoglobulin secreted in the MALT whereas IgG is the most common in the systemic system [169, 172]. IgA isotype class switching is induced by IL-4 and in the PP by IL-6 [150, 173, 174]. Furthermore, unlike serum IgA that is monomeric the IgA in the mucosa is polymeric, found as a dimer [175]. Since IgA functions to neutralize viruses [176-179], inhibit bacterial translocation to the lamina propria [180-182] and can regulate bacterial growth [183] its dominance in the mucosa is not surprising. Hence, despite the preference towards Th1 polarization, IgA production is the preferred isotype.

In order for B cells to produce IgA they must undergo SHM [184] and CSR. These two processes rely on the cytokines present and the immune microenvironment which is shaped by other immune cell populations such as T cells and DC's. Both of these immune cell populations are present in the PP, which are primary IgA induction sites in the gut [185]. For example, in the small intestine DC's produce retinoic acid, a vitamin A metabolite, that induces expression of the gut homing receptors integrin α4β7 and CCR9 on activated T and B cells [186, 187]. Furthermore, treatment of activated B cells with retinoic acid led to IgA production without T cell help [187].

Maintaining immune homeostasis in the GALT

In the gut IgA has two distinct functions, if it is secreted as a high affinity antibody then it neutralizes toxins and inhibits microbes, while low affinity IgA functions in a process known as immune exclusion [185]. Immune exclusion is a process that prevents neutralized agents that are not inflammatory in nature from coming into contact with epithelial linings thus maintaining local homeostasis of the tissue [188]. These two functions establish IgA as the master regulator of the immune induction/repression in the gut. In fact, IgA responses are highly dependent on the presence of commensal microorganisms in the gut. For example, germ-free animals and neonates both exhibit dramatically reduced numbers of IgA secreting B cells in the gut [189].

In addition to secreting antibodies B cells also secrete certain cytokines. The production of IL-10 by B cells was first noted in a B cell lymphoma [49]. This observation suggests that it may also participate in dampening the immune response against tumors. Since its identification IL-10 has been recognized for its anti-inflammatory properties. It was identified as an inhibitor of type 1 (pro-inflammatory) cytokines including: IL-1, TNF-α, and IL-12 [50-53]. It can also down-regulate MHC class II expression on immune cells [50]. In the gut IL-10 serves to down-regulate inflammation through various mechanisms.

An IL-10$^{-/-}$ mouse exhibits an exaggerated inflammatory response to microbial challenges; however, other pathogens are better cleared in absence of IL-10 [190]. This dichotomy is illustrated by the spontaneous development of IBD observed in IL-10$^{-/-}$ mice [191] as a consequence of their gut being colonized with enteric bacteria [192]. This *in vivo* phenotype mirrors *in vitro* data and indicates that IL-10 is a non-redundant cytokine, that is, there is no alternative regulatory mechanism to compensate for its ablation [193].

Recently, a unique B cell subpopulation, identified by its ability to secrete IL-10, has been characterized in mice. These B cells are phenotypically $CD1d^{hi}CD5^{+}$ and represent only 1-2% of splenic $B220^{+}$ cells in wild-type (WT) mice. Furthermore, adoptive transfer these IL-10-producing B cells can reverse inflammation in mice lacking $CD20^{+}$ B cells and $CD19^{-/-}$ mice. The negative regulation of inflammation is mediated by IL-10, produced by this B cell subpopulation. Because of this they are now known as B regulatory or B10 cells [54].

In the mouse, B10 cells are also found in the peritoneal cavity and are similar to their spleen counterparts: same surface marker phenotype and both secrete IL-10. However can peritoneal B10 cells modulate inflammation? To investigate this, a $RAG2^{-/-}$ mouse (a mouse that lacks both B and T cells), received $CD25^{-}CD45RB^{hi}CD4^{+}$ T cells, and either $IL\text{-}10^{-/-}$ $CD19^{+}$ B cells or WT $CD19^{+}$ B cells [71] . The $RAG2^{-/-}$ mice that received the $IL\text{-}10^{-/-}$ $CD19^{+}$ B cells developed higher colitis scores [194]. Taken together, these results suggest that peritoneal B10 cells are important in gut homeostasis and can modulate T cell function during inflammatory conditions such as colitis.

In 2010, a human B cell subset with the ability to produce IL-10 and capacity to suppress the differentiation of Th1 cells was described [195]. This regulatory capacity was IL-10 dependent and ablated by the addition of either anti-CD80 or anti-CD86 antibodies suggesting that T cell help is required for B reg function. This regulatory B cell subset is phenotypically defined as $CD19^{+}CD24^{hi}CD38^{hi}$ and was found in both normal and SLE patients; however, the suppressive capacity of these cells was diminished in cells from SLE patients [195]. Taken together, this indicates that a breach in the interplay of between T and B cells may lead to autoimmunity.

In humans, the inflamed gut leads to the differentiation of Breg cells that negatively regulate inflammation via the release of IL-10 [196]. Although the mechanism behind the differentiation of the B reg cells has not been completely elucidated, IL-1β and IL-6 are important cytokines in the induction of these regulatory B cells. Interestingly, both IL-1β and IL-6 are pro-inflammatory cytokines; hence overt immune responses would be regulated through the production of IL-10. These data are reminiscent of animal studies where B reg cells were shown to lead to the resolution of colitis in mice [194].

While there are cell surface markers that define the B10 population, $CD1d^{hi}CD5^{+,}$ a specific transcription factor has yet to be associated to this cell subset. However, Blimp1 and IRF4 are upregulated while pax5 and Bcl6 are down-regulated [197]. Given that these transcription factors are linked to plasma cells, these observations suggest that B10 cells have the ability to differentiate into plasma cells capable of secreting polyreactive IgM and IgG antibodies. This may be reflect their ability to dampen inflammation through the clearance of potentially threatening antigens. The B10 cell lineage has yet to be well defined. B10 cells share some phenotypic characteristics with B1a cells of the peritoneal cavity, T2-MZ, transitional-2, precursors and MZ B cells [198-200].

The Mammary Gland is an important site of IgA production in the MALT

The mammary gland (MG) is an exocrine gland responsible for the production and secretion of milk. However, unlike other mucosal immune organs the MG develops in stages. The initial stage of development occurs during early puberty and is characterized by the presence of small rudimentary ducts and terminal end branches, TEB. As the gland matures and enters the second stage of development, during late puberty, the TEB elongate and begin to branch. The third stage of development occurs during pregnancy and is characterized by the TEB's growing into alveolar buds. The final stage of development occurs during lactation when the alveolar buds become mature alveoli [201]. Importantly, as the mammary gland ductal epithelia enters lactation, the last stage of MG developed, CCL28 begins to be expressed. Therefore, the full maturation of this gland is required for the expression of CCL28. Moreover, expression of CCL28 in the MG parallels the accumulation of IgA antibody secreting cells in the mammary gland, a process that can be inhibited by anti-CCL28 antibodies [202]. Taken together, these results indicate that the CCL28/CCR10 axis regulates the recruitment of IgA secreting plasma cells to the mammary gland (Figure 1.8).

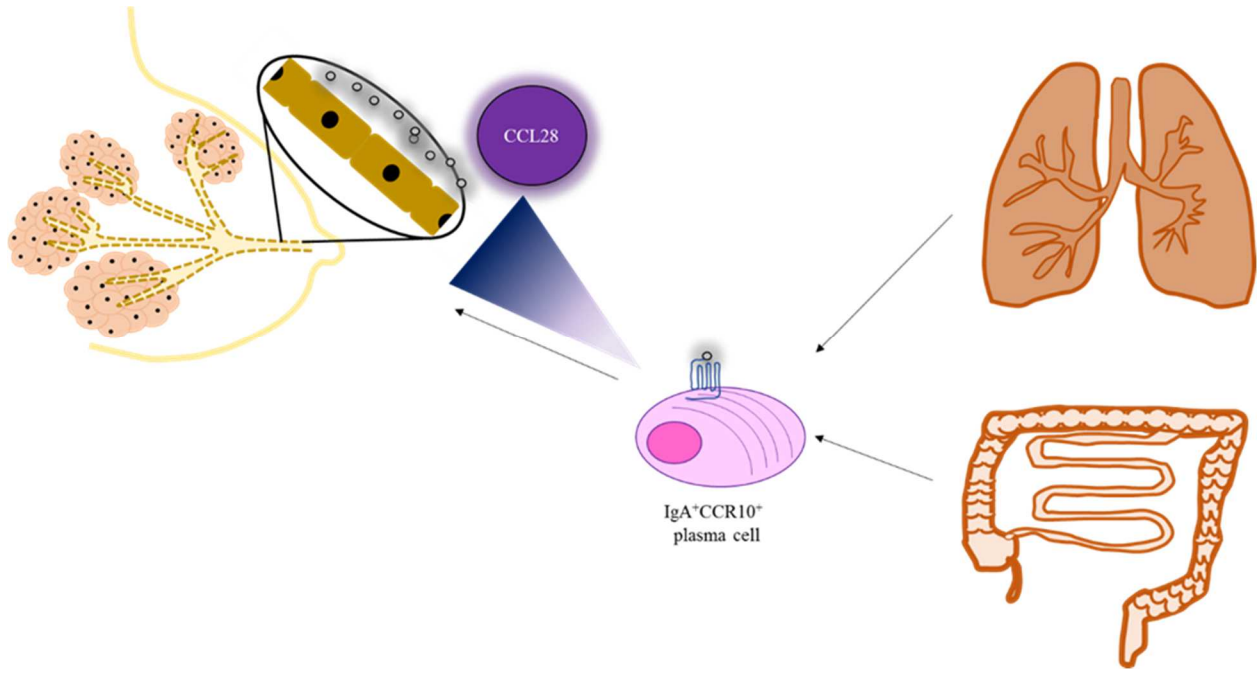

Figure 1.8. CCL28/CCR10 axis recruits IgA$^+$CCR10$^+$ plasma cells to the lactating mammary gland (MG). CCL28 recruits IgA$^+$CCR10$^+$ plasma cells from the respiratory and gastrointestinal tract. In this manner, neonates receive passive immunity against respiratory and gut pathogens via breast milk.

Abnormalities in B cell development and pathological implications

The BCR is an integral component of the B cell. It functions as a check point in every step of the B cell journey: from B cell development in the bone marrow, in B cell functions such as antigen presentation and even has an impact on future immunoglobulin production. For these reasons its importance cannot be underscored.

B cells developing in the bone marrow rely on the BCR for their survival and differentiation [99]. The importance of pre-BCR genes and down-stream signaling that leads to a mature BCR was demonstrated through the use of mice deficient in these genes, these data has been recapitulated in human patients that have primary immunodeficiencies. The most studied gene involved in BCR development is Burton tyrosine kinase, *BTK*. This gene is an integral component of down-stream signaling that occurs after the formation of the pre-BCR, it is mainly involved in promoting proper calcium-flux [203]. A mutation in this gene leads to X-linked globunemia, XLA. This phenotype was first clinically observed in a single male patient that suffered recurrent sepsis and had no detectable serum immunoglobulins [204]. Since then 600 other mutations of this gene have been noted, all of which have an effect on B cell development in the bone marrow [205]. The mouse models of XLA, which bear a mutated copy of the *Xid* gene or *Btk* gene, exhibit a much milder phenotype than human patients, this may be a direct consequence of mice being congenic, thus having no other variations in their genome, whereas the human population is diverse and many other genetic factors may lead to an exacerbated phenotype [206, 207].

XLA patients exhibit a higher susceptibility to developing inflammatory autoimmune diseases like RA and type I diabetes which suggests that Th1 responses are dominant in these patients

[208, 209]. Recently, the *BTK* gene has been implicated in the signaling via several TLR (toll like receptors) ligands including LPS, detected by TLR-4 [210, 211]. These observations have led to a flurry of publications that attempt to investigate the production of inflammatory cytokines of XLA patients, all of which report that XLA patients exhibit impaired secretion of pro-inflammatory cytokines following LPS stimulation [212-214].

However, these results are plagued with inconsistencies that stem from the methodology, both from the acquisition of the cells and the cells types utilized in the assays, and techniques, the actual assays utilized [215]. For instance, PBMC's collected from XLA patients activated with LPS produced significantly higher amounts of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 compared to B cell depleted PBMC's from healthy controls [215]. Interestingly, IL-10 was also upregulated by XLA patients compared to healthy controls [215]. The observed up-regulation of the cytokines above may be a direct consequence of the absence of the regulation normally provided by the presence of a functional *BTK* gene.

Other abnormalities in B cells can negatively impact their functionality later in B cell differentiation. For example, Common variable immune deficiency, CVID, is the result of mutation in various genes including inducible T cell co-stimulator (ICOS), CD19, a B cell surface marker, and TACI, a shared receptor for BAFF and APRIL [216]. CVID patients exhibit low levels of serum immunoglobulins, reduced numbers of memory B cells, a decrease in CSR, and impaired B cell activation [99]. Luckily, only a small percentage of CVID patients have all three mutations.

Patients with CVID exhibit a wide array of defects including alteration in the proliferative and activation pathways of T cells, irregular cytokine production, and decreased expression of co-stimulatory molecules like CD40L. The combinatorial effect of these defects result in drastically

noticeable B cell defects that hinder the B cells ability to undergo somatic hypermutation and class switch recombination [218]. The overall effect of CVID leads to autoimmunity, inflammation, and lymphoproliferative malignancies [219, 220].

The high degree of clinical heterogeneity observed in patients with CVID is a result of the number of mutations they have in specific genes. To better understand the pathology of this disorder and to find a possible classification system to be utilized in the diagnosis of CVID Berron-Ruiz et al. performed a characterization of CVID patients that included various parameters such as analysis of lymphocyte populations, memory B cells, and expression co-stimulatory molecules [218]. Adult CVID patients had the most pronounced phenotype. All measured lymphocyte populations, B, T, and NK cells, in adult CVID patients were drastically reduced. Memory B cells, defined as $CD19^{+}CD27^{+}$, were also lower in adult CVID patients compared to healthy controls. Interestingly, only the expression of ICOS, a costimulatory molecule expressed on the surface of activated T cells, was reduced in adult CVID patients compared to healthy controls [218].

IgA deficiency is the most common primary immunodeficiency [221]. In spite of being the most common immunodeficiency most patients are asymptomatic and are diagnosed serendipitously. However, some patients exhibit recurrent infections in mucosal tissues of the MALT including respiratory and gastrointestinal tracts. IgA is main isotype secreted at these sites therefore it is not surprising that patients with a phenotype exhibit symptoms in the MALT [222]. This pathology is a result of a maturation defect in B cells that results in a failure to produce IgA [223, 224]. Immature B cells are able to produce low levels of IgA, in conjecture with IgM and IgD, but they do not become IgA secreting plasma cells [225, 226]. Various immune players have been implicated with this deficiency. For example, a deregulated cytokine network has been

proposed as a potential factor in IgA deficiency. Lack of IL-4, IL-6, IL-7, IL-10, IL-21, and TGF-β have been associated to this disorder [223, 226-229]. Not surprisingly, all of the aforementioned cytokines have a critical role in B cell differentiation, proliferation and survival, and immunoglobulin secretion specifically IgA secretion. At the genetic level mutations in TACI have been observed, but the causal relationship between the two is yet to be defined [229, 230]. Recently a shared mutated locus was identified for IgA deficiency and CVID [231]. Moreover, IgA deficiency has been reported to progress to CVID suggesting that IgA deficiency lies on the spectrum of CVID pathologies [232, 233].

Hyper-IgM, over production of IgM, results from a mutation in CD154 the ligand of CD40. This mutation leads to inadequate B cell stimulation, the B cell does not receive the secondary co-stimulatory signal from the T cell, which results in an absence of CSR and SHM [217]. Patients with hyper-IgM exhibit high levels of serum IgM, an absence of other immunoglobulin isotypes, and recurring infections [217]. This disorder is often seen in conjecture with IgA deficiency.

Conclusions

The role of B cells within the adaptive immune response continues to be modified and refined. It is evident that although they require differentiation and special activation to produce cytokines, B cells are active immune response modulators with the ability to either augment or suppress a response depending on cytokines they secrete. Furthermore, the identified effector subtypes, Be1 and Be2, are of critical importance to the initiation and propagation of either type I or type II responses because their products not only stimulate either response, but also participate in cross regulation mechanisms that inhibit opposite responses [254]. Overall, I conclude that B cells are an integral component of the adaptive immune system and possess interesting characteristics that have been recognized only recently. Their ability to produce cytokines likely reflects their potentially important role as regulatory cells of the immune system. I am confident that in the future B cells will become a very active area of research in immunoregulation.

IL-40: A novel cytokine secreted by activated B cells

The identification of cytokines and their receptors has been pivotal in elucidating the mechanisms through which the immune system responds to external antigens. Cytokines are involved in the induction, modulation, and resolution of the immune response [234, 235]. Furthermore, their deregulation can lead various pathologies [236-238]. Given their importance, there has been a strong interest in the identification and functional characterization of cytokines. Most known cytokines belong to superfamilies that arose during evolution through gene duplication. The presence of common structural features in their sequences facilitated the identification of cytokine family members. However, IL-40 does not belong to any of these superfamilies and was identified using a bioinformatics approach.

IL-40 was identified as an ISAG (immune system associated gene) after a thorough analysis of a comprehensive database of human gene expression that includes 105 different human tissues or cells (BIGE: Body Index of Gene Expression) [239, 240]. IL-40, currently an unannotated open reading frame: *C17orf99*, has been previously reported on. One these publications reported *C17orf99* as a gene encoding a secreted protein after performing a survey looking for predicted signal sequences [241]. Another publication, generated a mouse knock-out library of secreted and transmembrane proteins, one of these proteins was encoded by *C17orf99* [242].

In my work as a graduate student, I have contributed to the exciting field of cytokine identification by characterizing a novel activated B cell cytokine, Interleukin 40 (IL-40). My dissertation work focused on characterizing the function of IL-40 *in vivo.* To properly elucidate the physiological function of this new cytokine I obtained a mouse deficient in (6030468B19Rik), the homolog of *C17orf99.* Besides the identification of *C17orf99* as a secreted protein and the mouse knock-out library in which a mouse deficient in *C17orf99* was

generated there was little other information available on this molecule. From the BIGE database, I knew that this gene was associated with the immune system because of its high expression in the fetal liver, bone marrow, and activated B cells. Hence, I began by performing a thorough immunophenotype to compare the immune compartments of the IL-40-deficient mouse (*Il-40*$^{-/-}$) to a WT control. I continued my analysis of the *Il-40*$^{-/-}$ mouse by performing other experiments aimed to identify a possible abnormalities in the immune system of the mouse. My initial characterization of IL-40 has revealed that this cytokine is involved in IgA production, both systemic and mucosal, and that its expression plays a role in mammalian specific immune functions. Furthermore, my doctoral thesis work has opened up a new field in cytokine and B cell biology.

CCL28: A chemotactic cytokine that regulates MALT homeostasis

As described above, chemokines are chemotactic cytokines that are responsible for recruiting given cell types to a given location by means of a concentration gradient. The production of the chemokine ligand by a particular cell induces a chemotactic response on the cell that bears the cognate receptor for the ligand secreted. This mechanism is used in throughout the various systems including the immune system. Within the immune system chemokine ligand/receptor pairs function in every aspect of a response from inducing CCR7 on B cells that results in their migration toward CCL19/CCL21 expressing T cells to recruitment of long lived plasma cells to bone marrow through the CXCR4/CXCR12 axis [122, 243]

CCL28 was identified along with its receptor, CCR10, in the laboratory of my mentor Dr. Zlotnik, as a product of epithelial cells that line the mucosa and induces calcium mobilization in both $CD4^+$ and $CD8^+$ T cells [244]. It has a high level of homology with CCL27 which explains their shared a receptor, CCR10 and indicates that these two genes arose through a gene duplication event. Interestingly, CCL27 is mainly expressed in the skin and directs T cells to cutaneous sites; hence the gene duplication led to selective chemokine differentiation and specialized tissue/cell type expression [245]. We should note that both the mucosa and skin are barrier tissues, an observation that may account for the specialization of their functions and may explain their expression patterns. Besides its chemotactic properties, CCL28 also exhibits broad spectrum antimicrobial activity against gram positive and gram negative bacteria, and fungi [246]

Various reports and the BIGE reveal that the highest site of human CCL28 expression is the salivary gland followed by other mucosal sites including small intestine and colon [247]. Deregulated levels of CCL28 have been correlated to various pathologies including salivary

gland tumors, Hodgkin's disease, and Sjögren's syndrome [247-249]. Its strong expression in the mucosa links this chemokine to the maintenance of homeostasis of the MALT.

During the course of my doctoral dissertation work I worked to functionally characterize IL-40, in addition to elucidating the physiological consequences of CCL28 ablation in a mouse model. Although this chemokine has been studied, see above, there are no reports utilizing a CCL28 deficient mouse. At this point I would like to mention that I have been extremely privileged to work with not one, (*Il-40*$^{-/-}$), but two, (*Ccl28*$^{-/-}$) of the first generated knock-out murine models of each of these molecules.

The *Ccl28*$^{-/-}$ mouse recapitulates the phenotype of the CCR10-deficeint mouse, namely a decrease in IgA in the mucosal secretions [250]. In addition to a striking IgA deficiency, I have observed interesting mating behavior and an increased susceptibility to salmonella infection. This implies that CCL28 is involved in much more than just the mucosal immune system and that it may have a role in development. The salmonella infection data was a result of a collaboration with the laboratory of Dr. Manuela Raffatellu and provides concreate evidence that CCL28's antimicrobial abilities are extremely important in resolving mucosal infections.

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CHAPTER 2

IL-40: A novel cytokine produced by activated B cells

Abstract

Cytokines are key effector molecules that regulate immune and inflammatory responses. Here, I describe a novel B cell cytokine encoded by an uncharacterized gene (*C17orf99*), whose expression is strongly induced in B cells upon activation. A *C17orf99* deficient mouse exhibits an altered B cell phenotype including smaller Peyer's patches, reduced levels of serum immunoglobulins, and a restricted antibody response to heat-inactivated (HI) vaccinia virus (VACV). *C17orf99* is only present in mammalian genomes, suggesting that it plays a role in mammalian immune responses. Accordingly, *C17orf99* expression is induced in the mammary gland upon the onset of lactation and a *C17orf99*$^{-/-}$ mouse exhibits reduced levels of gut and mammary gland IgA. I conclude that *C17orf99* encodes a novel B cell-associated cytokine (Interleukin-40) that plays an important role in the development of humoral immune responses.

Introduction

Cytokines are small secreted proteins that have pleiotropic roles throughout various organ systems. In the immune system they are active participants that play an essential role in host defense and development of immune responses. In essence cytokines are the orchestrators of the immune response elicited because their secretion or lack thereof has a tremendous effect throughout the duration of a response. For example, IL-4 is an integral component to B cell responses. It functions as a survival/differentiation factor, it synergizes with activation signals received such as CD40 ligation, and induces the preferential secretion of certain immunoglobulin. Moreover, it induces the preferential secretion of certain isotypes, and the cells that respond to it function in a cross-regulation mechanism that serves to polarize the response to a Th2 response while at the same time inhibiting a Th1 type response. Like IL-4, IFN-γ functions in a similar fashion however directs the immune response in the opposite direction, towards Th1 polarization. These two cytokines are just one example of how cytokines modulate the immune response by exerting their effect on B cells.

The recent discovery of cytokine producing B cells such as regulatory B cells (Breg/B10) and effector B cells: Be1 and Be2 has given credence, through experimental data, to B cells being cytokine producers in addition to producing antibodies and presenting antigen. This newly attributed function to B cells provides evidence that beyond just responding to T cell help B cells are able to modulate and polarize immune responses based on their cytokine secretion profile (Figure 2.1).

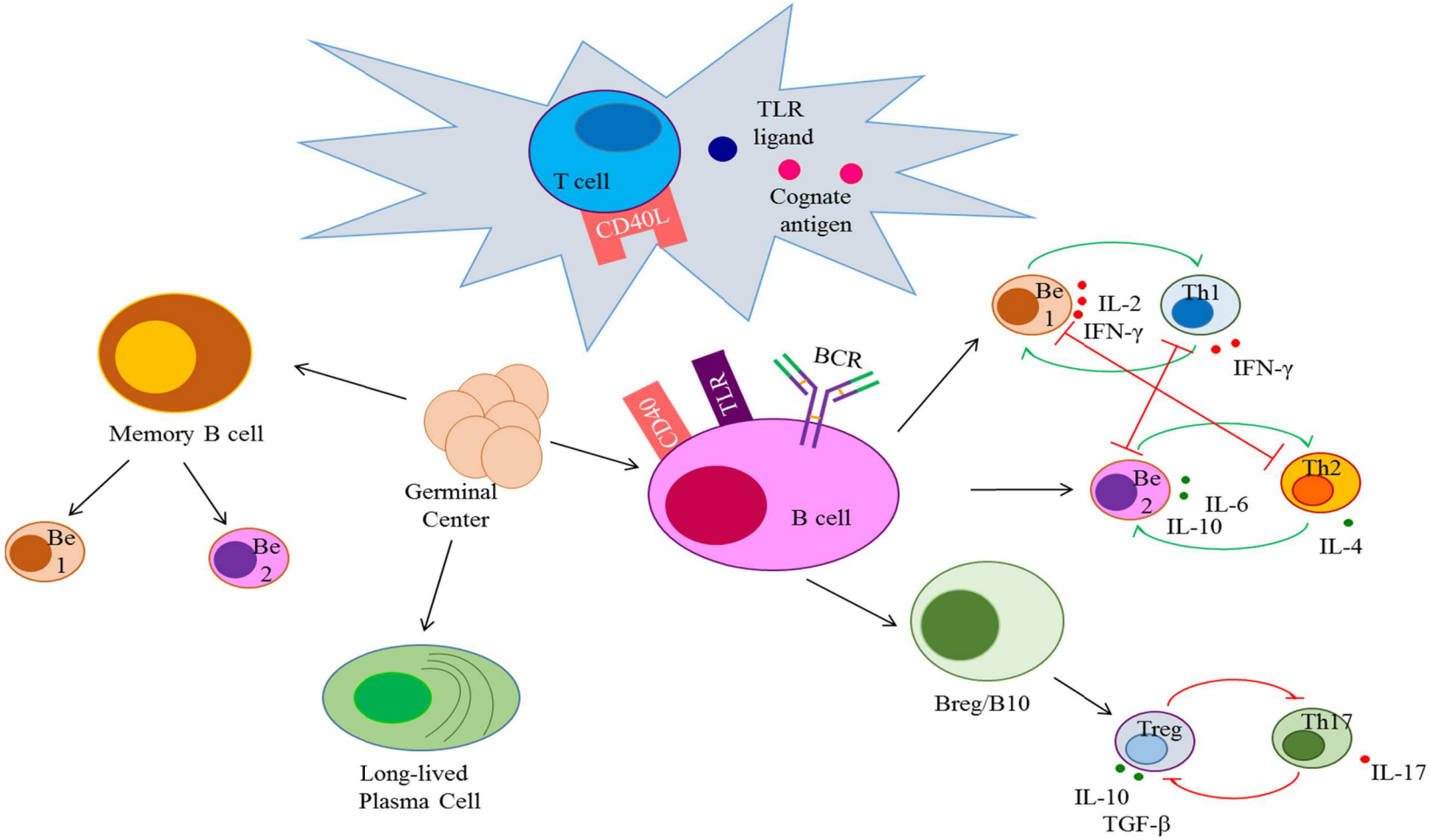


Figure 2.1. Regulation of immune response through B cell cytokine production. In a steady-state there is a balance between pro-inflammatory and anti-inflammatory cytokines produced by B and T cells. As a naïve B becomes activated via T cell help (CD40 engagement), TLR ligands (LPS in mice or CpG in mice/humans) or BCR crosslinking via cognate antigen the B cell can differentiate into one of the cytokine producing subsets. The cytokine profile secreted then modulates the type of response elicited and concurrently inhibits the opposing response. Pro-inflammatory cytokines depicted as red circles. Anti-inflammatory cytokines depicted as green circles.

Given their pivotal role in both the development and function of the immune system, cytokine discovery and characterization has been a fertile research field. The common structural features in their sequences facilitates the identification of additional cytokine family members. A corollary is that cytokines that do not share structural features with known cytokines are less likely to have been identified. This is exemplified by the most recent cytokine to be identified, Meteorin-like (IL-39) that does not belong to an established cytokine family and is expressed in mucosal tissues, skin and alternatively activated macrophages [4].

A Bioinformatics approach to the identification of a novel activated B cell cytokine: Interleukin 40 (IL-40)

The sequencing of the human genome led to the identification of thousands of genes with no known function, many of which are poorly annotated and for which there is little or no information. The poorly characterized or completely unannotated genes is precisely where the interest of the Zlotnik lab lies. We are interested in the identification of ISAG's that encode either secreted or transmembrane proteins. To this end, we analyzed our comprehensive microarray database of human gene expression that includes 105 different human tissues or cells (BIGE: Body Index of Gene Expression) [1, 2]. This database is a very powerful tool that allows us to search for highly expressed genes in specific cells or tissues. For example, the output for Kallikrein 3 (*KLK3*), a prostate specific antigen, is exclusively expressed in the prostate gland (Figure 2.2).

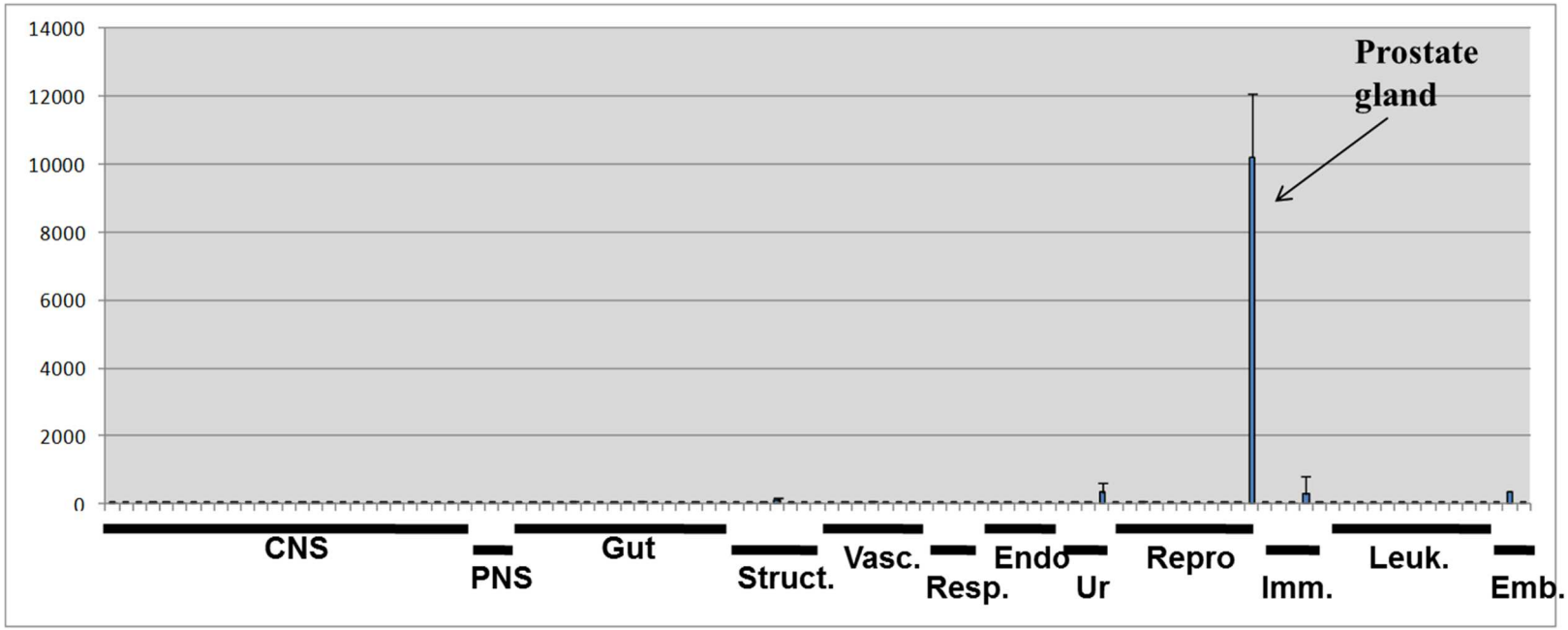


Figure 2.2. BIGE provides expression profile of genes in highly specific tissues or cells. Kallikrein 3 (*KLK3*), a prostate specific antigen, expression is restricted to the prostate gland. Data from the BIGE (Body Index of Gene Expression) database (human microarray data using the Affymetrix gene array (U133 2.0)). X-axis is organized by organ systems: CNS (central nervous system), Gut (gastrointestinal), Struct (structural), Vasc (vasculature), Resp (respiratory), Endo (endocrine), Ur (urinary), Rep (reproductive), Imm_T (immune tissue), Imm_C (immune cells), and Dev (developmental).

Because the BIGE provides a profile of gene expression in specific organ systems, it also provides insight into possible function of the gene of interest. Furthermore, it identifies genes with restricted expression, as exemplified by the expression profile of *KLK3*. In our efforts to identify novel ISAG's with a potential role within the immune system we searched for genes expressed in lymphoid and myeloid cells or tissues of the immune system such as: fetal liver, bone marrow, spleen, lymph nodes, and thymus. This screen yielded over 1500 genes, most of which are well known immune system associated genes that have been characterized over the last few decades including: cytokines, chemokines, transcription factor and clusters of differentiation (CD's). Interestingly, of this extensive output we identified 35 poorly characterized genes predicted to encode either transmembrane or secreted proteins expressed by either leukocytes or immune organs. We have recently reported three of these ISAGs. The first is a poorly characterized secreted protein (Isthmin 1) expressed by barrier tissues and natural killer (NK), natural killer T (NKT) and Th17 cells [3]. The second is tetraspanin 33 (*TSPAN33*), a transmembrane protein that represents a novel biomarker of activated B cells [4]. The third is one is IL-39/Meteorin-like, a novel cytokine produced by barrier tissues and alternatively activated macrophages [5]. Of special importance to me is another identified gene: *C17orf99 (Chromosome 17 open reading frame 99*).

IL-40, an unannotated gene formerly known as *C17orf99*

C17orf99 encodes a small secreted protein that is highly expressed in the fetal liver, bone marrow, and activated B cells. Its unique expression pattern caught our attention because it is restricted to sites of hematopoiesis (fetal liver and bone marrow) and produced by an activated lymphocyte that is not generally known for their cytokine production (activated B cells). In addition, this gene has a signal sequence, indicating that it encodes a secreted protein, however the *C17orf99* protein does not exhibit any other known domains. Since it is a product of an activated lymphocytes we have renamed this gene Interleukin 40 (IL-40). IL-40 is produced by B cells upon activation and is associated with type 2 cytokines (induced by IL-4 or IL-13) and inhibited by type I cytokines (IFN-γ). Interestingly, IL-40 is induced in the lactating mammary gland, suggesting that it has a role in mammalian specific immune functions.

A mouse deficient in IL-40 *(Il-40$^{-/-}$)* exhibits more B cells in the resting spleen and decreased levels of serum and mucosal IgA, while maintaining normal serum levels of IgM and total IgG. To investigate the possibility of defective antibody repertoire we utilized the vaccinia virus infection as a means to quantitatively assess the ability of the *Il-40$^{-/-}$* to produce antibodies against a specific pathogen. Compared to the WT control mouse the *Il-40$^{-/-}$* mouse was not able to elicit a response of the same magnitude as measured by antibody diversity, epitopes recognized. This suggests that IL-40 is important to the diversification of the antibody repertoire generated by plasma cells. The IgA defect is may be a result of a reduction in the number and size of PP in the small intestine. Additionally, there is a reduction in both germinal center B cells and IgA secreting B cells within the PP. Since, we noted an IgA defect associated to tissues of the MALT, we infected the *Il-40$^{-/-}$* with a mouse model of Salmonella, however because this was

an acute infection we did not see any significant differences. Finally, IL-40 is also produced by human lymphomas, suggesting that it may have a role in cancer pathogenesis. Taken together, IL-40 is a novel B cell cytokine whose function is associated to B cell homeostasis, IgA production, plasma cell development, and may be involved in the evolution of lymphomas.

Materials & Methods

Body Index of Gene Expression [6] Database

The construction of the BIGE database has been previously described [1, 2]. Briefly, 105 different tissues and cells were collected from 8 healthy donors (4 male, 4 female) within 5 hours post-mortem. RNA was extracted from the tissues and cells and used to synthesize cDNA for hybridization to Affymetrix U133 2.0 gene arrays (Affymetrix, Santa Clara, CA). A probe set corresponding to *C17orf99* was used to show the expression of this cytokine within the entire body panel of tissues and cells.

Cell lines

The human B cell line 2E2 has been described [7]. The HEK 293 cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA). The human lymphoma lines were acquired through a collaboration with the Fruman laboratory (Dr. David Fruman Laboratory, UCI, Irvine, CA). The murine cell A20-2J has been described [8].

q-PCR

Quantitative real-time PCR (q-PCR) data was generated with a Roche Lightcycler 480 system using the Universal Probe Library (UPL) based system (Roche, Indianapolis IN). Total RNA was extracted from mouse tissues or cells using the Qiagen RNeasy® kit according to manufactures instructions (Qiagen, Valencia, CA). Equal concentrations of RNA were used for each sample in a reverse transcription reaction to synthesize cDNA. qPCR results were processed in Excel and analyzed using GraphPad Prism (http://www.graphpad.com).

Mice

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC), of the University of California, Irvine. C57BL/6J (stock number 000664) were obtained from the Jackson Laboratory (Bar Harbor, ME). *Il-40*$^{-/-}$ mice (B6/129S5-6030468B19Rik$^{-/-}$) were obtained from the Knockout mouse repository (KOMP, UC Davis, Davis, CA). The B6/129S5-6030468B19Rik$^{-/-}$ mouse obtained were backcrossed to C57BL/6 mice at least 5 times. Serum samples were obtained through sub-mandibular cheek bleed as described [9]. Fecal samples were collected as described [10]. Breast milk samples were obtained by collecting the stomach contents of neonate mice. Briefly, stomach contents were centrifuged at max speed for 15 minutes and supernatants were collected.

ELISA

ELISA was performed according to manufacturer's protocol (Ebioscience, San Diego CA). Plates were read at 450nm. For fecal IgA ELISA plates (Sigma Aldrich, St Louis, MO) were coated with anti-IgA mAb (clone RMA-1) (Biolegend, San Diego CA) at 2ug/ml. 5% milk in PBS-T was used as blocking buffer. Plates were washed 3 times with PBS-T after each step. AP conjugated anti-mouse IgA (Southern Bio, Birmingham, AL) used as secondary antibodies for 1 hr at room temperature and washed 3 times with PBS-T. SureBlue substrate (KPLGaithersburg, MD) was added, reaction was stopped with 2N H_2SO_4. Plates were read at 630nm.

In vitro CSR induction

Spleen cells were suspended in RPMI with FBS (10%), 50 mM β-mercaptoethanol and 1X antibiotic/antimycotic mixture (Invitrogen, Grand Island, NY) at 37°C in 48-well plates and stimulated with the following reagents: LPS (5µg/mL) from E. coli (Sigma-Aldrich) plus TGF-β (2ng/mL R&D solutions) and anti-IgD dextran (Fina Biosolutions) for CSR to IgA. Additionally,

LPS was used in combination with, rmIL-4 (5 ng/ml) for CSR to IgG1, and IFN-γ (50 ng/ml; PeproTech Inc.) for CSR to IgG3. Cells were collected on day 4 for surface Ig analysis, after staining with FITC-anti-mouse IgG1 (clone A85-1), anti-mouse IgG2a (clone R19-15), anti-mouse IgG2b (clone R12-3) anti-mouse IgG3 (clone R40-82) or anti-mouse IgA (clone C10-3) rat mAb and PE-anti-mouse CD45R (B220) (clone RA3-6B2) rat mAb (BD Biosciences). Cells were fixed with 1% paraformaldehyde in PBS and analyzed by flow cytometry using BD FACSCalibur (BD Biosciences) using Flojo data analysis software (TreeStar Inc.).

Peyer's Patches- isolation and staining

Peyer's patches were isolated from the small intestines of WT and *Il-40*$^{-/-}$ mice. Peyer's patch B cells were stained with phycoerythrin (PE)-Labeled anti-mouse CD45R (B220), rat mAb (RA3-6B2, eBioscieces) and FITC-labeled PNA (E-Y Laboratories, San Mateo, CA), 7AAD (Biolegend), and APC-labeled IgM (Biolegend) and analyzed by flow cytometry using BD FACSCalibur (BD Biosciences San Jose CA) using Flojo data analysis software (TreeStar Inc., Ashland OR).

HI-VACV- Mouse Vaccination and Sera Collection

WT and *Il-40*$^{-/-}$ mice were vaccinated with 2 x 10^6 PFU heat-inactivated Vaccinia virus Western Reserve (VVWR) strain with Alum adjuvant administered intramuscularly. Mice received a vaccine boost at day 14. Longitudinal sera samples were collected from mice by saphenous vein bleed at days 0, 7, 14 and 28 post-vaccination.

Viruses

VVWR stocks were grown on HeLa cells in T175 flasks, infecting at a multiplicity of infection of 0.5. Cells were harvested at 60 h, and virus was isolated by rapidly freeze-thawing the cell pellet three times in a volume of 2.3 ml RPMI plus 1% fetal calf serum (FCS). Clarified

supernatant was frozen at -80°C as virus stock. VVWR stocks were titrated on Vero cells (2 x 108 PFU/ml). Heat-inactivated VVWR stock was prepared by incubating virus on a water bath at 65°C for 1 h.

Results

A bioinformatics approach to identifying IL-40 (*C17orf99)*, a novel B cell cytokine

Our initial screen of the BIGE yielded over 1500 genes associated with the immune system. Most of these gene encode known transmembrane or secreted proteins associated with the immune system including most known cytokines and CD molecules. Importantly, we also identified 35 gene encoding poorly characterized or completely unannotated secreted or transmembrane proteins. *C17orf99* was among the unannotated genes and its expression profile made it an attractive candidate worthy of future studies. *C17orf99* is highly expressed in the fetal liver, bone marrow, and activated B cells (Figure 2.3a). A table with the top 20 sites of *C17orf99* expression is seen in Table 2.1. At the onset of my studies on *C17orf99* only two other publications had mentioned it. The first was a publication that reported a survey of predicted secreted and transmembrane proteins, one of these being *C17orf99* [11]. The second publication reported a library of 472 mouse knock-outs encoding poorly characterized, secreted or transmembrane, one of which was *C17orf99* [12].

The human *C17orf99* gene encodes a 265 amino acid protein with a predicted N-terminus signal peptide of 20 amino acids (Figure 2.3b). The homolog, *6030468B19Rik*, is present in various mammalian species including primates, dogs, and mice, however it is absent in chicken and zebrafish (Figure 2.3c). We also performed end-point PCR to confirm that IL-40 expression is restricted to hematopoetic tissues (2.3d).

This gene encodes a small protein of 265 amino acids with a predicted 20 amino acid signal peptide (Figure 2.3b). To confirm that it encodes a secreted protein, we inserted the predicted human *C17orf99*open reading frame cDNA from human 2E2 B cells [7], into the plasmid

pTT5V5H8 [13] resulting in a recombinant gene encoding a *C17orf99*-$(His)_8$ tag fusion protein. HEK 293 cells were transiently transfected with the pTT5V5H8-*IL40* construct, or empty vector, and supernatants were collected on days 1 and 3 and analyzed for the presence of recombinant *IL-40* protein. Western blot analysis of affinity-purified supernatants using an anti-His antibody detected an approximately 27kDa protein in cell cultures transfected with the pTT5V5H8-*C17orf99* construct, (Figure 2.3e) confirming that *IL-40* encodes a secreted protein.

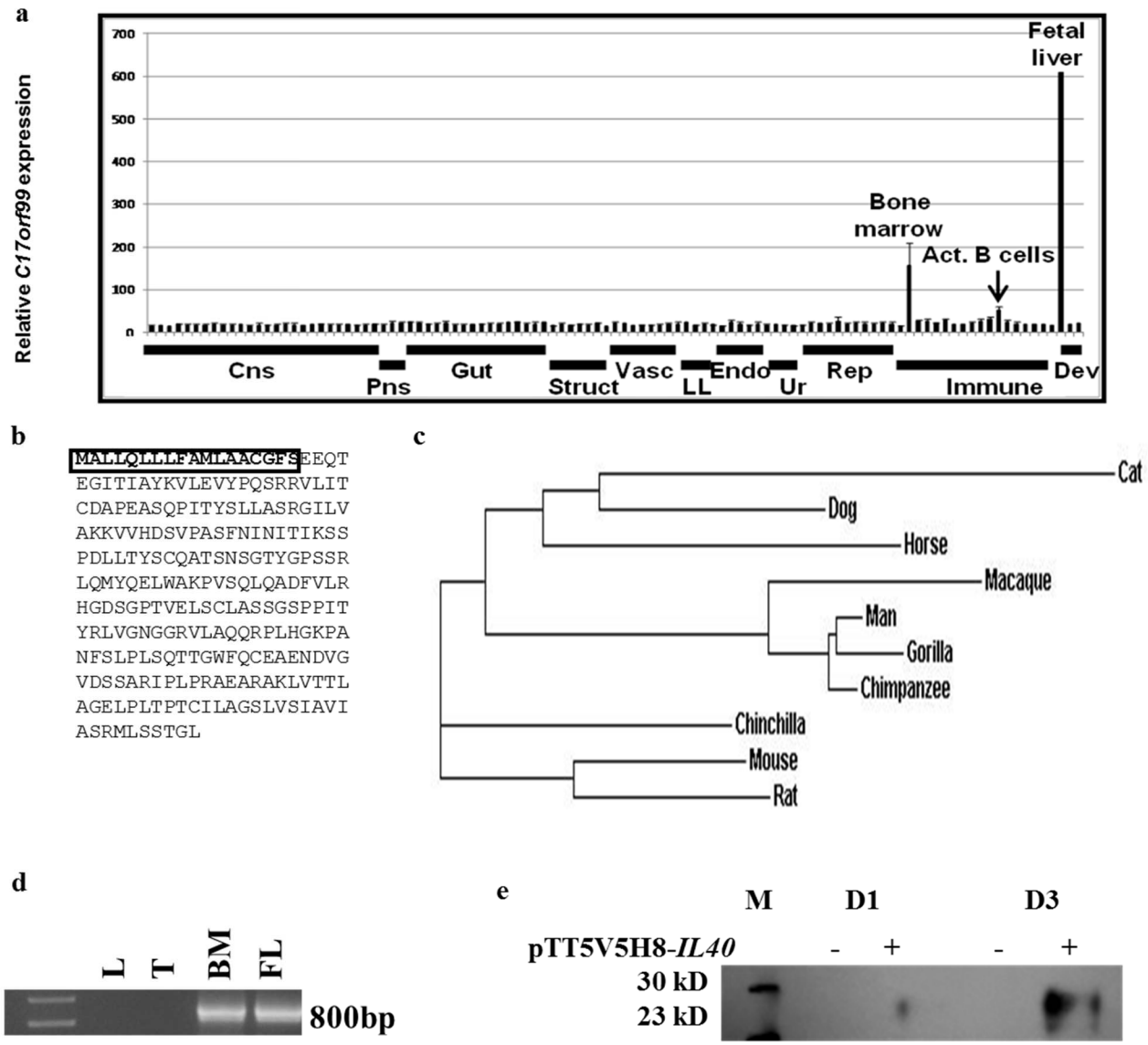
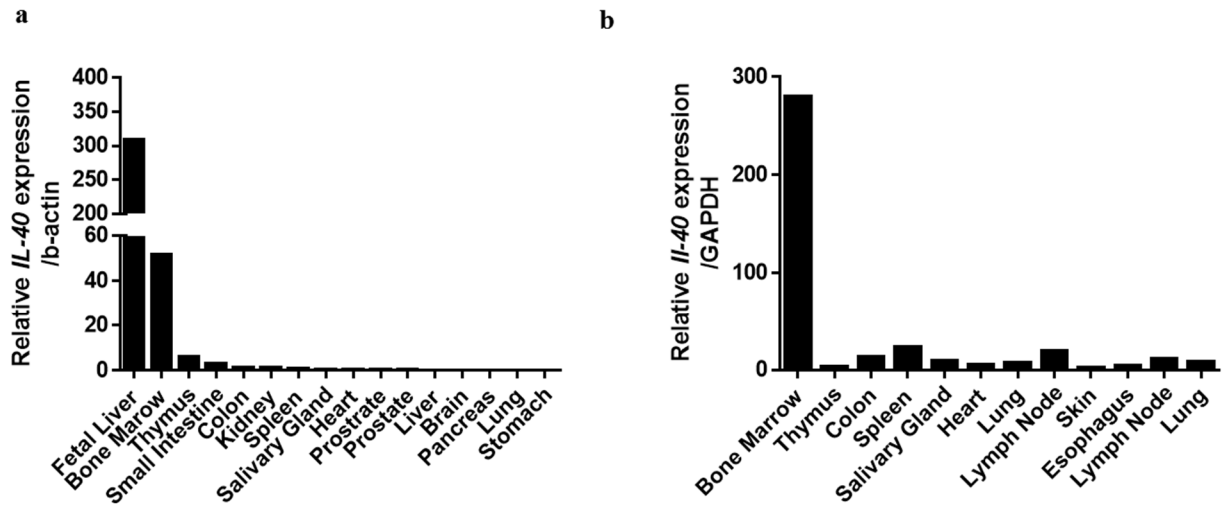

a
Relative C17orf99 expression
700
600
500
400
300
200
100
0
Fetal liver
Bone marrow
Act. B cells
Cns
Pns
Gut
Struct
Vasc
LL
Endo
Ur
Rep
Immune
Dev
b
MALLQLLLFAMLAACGFSEEQT
EGITIAYKVLEVYPQSRRVLIT
CDAPEASQPITYSLLASRGILV
AKKVVHDSVPASFNINITIKSS
PDLLTYSCQATSNSGTYGPSSR
LQMYQELWAKPVSQLQADFVLR
HGDSGPTVELSCLASSGSPPIT
YRLVGNGGRVLAQQRPLHGKPA
NFSLPLSQTTGWFQCEAENDVG
VDSSARIPLPRAEARAKLVTTL
AGELPLTPTCILAGSLVSIAVI
ASRMLSSTGL
c
Cat
Dog
Horse
Macaque
Man
Gorilla
Chimpanzee
Chinchilla
Mouse
Rat
d
L
T
BM
FL
800bp
e
M
D1
D3
pTT5V5H8-IL40
-
+
-
+
30 kD
23 kD

Figure 2.3. ***C17orf99* encodes a novel cytokine expressed in fetal liver, bone marrow, and activated B cells**. (a) *C17orf99* expression in normal human tissues and immune cells. Data from the BIGE (Body Index of Gene Expression) database (human microarray data using the Affymetrix gene array (U133 2.0)) showing the expression profile of *C17orf99*mRNA. X-axis is organized by organ systems: CNS (central nervous system), Gut (gastrointestinal), Struct (structural), Vasc (vasculature), Resp (respiratory), Endo (endocrine), Ur (urinary), Rep (reproductive), Imm_T (immune tissue), Imm_C (immune cells), and Dev (developmental). (b) *C17orf99*aminoacid sequence showing the signal peptide. (c) Clustal Omega analyses of the amino acid sequence of *C17orf99*in 10 mammalian species. *C17orf99* homologs were restricted to placental mammals (eutheria), marsupials and monotremes. (d)RT-PCR confirmation of *C17orf99* expression in human fetal liver, bone marrow, thymus (T) and lung (L), representative of three independent experiments (e) Western blot of supernatant from HEK 293 cells that were transfected with either pTT5V5H8-*IL40* or empty vector. Supernatants were collected on day 1 and 3, purified using a GE HisTrap purification column. Western blot was performed using an anti-His antibody followed by an anti-rabbit HRP secondary antibody.

#	Tissue Type	Mean Expression	#	Tissue Type	Mean Expression
1	Fetal Liver	609	11	Vena cava	25
2	Bone Marrow	156	12	Small intestine (duodenum)	24
3	Activated B cells	52	13	Resting T cells	24
4	Resting B cells	32	14	Oral mucosa	23
5	Tonsil	28	15	Small intestine (jejunum)	23
6	Thymus	28	16	Colon	23
7	Activated Monocytes	28	17	Nipple	23
8	Endometrium	26	18	Fallopian tube	23
9	Lymph node	26	19	Resting Monocytes	22
10	Pancreas	25	20	Placenta	21

Table 2.1. Top 20 sites of *IL-40* (*C17orf99*) expression from BIGE database. For a complete listing of tissues included in this database see [1, 2]

Next, we compared human and mouse expression. First, we confirmed our BIGE database output by performing qPCR on a panel of commercially available RNA from both human and mouse. In both species, IL-40 is specifically expressed by hematopoetic tissues: fetal liver and bone marrow (Figure 2.4a and 2.4b). Second, we analyzed the protein products and found that at the amino acid level the protein exhibits 72% homology. Furthermore, its expression is homeostatic, seen at steady state levels in hematopoetic tissues and upon induction by activated



c

```
Human  23   EEITPVVSIAYKVLEVFPKGRWVLITCCAPQPPPPITYSLCGTKNIKVAKKVVKTHEPAS  82
            EE T  ++IAYKVLEV+P+ R VLITC AP+   PITYSL  ++ I VAKKVV    PAS
Mouse  19   EEQTEGITIAYKVLEVYPQSRRVLITCDAPEASQPITYSLLASRGILVAKKVVHDSVPAS  78
Human  83   FNLNVTLKSSPDLLTYFCWASSTSGAHVDSARLQMHWELWSKPVSELRANFTLQDRGAGP  142
            FN+N+T+KSSPDLLTY C A+S SG +  S+RLQM+ ELW+KPVS+L+A+F L+   +GP
Mouse  79   FNINITIKSSPDLLTYSCQATSNSGTYGPSSRLQMYQELWAKPVSQLQADFVLRHGDSGP  138
Human  143  RVEMICQASSGSPPITNSLIGKDGQVHLQQRPCHRQPANFSFLPSQTSDWFWCQAANNAN  202
             VE+ C ASSGSPPIT  L+G  G+V  QQRP H +PANFS   SQT+ WF C+A N+
Mouse  139  TVELSCLASSGSPPITYRLVGNGGRVLAQQRPLHGKPANFSLPLSQTTGWFQCEAENDVG  198
Human  203  VQHSALTVVP  212
            V  SA   +P
Mouse  199  VDSSARIPLP  208
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Figure 2.4. IL-40 expression in mouse and humans. (a) qPCR analysis of human samples from commercially obtained RNA's. (b) qPCR analysis of mice samples from commercially obtained RNA's. (c) BLASTp sequence alignment of human and mouse IL-40. There is 72% conservation at the amino acid level.

IL-40 is produced by activated B cells

Cytokines exert their effects throughout every functional branch of the immune system. For example, IL-7 participates in B cell development within the fetal liver and later on B cell differentiation within the germinal center reactions [14, 15]. Additionally, in humans it is essential to for the development of T cells [16]. Due to the expression pattern of IL-40, I hypothesized that like IL-7, IL-40 would also have various effects upon the immune system from B cell ontogeny to B cell proliferation/homeostasis.

With this in mind we then proceeded to confirm the BIGE data for B cells. We obtained human immune cell populations purified from peripheral blood mononuclear cells (PBMCs) under resting or activated conditions. These cells were activated by the addition of anti-CD40 antibody (Ab) plus IL-4 for 24 hours, which resulted in a significant up-regulation ($p<0.0005$) of *IL-40* transcription (Figure 2.5a). Next, B cells from Wild Type (WT) C57BL/6 mice were activated with CD40 ligand (CD40L) and IL-4 and *Il-40* expression was measured by qRT-PCR, this too resulted in the up-regulation of *Il-40* ($p <0.00016$) (Figure 2.5b). Taken together, these data indicate that IL-40 is a gene expressed upon activation by both human and mouse B cells.

The recent identification of effector B cell subtypes capable of producing either Th1 or Th2 cytokines prompted an important question regarding the nature of IL-40, namely: is IL-40 a type 1 or type 2 cytokine? To address this important aspect of IL-40 biology, I activated whole splenocytes from WT mice under various conditions. Activation with anti-CD40 plus IL-4 and/or IL-13, both type 2 cytokines, lead to significant induction of IL-40 production, compared to that seen by activation with anti-CD40 alone. This induction was ablated by the addition IFN-γ, a type 1 cytokine. Interestingly, anti-CD40 plus TGF-β produced the highest levels of IL-40 production (Figure 2.5c). Taken together these data show that IL-40 production is induced in B

cells by CD40 engagement, is potentiated by Th2 cytokines or TGF-β, and is inhibited by IFN-γ. This suggests that IL-40 represents a novel B cell-derived cytokine that is produced upon activation and is potentiated by type 2 cytokines [17]

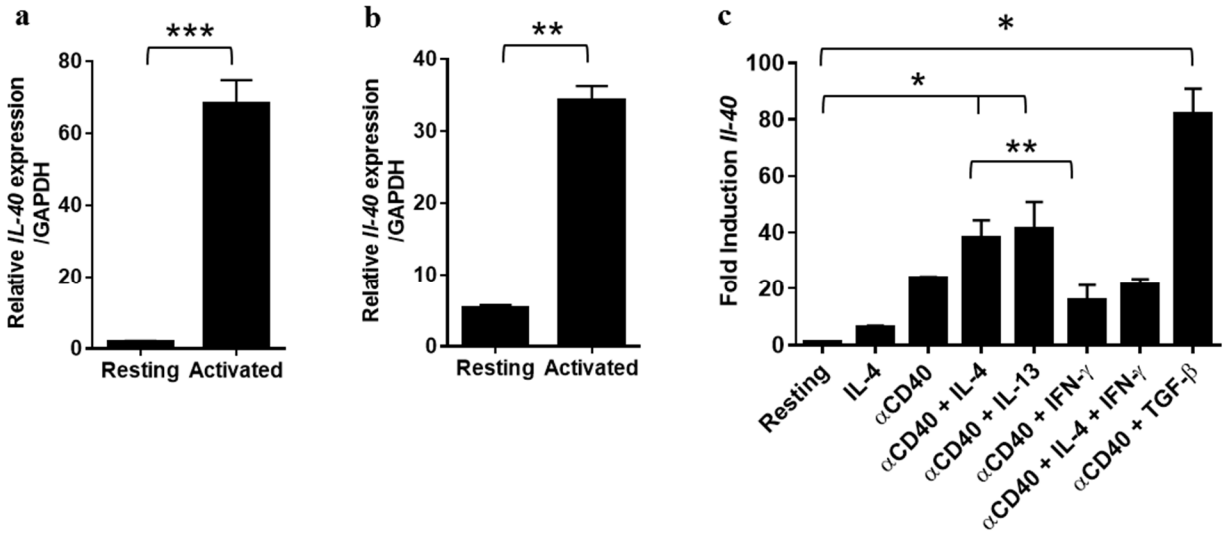

Figure 2.5. The *IL-40* gene is induced in activated B cells and its expression is potentiated by type 2 cytokines. (a) qRT-PCR analysis of *IL-40* expression in human resting and activated B cells. Cells were activated for 24h with anti-CD40 Ab + IL-4, representative of three independent experiments. (b) qRT-PCR analysis for *Il-40* expression from WT mouse spleen stimulated with CD40L+IL-4for 24h, n=3 mice per group, representative of three independent experiments.(c) qRT-PCR analysis of*Il-40*expression in mouse splenocytes stimulated with anti-CD40 Ab alone, or in combination with IL-4, IL-13, IFN-γ, or TGF-β, (all at 2ng/mL) for 3 days, n=3 mice per group, representative of three independent experiments.

Physiological Implications of IL-40 ablation *in vivo*

To properly characterize the functional role of IL-40 we obtained an *Il-40* deficient mouse (6030468B19Rik), where the IL-40 gene has been inactivated to produce an *Il-40*$^{-/-}$ mouse. This mouse was previously generated as part of a mouse knock-out library of secreted and transmembrane proteins [12]. The mice were generated either by homologous recombination or by gene trapping using preexisting ES clones. The *Il-40*$^{-/-}$ mouse was generated via homologous recombination (Figure 2.6). Upon receiving the *Il-40*$^{-/-}$ mouse, I ensured that the mouse had no defects in growth, determined by weight gain (Figure 2.7), or reproduction, as determined by breeding records.

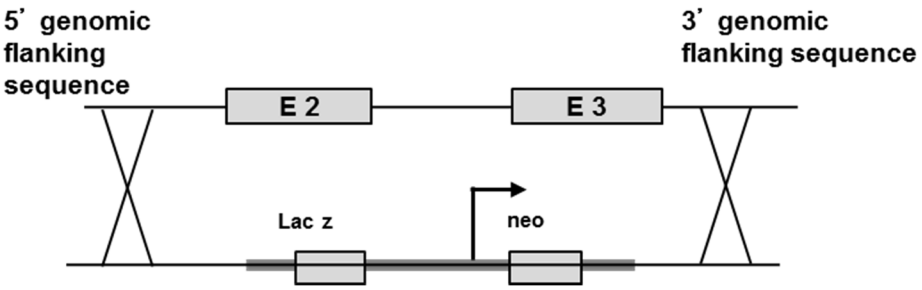

Figure 2.6. Confirmation of *Il-40* deletion. Target construct used to generate *Il-40*$^{-/-}$ mice.

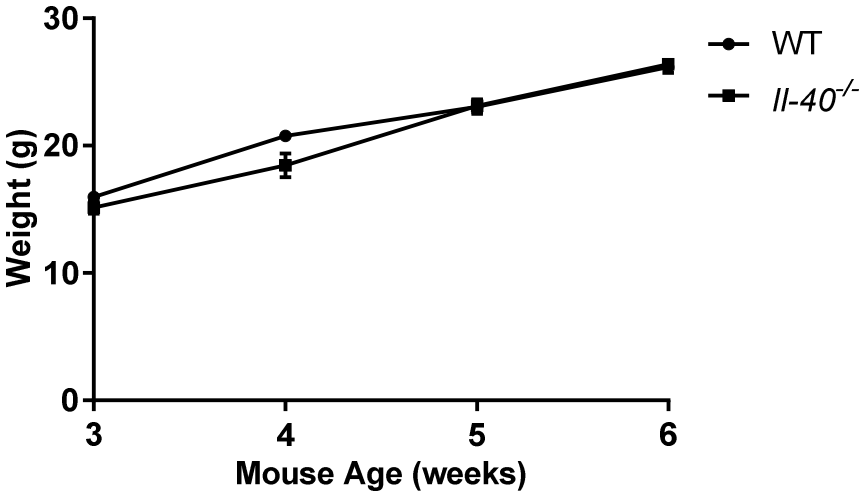

Figure 2.7. *Il-40*$^{-/-}$ mice grow normally as judged by weight. WT or *Il-40*$^{-/-}$ mice were weighed in grams from 3-6 weeks, n=5 mice per group.

IL-40 deficient mice (*Il-40*$^{-/-}$) mice exhibit an altered B cell phenotype

Since IL-40 is a homeostatic cytokine, I hypothesized that it may have other functions within the immune system. Hence, I sought to perform a comprehensive immunophenotypic analysis of various immune compartments including: thymus, bone marrow, and peritoneal exudate cells, but observed no significant differences in the immune cell populations present in these organs (Figures 2.8-2.10). The spleen, however, did exhibit differences between the WT and *Il-40*$^{-/-}$ mouse. Here, I observed an increase in B cells, compared to T cells (Figure 2.11)

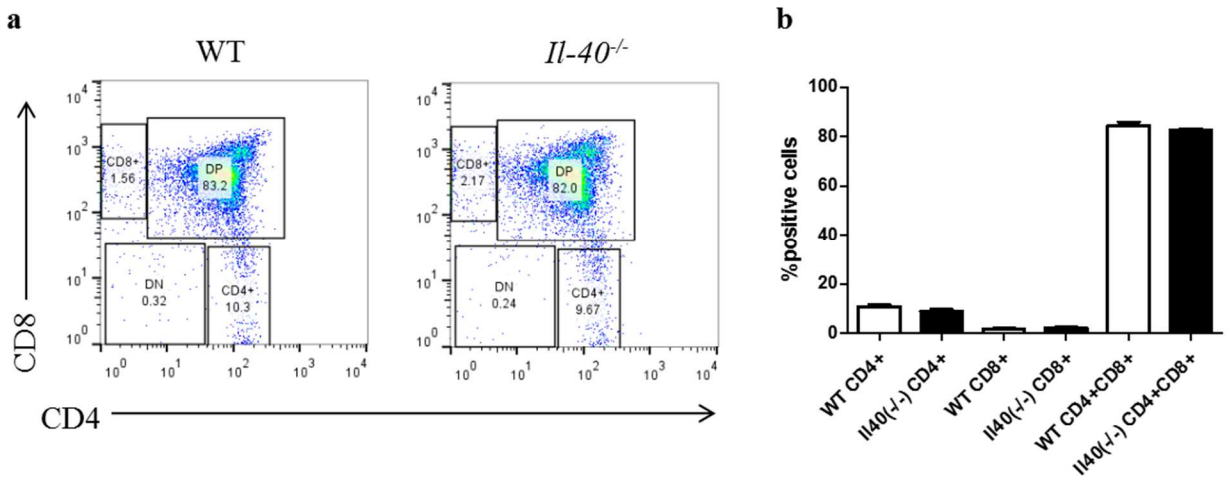

Figure 2.8. Thymus development is normal in the *Il-40*$^{-/-}$ mouse. (a). Thymocytes from WT and *Il-40*$^{-/-}$ mice were stained for CD8 and CD4 and analyzed by FACS. (b) Quantification of FACS data.

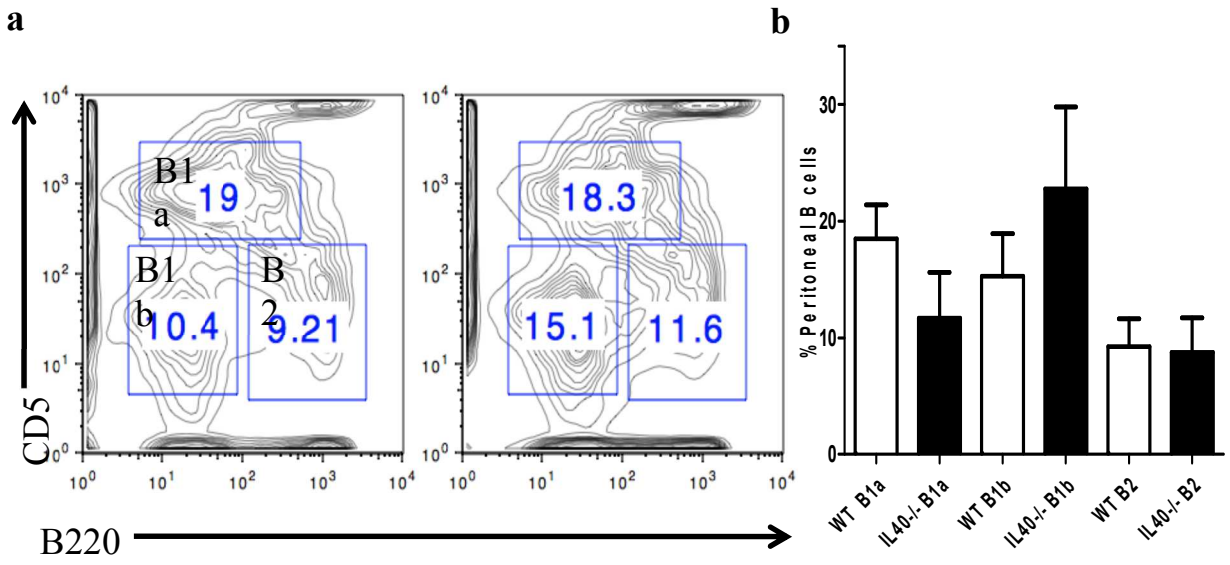

Figure 2.9. Peritoneal exudate cells (PEC) are normal in the *Il-40*$^{-/-}$ mouse. (a). PEC were isolated from WT and *Il-40*$^{-/-}$ mice were stained for CD5 and B220 and analyzed by FACS. (b) Quantification of FACS data.

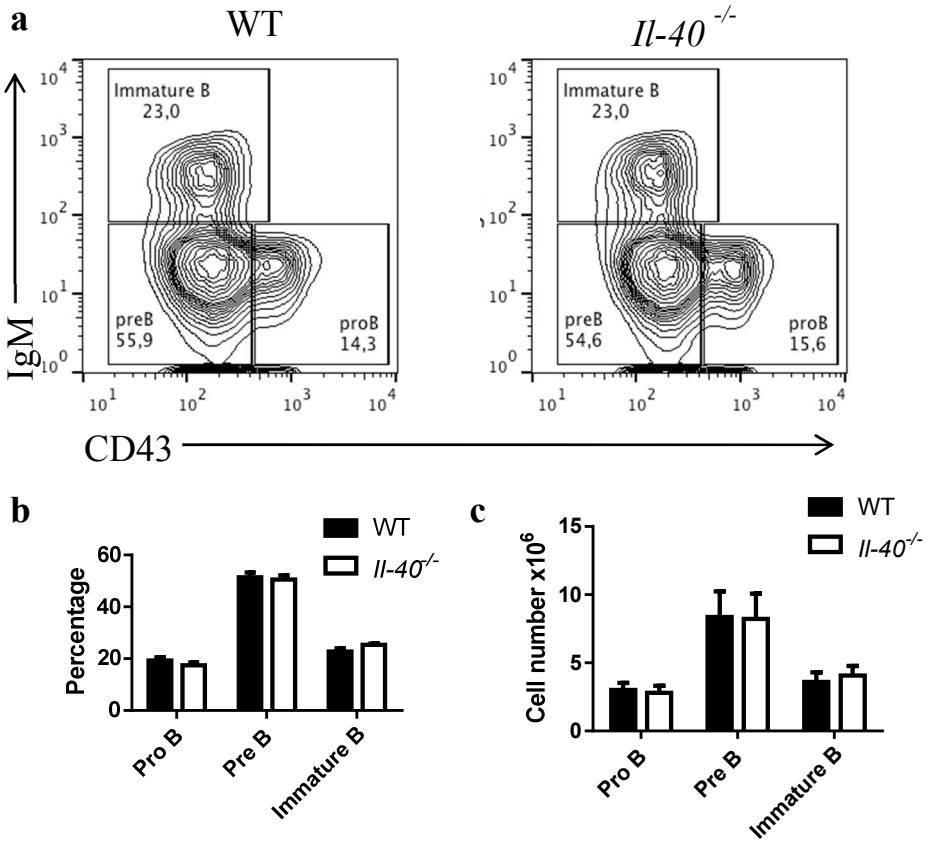

Figure 2.10. Bone Marrow B cell development is normal in the *Il-40*$^{-/-}$ mouse. (a). Bone marrow exudate cells from WT and *Il-40*$^{-/-}$ mice were stained for CD43 and IgM and analyzed by FACS. (b) Quantification of FACS data shown in percentage. (c) Quantification of data shown as absolute numbers.

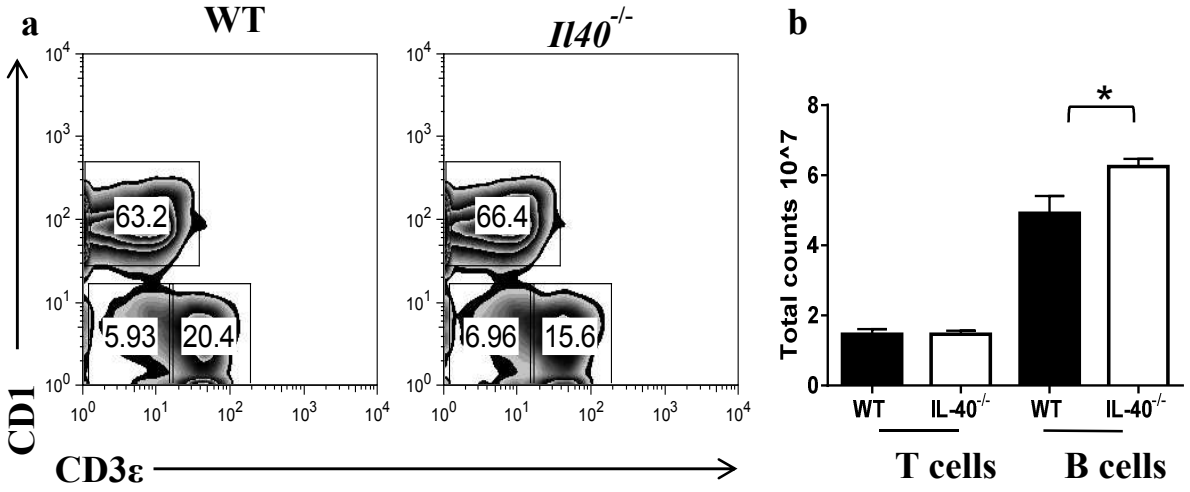

Figure 2.11. B cells are altered in the resting spleen of the *Il-40*$^{-/-}$ mouse. (a). Whole splenocytes were stained using CD19 and CD3ε and analyzed by FACS. (b) Ratios of B cells (CD19^{+}) and T cells (CD3ε^{+}) from FACS data.

The B cell defect appears to be restricted to B cells in the periphery. With this in mind, I began to think about B cell functions in the periphery, namely: antibody production. Hence, I performed various ELISA assays to quantify the production of serum immunoglobulins. The *Il-40*$^{-/-}$ mouse exhibits normal levels of serum IgM, total IgG, but serum IgA is reduced (Figure 2.12a-c). This phenotype is reminiscent of the *APRIL*$^{-/-}$ mouse, in which APRIL is not expressed, resulting in altered IgA production, while the other immunoglobulins remain unaffected. To further investigate the specific reduction of IgA in the serum we performed an *in vitro* CSR assay to elucidate two different questions. First, can the B cells from the *Il-40*$^{-/-}$ undergo class switching? Second, is this an intrinsic or extrinsic defect? Despite the significant reduction of IgA, B cells from the *Il-40*$^{-/-}$ mouse efficiently class switched to all of the immunoglobulins induced (Figure 2.13). These data suggests that the deficit seen in IgA production observed in the *Il-40*$^{-/-}$ mouse is extrinsic. Together, these data indicate abnormal B cell homeostasis in the *Il-40*$^{-/-}$ mice and strongly suggests that IL-40 plays an important role in humoral immunity.

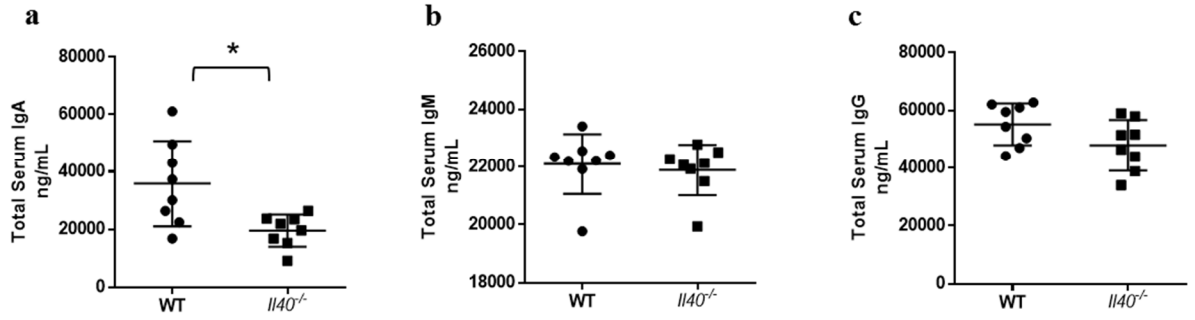

Figure 2.12. *Il-40*$^{-/-}$ mice have altered serum immunoglobulins. Levels of immunoglobulins in WT or *Il-40*$^{-/-}$ mice measured by sandwich ELISA (n=16) (a) IgA $p<0.0114$. (b) IgM not significant (n.s.). (c) Total IgG n.s.

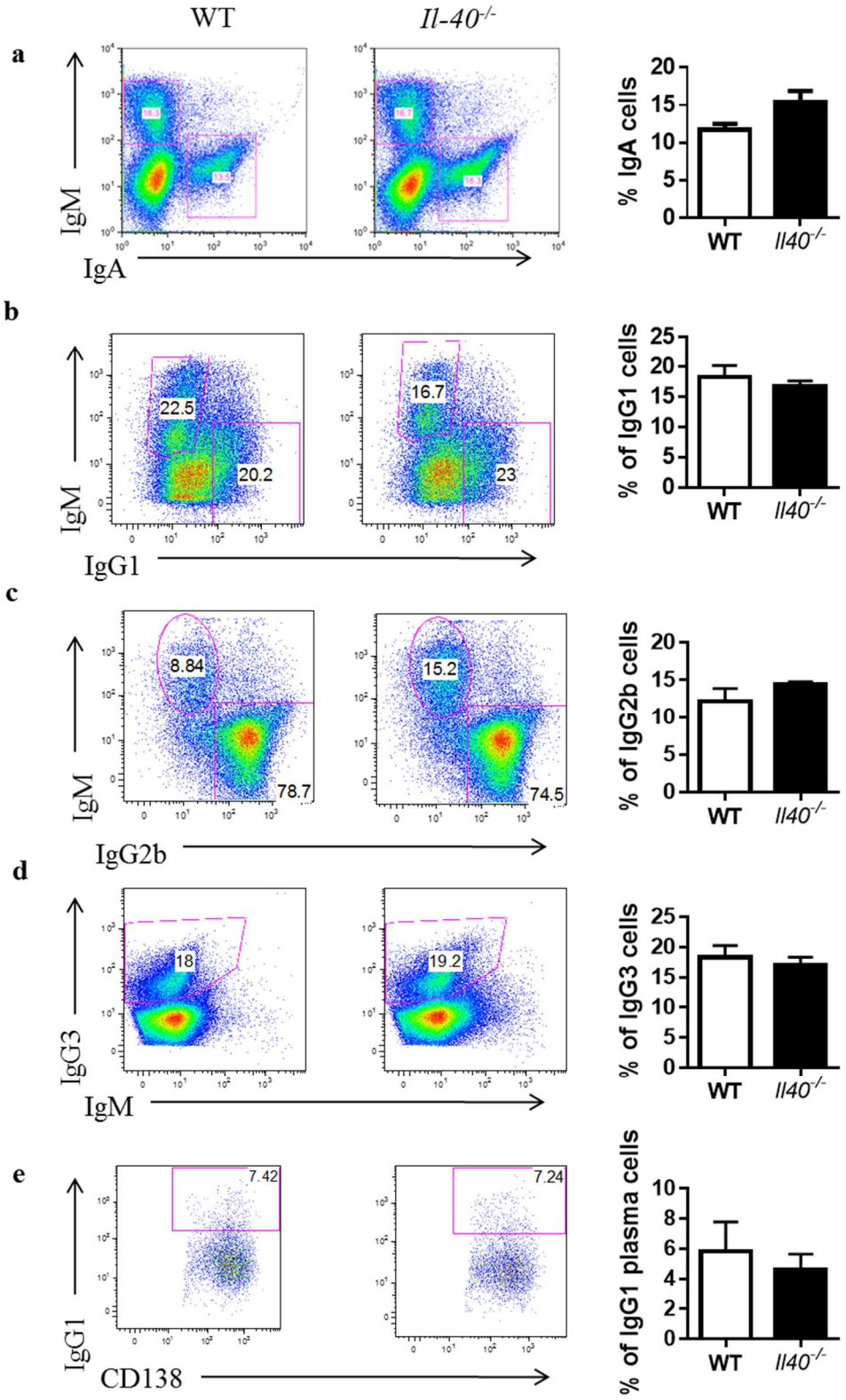
WT
Il-40-/-
a
IgM
IgA
% IgA cells
WT
Il40-/-
b
22.5
20.2
16.7
23
IgM
IgG1
% of IgG1 cells
c
8.84
78.7
15.2
74.5
IgM
IgG2b
% of IgG2b cells
d
18
19.2
IgG3
IgM
% of IgG3 cells
e
7.42
7.24
IgG1
CD138
% of IgG1 plasma cells

Figure 2.13. *Il-40*$^{-/-}$ mice do not have a defect in Class Switch Recombination (CSR). CSR induction of mouse splenocytes stimulated for 4 days, stimulated with A): LPS + anti-BCR + TGF-β (IgA switching), B): LPS + IL-4 (IgG1 switching), C): LPS + IFN-γ (IgG3 switching), measured by flow cytometry. D): Stimulation of IgG1 plasma cells with LPS + IL-4. E) Plasma cells (CD138^{+}) measured by flow cytometry. Representative data obtained from at least 3 independent experiments, with at least 3 groups. n=3 mice per group.

Il-40 Deficient Mice Exhibit a Decreased Antibody Response to Immunization with Heat Inactivated Vaccinia Virus (HI-VACV)

Given that the *Il40*$^{-/-}$ mouse exhibits altered levels of immunoglobulins in the serum, but can efficiently undergo *in vitro* class switching I sought to investigate whether the *Il-40*$^{-/-}$ mouse is able to mount an efficient humoral immune response. To this end, we immunized WT or *Il40*$^{-/-}$ mice with a heat-inactivated (HI) form of the mouse-adapted Vaccinia virus (VACV) WR strain.

The antibody response to immunization was monitored at different time points by testing serum samples using a VACV WR strain proteome microarray [18]. This allowed monitoring of isotype-specific immunoglobulin responses against viral antigens present in the VACV WR array. The average signal intensities representing VACV antigen specific IgG1 responses were plotted against days post immunization (Figure 2.14a and b). Both WT and *Il-40*$^{-/-}$ mice responded to the immunization, but the number of antigens to which the *Il40*$^{-/-}$ mice responded was significantly lower than in WT mice 28 days post vaccination (Figure 2.14 a and b).

The response to HI-VACV is skewed toward structural proteins, rather than proteins expressed in infected cells, which may be explained by the inability of the heat-killed virus to infect cells. WR187, a viral membrane glycoprotein, showed the highest reactivity in both WT and *Il-40*$^{-/-}$

mice (Figure 2.14a and b). Significantly, the number of antigens towards which the WT produced significant IgG1 responses was higher than in the *Il-40*$^{-/-}$ mouse (Figure 2.14c) indicating a deficient Ab response in the *Il-40*$^{-/-}$ mouse. Interestingly, IgG1 responses are driven by IL-4, a type 2 cytokine [19], which also up-regulates IL-40 production by activated B cells (Figure 2.5c). In contrast, there was no difference in the number of IgG2a antibodies produced against HI-VACV between the *Il-40*$^{-/-}$ or WT mice (Figure 2.15d). IgG2a responses are driven by IFN-γ, a type 1 cytokine that down-regulates IL-40 production (Figure 2.5c). These observations, along with the fact that different cytokines regulate the development of immune responses to different immunoglobulin isotypes, suggest that IL-40 may be involved in the development of humoral responses for certain immunoglobulin isotypes.

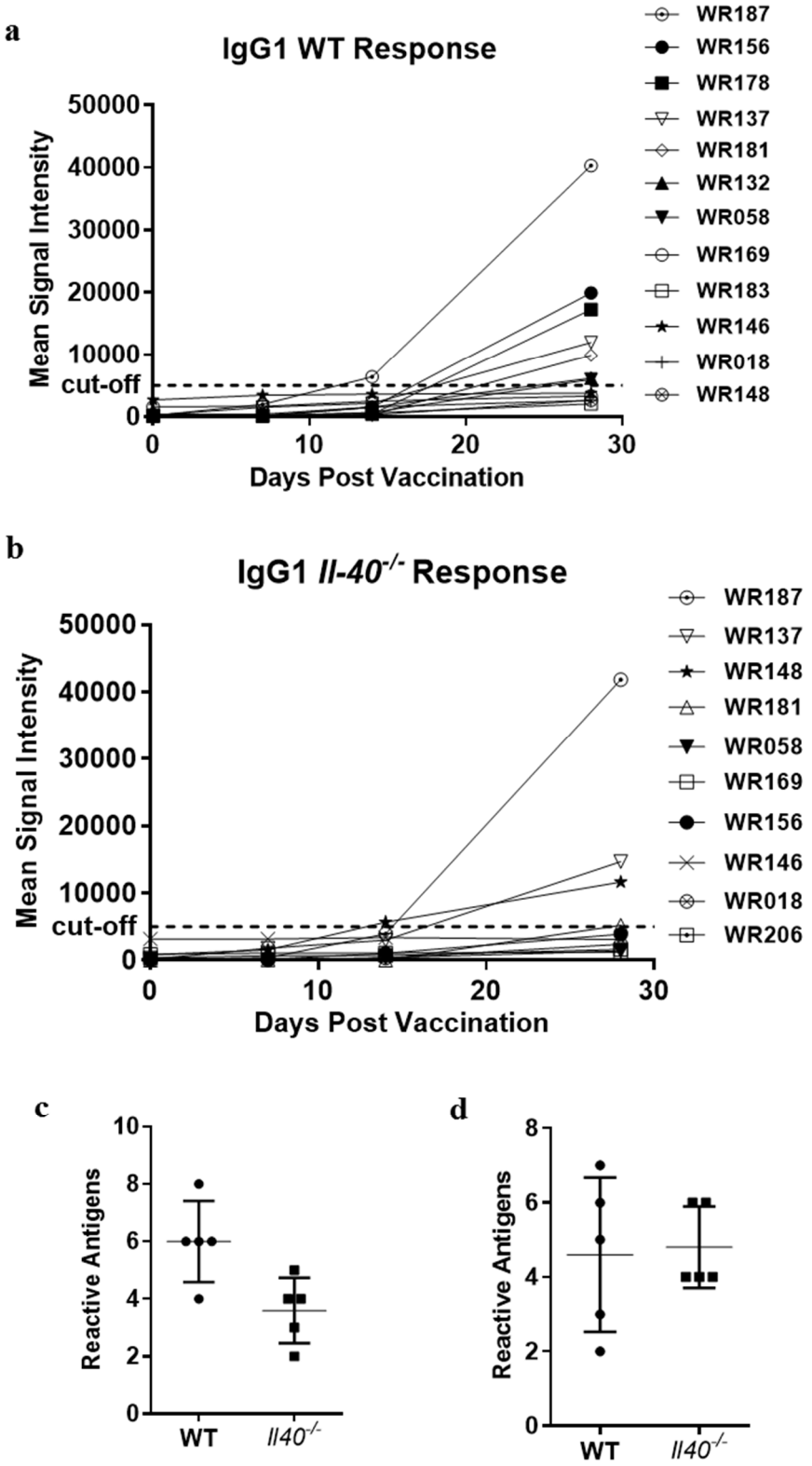
a
IgG1 WT Response
Mean Signal Intensity
50000
40000
30000
20000
10000
cut-off
0
0
10
20
30
Days Post Vaccination
WR187
WR156
WR178
WR137
WR181
WR132
WR058
WR169
WR183
WR146
WR018
WR148
b
IgG1 Il-40-/- Response
Mean Signal Intensity
50000
40000
30000
20000
10000
cut-off
0
0
10
20
30
Days Post Vaccination
WR187
WR137
WR148
WR181
WR058
WR169
WR156
WR146
WR018
WR206
c
Reactive Antigens
10
8
6
4
2
0
WT
Il40-/-
d
Reactive Antigens
8
6
4
2
0
WT
Il40-/-

Figure 2.14. *Il-40*$^{-/-}$ mice exhibit a blunted response to HI-VACV immunization. (a, b) Time-course of IgG1 response to immunization in WT and *Il-40*$^{-/-}$ mice (n = 5). Mice were immunized at day 0 and given a boost at day 14. Longitudinal sera samples were collected at days 0, 7, 14, and 28 and probed on a vaccinia proteome microarray. (c) Number of IgG1 vaccinia antigen-specific reactive antigens. (d) Number of Ig2a vaccinia antigen-specific reactive antigens, $p<0.05$. Reactive antigen spots were defined as antigen reactivity greater than a cut-off value of the mean of the IVTT control spots plus 3 standard deviations. Shown are representative experiments of 3 independent experiments.

Il-40$^{-/-}$ mice have an altered B cell phenotype in the GALT

To expand the results indicating that *Il-40*$^{-/-}$ mice exhibit altered immunoglobulin responses, I next explored whether IgA production is affected by the loss of IL-40. To this end, I analyzed components GALT in the *Il-40*$^{-/-}$ and WT mice. The GALT contains a large number of immune cells and is a preferred site of IgA responses [20]. PP represent gut-associated lymphoid nodules that constitute a major component of the GALT, participate in immune surveillance of the digestive tract, and contain both germinal center B cells and IgA secreting plasma cells [20].

The *Il-40*$^{-/-}$ mouse has fewer PP than WT mice ($p < 0.01$) (Figure 2.15a) and the PP present in the *Il-40*$^{-/-}$ mouse are smaller. This observation led to the quantification of the cells present in the PP itself. The PP is composed of various immune cell populations, but the most prevalent are germinal center B cells that ultimately differentiate into IgA secreting plasma cells. *Il-40*$^{-/-}$ mice exhibit a greater than 2-fold reduction in B220^{+}PNA^{+}, germinal center B cells (n=5, $p<0.0005$) (Figure 2.15b and c) [21], [22]. Since IgA is the primary immunoglobulin produced at this site, [21], [22] I measured the total IgA switched cells (B220^{lo-hi}PNA^{+}IgA^{+}) (Figure 2.16d) and found a 5-fold reduction in total IgA positive cells (n=5, $p<0.0001$) (Figure 2.15e). These data indicate that IL-40 participates in the development of IgA responses in the GALT, specifically in the PP.

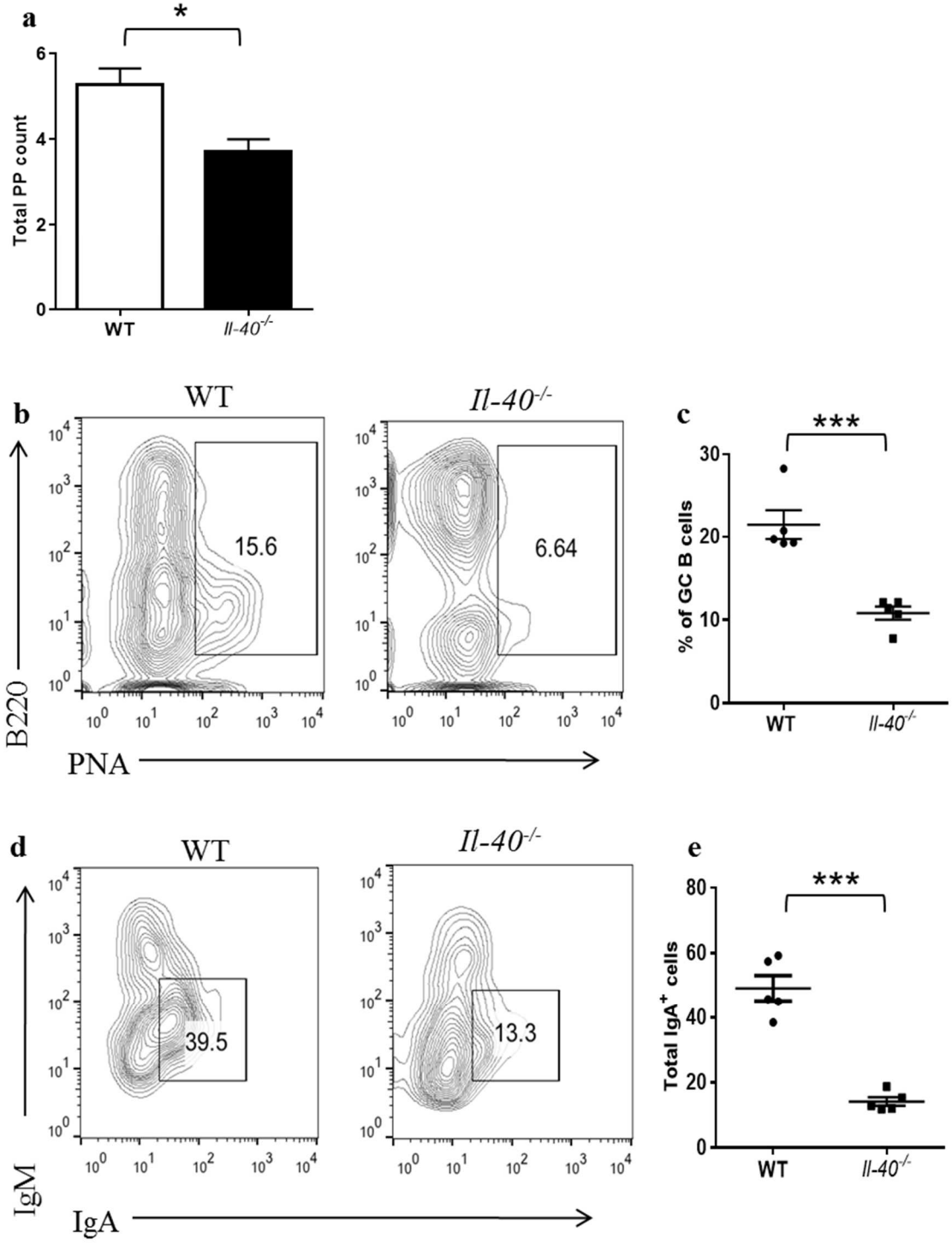
a
*
Total PP count
WT
Il-40-/-
b
WT
Il-40-/-
15.6
6.64
B220
PNA
c
***
% of GC B cells
WT
Il-40-/-
d
WT
Il-40-/-
39.5
13.3
IgM
IgA
e
***
Total IgA+ cells
WT
Il-40-/-

Figure 2.15. *Il-40*$^{-/-}$ mice exhibit a defect in GALT B cell populations. (a) Total counts of Peyer's patches from WT and Il-40$^{-/-}$ mice (n = 7) ($p<0.01$). (b) Measurement by flow cytometry of total germinal center B cells (B220^{+}PNA^{+}) in Peyer's Patches. (c) Percent quantification of total GC B cells (n=5) ($p<0.0005$). (d) Measurement by flow cytometry of total IgA switched cells, B220^{lo-hi}PNA^{+} lymphocytes. (e) Percent quantification of total IgA switched cells (n=5) ($p<0.0001$).Shown are representative experiments 3 independent experiments.

Interestingly, the most common human immunodeficiency is characterized by the absence of IgA in the serum and other secretions, with normal levels of other immunoglobulins isotypes such as IgG1 and IgM [23]; a phenotype similar to *Il-40*$^{-/-}$ mice. Although the human *C17orf99* gene has not been associated with any human disease to date, it should be interesting to study the status of this gene in IgA deficient individuals to establish a potential link between IL-40 and IgA immunodeficiency in humans. I predict that at least some human IgA immunodeficiencies may be linked to IL-40.

Il-40$^{-/-}$ mice have reduced levels of IgA in mucosal secretions.

Since the *Il-40*$^{-/-}$ mouse exhibits decreased levels of serum IgA immunoglobulin while maintaining normal levels of total IgG and IgM (Figure 2.12a-c), I sought to investigate whether the levels of secretory IgA are reduced in the *Il-40*$^{-/-}$ mice. The IL-40 sequence is conserved among mammalian genomes, see figure 2.3c, and missing in chicken and zebrafish. This observation led me to hypothesize that IL-40 may have a role specific to mammalian immune functions. A distinct hallmark of all mammals is that they feed their young milk secreted from the mammary gland. The mammary gland is an exocrine gland that is part of the MALT, mucosal associated lymphoid tissue. It is a very unique immune organ because it develops in

stages and is relatively quiescent from an immunological perspective, until the onset of lactation. The final differentiated stage of the mammary gland is achieved upon lactation. The morphological changes induced at the onset of lactation are accompanied by a complete redefinition of the immune microenvironment of the mammary gland. For example, CCL28, a chemotactic cytokine is induced at the onset of lactation (Figure 2.16a). This results in the recruitment of IgA producing plasma cells to the mammary gland, as seen by an increase in IgA (Figure 2.16b). Importantly, IL-40 is also induced upon the onset of lactation (Figure 2.16c), it is highest 1 week after parturition and seems to level off at 3 weeks post-partum. This suggests that it participates in the early stages of lactation.

However, as shown in Figure 2.16d, *Il-40*$^{-/-}$ mice exhibit a profound decrease in milk IgA levels compared to WT (WT n = 5 IL-40$^{-/-}$ n = 6, $p < 0.0001$). Additionally, IgA levels in fecal pellets of *Il-40*$^{-/-}$mice (Figure 2.16e) were reduced 2-fold compared to WT mice (n=16, $p<0.002$). Taken together, these results indicate that *C17orf99* encodes a novel B cell cytokine (interleukin-40) that is involved in secretory immune responses.

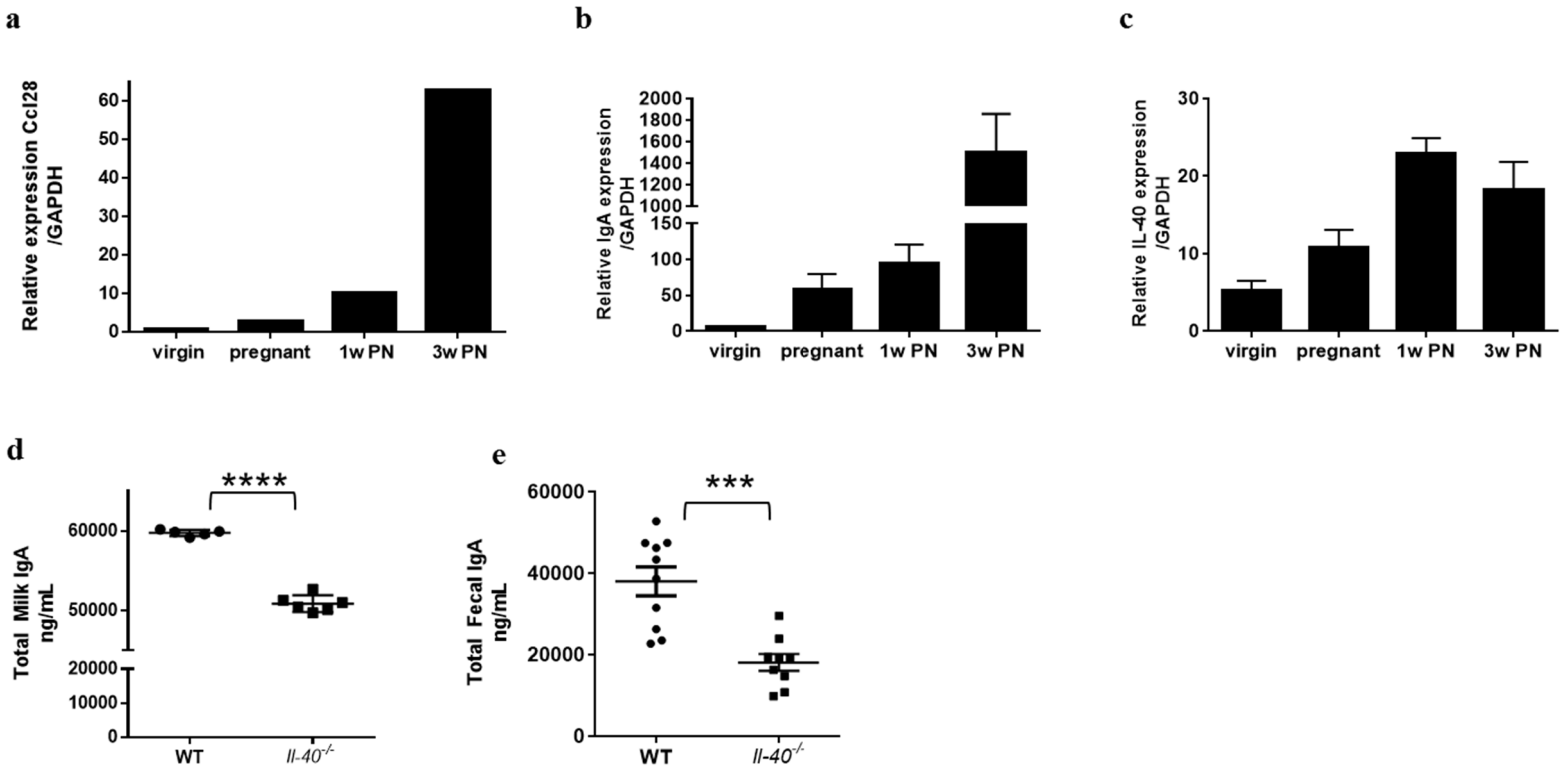

Figure 2.16. *Il-40*$^{-/-}$ mouse exhibits markedly decreased IgA levels in mucosal secretions. (a) qPCR analysis of *Ccl28* in virgin, pregnant, 1 week post-natal, PN, lactating, and 3 week PN lactating of WT mouse mammary glands. (b) qPCR analysis of IgA expression in WT virgin, pregnant, 1 week PN lactating, and 3 week PN lactating. (c) qPCR analysis of *Il-40* expression in WT virgin, pregnant, 1 week PN lactating, and 3 week PN lactating mouse mammary glands. (d) Measurement of total IgA in breast milk by sandwich ELISA of WT vs *Il-40*$^{-/-}$mice, (n= 10) ($p<0.0001$). (e) Measurements of total IgA in fecal pellets by sandwich ELISA of WT vs *Il-40*$^{-/-}$mice (n=10) ($p<0.006$) Experiments are representative of 3 independent experiments, each performed with at least 5 mice per group.

IL-40 in human disease

IL-40 is produced by human B cell lymphomas

Cytokines are responsible for generating a specific immune microenvironment that will either augment or inhibit certain immune responses. Similarly, the pathogenesis of various lymphomas and leukemia's depends on the immune microenvironment shaped by cytokine secretion. B cell lymphomas are often the result of deregulated B cell activation/proliferation. Since IL-40 is secreted upon activation, I hypothesized that B cell lymphomas may produce IL-40.

To this end I acquired various human lymphoma cell lines, through a collaboration with the laboratory of Dr. David Fruman, and analyzed their expression of *IL-40* using qPCR. Of the 13 human lymphoma cell lines, 2 of these expressed *IL-40* during the resting state, HL-2 and VAL (Figure 2.17).

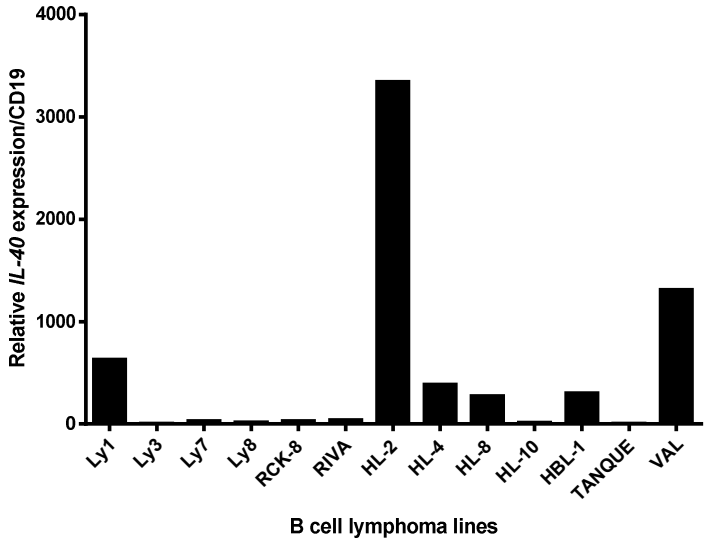

Figure 2.17. *IL-40* is expressed by human B cell lymphoma cell lines. RNA was extracted from resting B cell lymphomas, cDNA was synthesized and used for analysis of *IL-40* by qPCR. Shown are 13 B cell lymphomas, 2 of which produce *IL-40* under resting conditions.

The HL-2 lymphoma cell line is a Hodgkin Lymphoma characterized by the presence of Reed-Sternberg (RS) cells. Originally germinal center B cells, the RS cells have since lost their B cell phenotype as a consequence of gene reprogramming that accompanies cellular transformation. The VAL lymphoma line was derived from a bone marrow of a patient with B-acute lymphoblastic leukemia (B-ALL). This datum indicates that like other cytokines, IL-40 participates in various aspects of the immune system. Furthermore, it suggests that its expression is linked to the genesis of B cell lymphomas and that it may serve as a biomarker in the early detection of these pathologies.

Conclusions and Future Directions

Future Directions

The *Il-40*$^{-/-}$ mouse exhibits a profound defect in IgA both at the cellular and secretion level. I hypothesize that this may be a consequence of an *in vivo* block to IgA plasma cell differentiation or could possibly be a result of deregulated chemokine production that inhibits the migration of B cells or IgA plasma cells to given sites of IgA production. With this in mind, I have begun to study the chemokines responsible for B cell and plasma cell migration. I activated whole splenocytes from both *Il-40*$^{-/-}$ and WT controls for 24 hours and performed qPCR and checked a panel of various chemokines important to B cell and plasma cell migration. Interestingly, CXCR5 which binds to CXCR13 and plays a role in defining the structure of secondary lymphoid follicles, was not upregulated upon activation in the *Il-40*$^{-/-}$ mice compared to WT (Figure 2.18a).

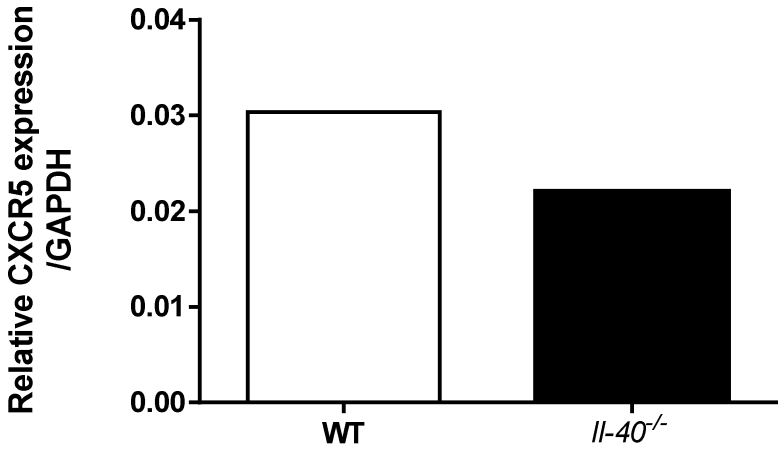

Figure 2.18. CXCR5 is not properly upregulated upon activation in the *Il-40*$^{-/-}$ mouse. (a) q-PCR analysis of activated whole splenocytes harvested from *Il-40*$^{-/-}$ and WT mice. Cells were activated for 24h with anti-CD40 Ab + IL-4.

The CXCR5/CXCR13 axis is responsible for properly positioning B cells in order to receive T cell help within the follicles of secondary lymphoid organs. This data suggests that the B cells in the *Il-40*$^{-/-}$ mice are not being recruited into the secondary lymphoid follicles hence will not enter a GC reaction that results in production of antibodies. This may partially explain the blunted response seen against HI-VACV. This data is preliminary and needs to be repeated with more mice and using more methods of activation, nevertheless it provides a possible route to elucidate the mechanism behind the observed humoral defects of the *Il-40*$^{-/-}$ mouse.

Next, I wanted to investigate whether plasma cell differentiation is intact. To this end, I performed a plasma cell induction using whole splenocytes that were enriched for B cells and activated in the presence of IL-4 and IL-5 for four days [24]. I checked a panel of genes associated with the plasma cell differentiation program including: Blimp-1, CD138, Pax5, XBP-1, J-chain, and CIITA. All of these genes are involved in the transition from B cell to plasma cell. For instance, Blimp-1 is the transcription factor associated with plasma cells, Pax5 is a B cell transcription factor that must be repressed in order for Blimp-1 to be transcribed. Other genes such as XBP-1 and CIITA are other transcription factors that are upstream of Blimp-1. The J-chain functions as control gene to ensure that plasma cells induced are capable of secreting antibodies. CD138 represents a plasma cell surface marker.

The *Il-40*$^{-/-}$ mouse exhibited no significant differences compared to the WT control in the expression of Pax5, Blimp-1, or XBP-1 (Figure 2.19a-c). However, CIITA, a gene upstream Blimp-1 was not up-regulated at the same level as it was in the WT ($p<0.0006$***) (Figure 2.19d). Interestingly, the expression of the J-chain was also reduced in the *Il-40*$^{-/-}$ mouse ($p<0.0011$**) (Figure 2.19e). Although this is a preliminary experiment, this datum suggests that IgA, which requires a J-chain, is specifically affected, but that the B cell of the *Il-40*$^{-/-}$ mouse can

differentiate into plasma cells. Moreover, the deregulation of CIITA, a gene located upstream of Blimp-1 and the master regulator in adaptive responses because of its role in the induction of MHC class II expression [25], in the *Il-40*$^{-/-}$ mouse further reinforces IL-40's role in the development of humoral immune responses.

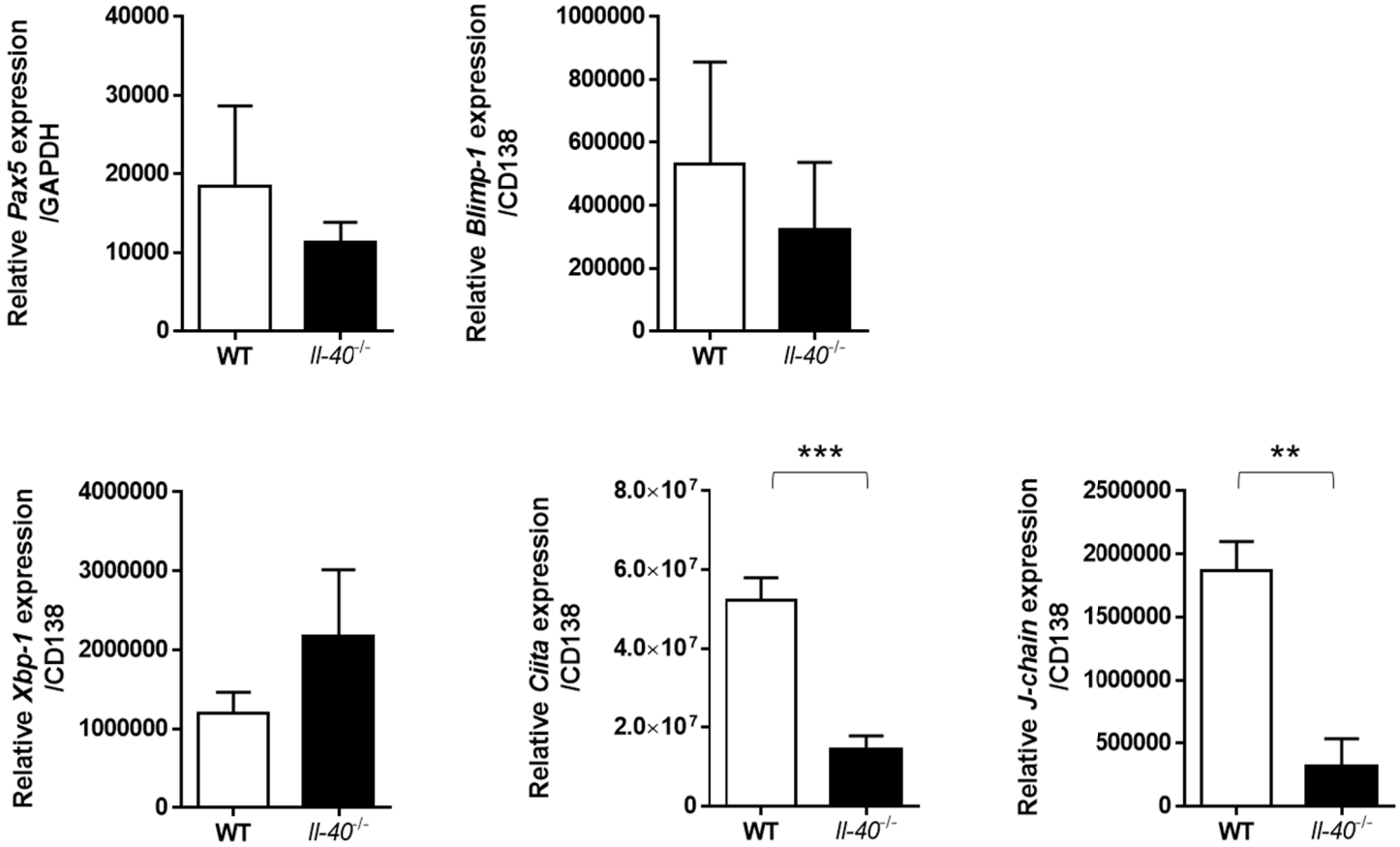

Figure 2.19. B cells in the *Il-40*$^{-/-}$ mouse can be induced to become plasma cells. (a) qPCR analysis of Pax5 in B cells induced into plasma cell differentiation. (b) Blimp-1 (c) XBP-1 (d) CIITA (e) J-chain. Whole splenocytes were activated with IL-4, IL-5, and anti-CD40 for 4 days.

Conclusions

Here I report a novel ISAG, *C17orf99*, which we have renamed Interleukin 40 (IL-40). This study began with the analysis of our database of human gene expression, BIGE. Initially our screen yielded most known immune system genes such as transcription factors, clusters of differentiation (CD's), along with known cytokines and chemokines. However, this screen also identified 35 other gene that encode either unknown or poorly characterized transmembrane or secreted proteins. We recently reported three of these: Isthmin 1 (ISM1) [3], TSPAN33 [4], and IL-39/Meteorin-like. These three genes had been reported, but their association with the immune system was unknown. In contrast, for *C17orf99* there is no prior information except for a report that identified it as a possible secreted protein [11] and another publication that generated a mouse knock-out library of secreted and transmembrane proteins, one of which was *C17orf99* [12].

C17orf99 has a unique expression profile (Figure 2.3a), with significant expression in the bone marrow and fetal liver. Its sequence predicts a small secreted protein with a 20 amino acid signal peptide (Figure 2.3e), which we confirmed by Western blot analysis of supernatants collected from transfected HEK 293 cells. Sequence analyses revealed that *C17orf99* does not belong to any currently known cytokine family. Interestingly, a phylogram of *C17orf99* indicates that it is only present in mammalian genomes, suggesting that it may participate in a mammalian specific immune function. *C17orf99* is strongly up-regulated in B cells upon activation (Figures 2.5 a and b), and its expression is induced by type 2 cytokines such as IL-4 and IL-13, inhibited by type 1 cytokines (IFN-γ), and potentiated by TGF-β (Figure 2C). Taken together, these data indicate that *C17orf99* encodes a novel B cell derived cytokine that we have called interleukin-40 (IL-40).

We obtained the *C17orf99*$^{-/-}$ mouse (*Il-40*$^{-/-}$ mouse) which facilitated the functional characterization of this novel cytokine. The *Il-40*$^{-/-}$ mouse has more splenic B cells (Figure 2.12), and altered levels of immunoglobulins, specifically a reduction in IgA. The GALT of the *Il-40*$^{-/-}$ mouse is also affected as exemplified by a reduction Peyer's Patches, both count and size (Figure 2.15). IgA responses seem to be especially affected as seen by a reduction in IgA secreting plasma cells and reduced levels of secretory IgA in mucosal secretions. Immunization with heat inactivated vaccinia virus revealed a blunted antibody response in the *Il-40*$^{-/-}$ mouse compared to the WT control. Taken together, these observations indicate that *C17orf99* encodes a novel B cell derived cytokine, which we have named interleukin-40 (IL-40), that is involved in humoral immunity, B cell homeostasis and proliferation, and may participate in the pathogenesis of certain lymphomas.

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CHAPTER 3

CCL28: A chemokine involved in the MALT of the immune system

Abstract

CCL28 is a chemokine expressed in the salivary and by epithelial cells in various mucosal tissues including the mammary glands, trachea, bronchi and colon. In the mammary gland, CCL28 expression is induced *de novo* upon the onset of lactation and plays a major role in IgA production through the recruitment of $CCR10^{+}$ plasma cells, which include IgA antibody secreting cells. Here, I show that CCL28 expression alters the immune system of the mammary gland. To properly elucidate the physiology of CCL28, we obtained a mouse deficient in Ccl28. The *Ccl28*$^{-/-}$ mouse exhibits deregulated levels of IgA in mucosal secretions. Infection of the *Ccl28*$^{-/-}$ mouse with *S. Typhimurium* showed that this mouse is extremely sensitive to infection with this pathogen. This result may be due to either the defective immune cell recruitment to the gut of the *Ccl28*$^{-/-}$ mouse or, alternatively, to the antimicrobial activity exhibited by CCL28. Future experiments will address this question.

Introduction

Chemokines are small secreted proteins that mediate cell migration to various sites throughout the organism. These chemotactic cytokines participate is a diverse array if physiological and pathological processes including embryogenesis, immune system development and homeostasis, inflammation, tumorigenesis and cancer metastasis [3-5]. In the immune system, chemokines and their receptors are a vital component of various immune associated functions as evidenced by their participation in nearly every facet of the immune response (Figure 3.1).

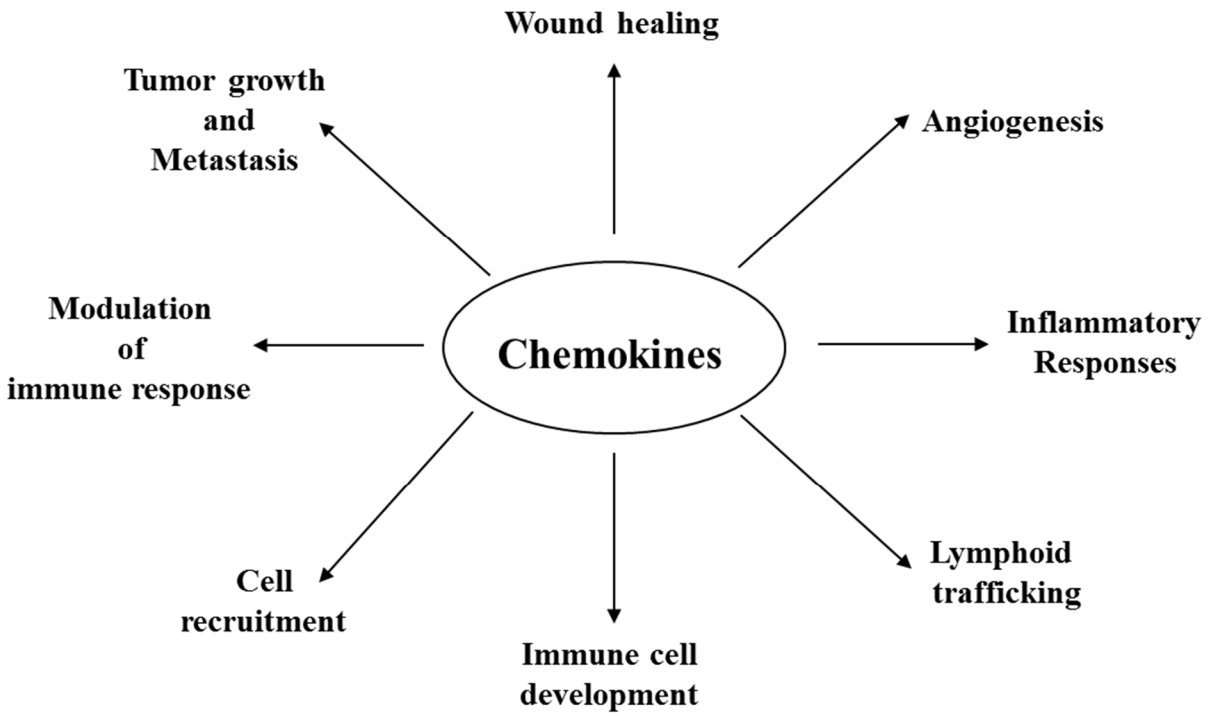

Figure 3.1. Biological functions of chemokines and chemokine receptors. Chemokines participate in various aspects of the immune system.

The chemokine superfamily is made up of 48 chemokine ligands and 21 chemokine receptors. Due to the large number of total chemokines and to facilitate their study this family has been subdivided based on the position of the cysteine residues in their N-terminus. There are currently four subfamilies: 1.) CXC chemokines, α-chemokines, have two cysteine residues separated by one amino acid, 2.) CC chemokines, β-chemokines, have two consecutive cysteine residues, 3.) C chemokines, γ-chemokines, have one cysteine residue, and 4.) CX_3C chemokines, δ-chemokines, have two cysteine residues separated by three amino acids [6]. This classification system also applies to chemokine receptors. Table 3.1 summarizes the chemokine subfamilies and their cognate chemokine receptors.

In addition to being subdivided according to cysteine residues present in the N-terminal region chemokines are also grouped based on their expression characteristics: homeostatic or inflammatory. Homeostatic chemokines are expressed in normal tissues in the absence of inflammatory stimuli, whereas inflammatory chemokines are expressed specifically under inflammatory conditions in a similar way as inflammatory cytokines (IL-1β, TNF-α, etc) or LPS stimulation [3]. A third category includes chemokines expressed under both homeostatic and inflammatory conditions. These chemokines are known as 'dual' chemokines.

Inflammatory chemokines are typically represented by many ligands that can bind a single receptor (ie they are 'promiscuous'), and often do not correspond well between species. These characteristics derive from their evolution, where a single ancestral chemokine gene duplicated that bound to a single receptor duplicated several times to give rise to various ligands, which then specialize to optimize the host's resistance to particular infectious agents. Examples of inflammatory chemokines include CCL2 and CCL5. Compared to inflammatory chemokines, homeostatic chemokines are much less promiscuous and exhibit restricted ligand/receptor

pairing. For example, CCR7 has two ligands: CCL19 and CCL21, but only CCL21 is expressed in high endothelial venules (HEV) and only CCL19 can desensitize and internalize the receptor [7, 8]. Hence, CCL21 is responsible for guiding the lymphocytes to secondary immune organs and CCL19 is responsible for desensitization in order to override CCL21 directed cell migration [9].

Subfamily	Receptor	Ligand
CXC	CXCR1	CXCL6, CXCL8
	CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8
	CXCR3A	CXCL9, CXCL10, CXCL11
	CXCR3B	CXCL9, CXCL10, CXCL11, CXCL14
	CXCR4	CXCL12
	CXCR5	CXCL13
	CXCR6	CXCL16
	CXCR7	CXCL11, CXCL12
	CXCR8	CXCL17
CC	CCR1	CCL3, CCL3L1, CCL5, CCL7
	CCR2A	CCL2, CCL7, CCL8, CCL13
	CCR2B	CCL2, CCL7, CCL8, CCL13
	CCR3	CCL3L1, CCL5,CCL7,CCL11,CCL13,CCL28
	CCR4	CCL17, CCL22
	CCR5	CCL3,CCL3L1, CCL4,CCL4L1, CCL5
	CCR6	CCL20
	CCR7	CCL19, CCL21
	CCR8	CCL1
	CCR9	CCL25
	CCR10	CCL27, CCL28
C	XCR1	XCL1, XCL2
CX_3CR1	CX_3CR1	CX_3CL1

Table 3.1. The Chemokine and chemokine receptor superfamily.

Chemokines exert their chemotactic abilities by signaling via a chemokine receptor. Chemokine receptors are GPCR's that upon ligation initiate signaling in the target cell via a Gαi subunit [6]. To be chemokine receptor, the protein must contain the following distinguishing characteristics of a GPCR: 7 transmembrane domains, a ligand binding domain in the N-terminus region, and a C-terminal region rich in serine/threonine residues [10]. Another structural motif of critical importance in signaling that is shared by chemokine receptors is a 'DRY box', which is required for G-protein coupling [11]. Figure 3.2 depicts the structures associated with a chemokine receptor.

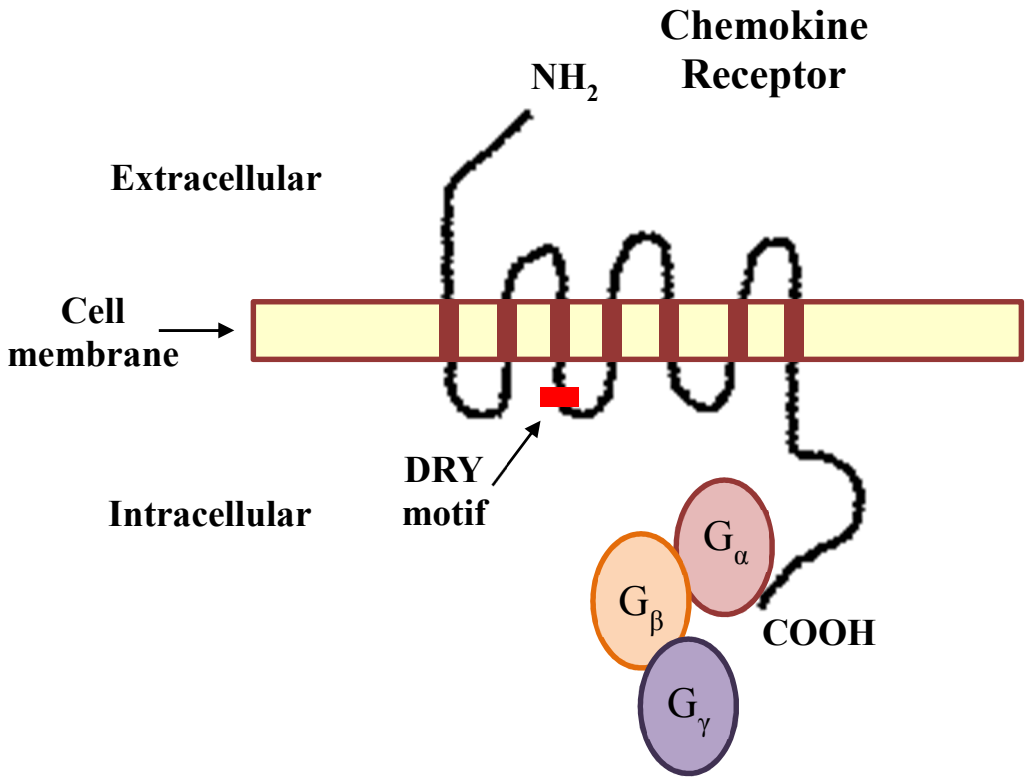

Figure 3.2. Common features of a chemokine receptor. Depiction of a chemokine receptor including 7 transmembrane domains – brown bars, DRY motif – red box, and signaling component Gα – pink oval.

The development of EST (expressed sequence tags) databases and the expanding field of bioinformatics have facilitated the identification of chemokines and their receptors [3]. The last two decades have witnessed the characterization of several of these ligand-receptor pairs. Our laboratory has been an active participant in the field of chemokine ligand-receptor discovery as well. We recently reported CXCL17 a novel chemokine expressed in the mucosa [12, 13]. Shortly thereafter we reported its receptor, CXCR8, previously known as orphan G-coupled receptor, GPR35 [11]. The identification of a ligand-receptor pair represents a significant contribution to the chemokine field, especially considering that the last receptor to be characterized was identified over a decade ago.

My studies on the immune system have strongly biased my interests to a specific component: the MALT. I have become very interested in the mucosa and cytokines that participate in its regulation, likely a consequence of IL-40's strong correlation with the mucosa as exhibited by reduced levels of IgA in mucosal secretions, PP count, and IgA secreting cells. Here, I will focus on CCL28, a mucosa associated chemokine that is expressed by epithelial cells that line mucosal surfaces and recruits IgA secreting plasma cells to mucosal sites. Although CCL28 has been part of the chemokine field for over a decade, there are no reports that characterized a mouse deficient in CCL28, (*Ccl28*$^{-/-}$ mouse).

CCL28: the discovery process that ultimately led to my project

My interest in the identification of novel ISAGs was instilled in me by my mentor, Albert Zlotnik. As a new graduate student he once told me, "Look at what everyone has looked at before and see what no one before has seen." This quote inspired me and I adopted this mentality in my approach to the study of CCL28. Although at the time I started this project CCL28 was not a new chemokine, it was originally discovered in the lab of Albert Zlotnik (then at DNAX research institute); his publication record provides evidence that his innovative nature and pioneering spirit have paved the field of cytokine and chemokine discovery.

The initial characterization of CCL28 was published 15 years ago. True to form, in this report the Zlotnik group took advantage of the recent avalanche of data available in various databases including HGS and Genbank™ databases. Using this bioinformatics approach they were able to identify three EST's which encoded a novel chemokine belonging to the β-subfamily, a CC chemokine. One of these identified EST's contained the entire open reading frame and revealed a novel CC chemokine of 127 amino acids with a predicted signal sequence of 22 amino acids at the N-terminus. Furthermore, the untranslated 3' region of the mRNA contains an instability sequence, a hallmark of cytokine mRNA's. The mouse homolog was also identified and cloned from a Rag-1$^{-/-}$ kidney cDNA library. Importantly, human and mouse CCL28 are highly conserved, 83% and 76% identity at the amino acid and nucleic acid levels respectively. This degree of shared identity indicates that CCL28 is a homologous protein in these species.

The initial studies of the biology of CCL28 began by identifying cells that respond to this chemotactic cytokine. Both CD4^{+} and CD8^{+} T cells migrated toward a CCL28 chemokine gradient under resting conditions, however this effect was abrogated upon activation utilizing anti-CD3. Next, they were interested in the identification of CCL28's receptor. CCL28 has a

high level of homology with a previously characterized chemokine, CCL27 (also discovered in the Zlotnik lab). This latter chemokine is responsible for the recruitment of T cells to cutaneous sites via its receptor GPR2. Because of the shared homology between the two chemokine ligand they hypothesized that these two chemokines may share a receptor, GPR2. Accordingly, they transfected Baf/3 cells, a mouse B cell line, with GPR2, and observed that CCL28 induced robust calcium signaling in GPR2 transfected Baf/3 cells. Moreover, the receptor was desensitized by CCL27, and vise-versa, indicating that CCL27 and CCL28 share a common receptor, GPR2. The shared receptor GPR2 was renamed CCR10 based on the nomenclature that had recently been implemented. Similar to CCL28, CCR10 is also highly conserved between human and mouse, 87% identity at the amino acid level, which indicates that it is a homologous protein in both species.

CCL28 is expressed in a variety of mucosal tissues under both homeostatic and inflammatory conditions. For example, they found that CCL28 is expressed in the colon under both normal and in a pathological conditions (such as ulcerative colitis). These data indirectly suggests that CCL28 is a dual chemokine, expressed under inflammatory and non-inflammatory conditions. Furthermore, CCL28 is up-regulated in IL-$10^{-/-}$ mice suggesting that it may participate in the mucosal immune system as a regulator of inflammation, perhaps in immune surveillance of the gut. Because CCL28 was expressed by various mucosal tissues, they performed immunohistochemistry on colon samples and found that epithelial cells are responsible for CCL28 production.

This initial characterization introduced a novel chemokine and its receptor into the field of chemokines. To discover both a novel chemokine ligand and its receptor concomitantly is a significant discovery that opened up the field of CCL28/CCR10 physiology. This

groundbreaking publication facilitated the study of this chemokine and its receptor and paved the way for the last 15 years of publications on the subject of CCL28 and CCR10.

CCL28 a mucosal chemokine that has a protective role in the MALT

My interest in CCL28 started when I saw the CCL28 expression profile from the BIGE database of human gene expression. These datum indicate that the main sites of CCL28 expression are the salivary and mammary gland. Immediately I began to consider the function of these two glands and the mucosal secretions each of these produce. The salivary gland produces saliva which coats the interior of the mouth and bathes the entire length of the digestive tract. It contains multiple enzymes that initiate the process of digestion, as well as many mucosal proteins that protect the delicate epithelial cells from coming into direct contact with potential pathogens [14]. The mammary gland is a rather inert immune gland until it reaches maturity, or lactation. However, upon the onset of lactation the mammary gland becomes responsible for the production of milk, which contains various enzymes, immune cells and immunoglobulins [15]. Both of these secretions (saliva and milk) ultimately have a protective role in the mucosa. The first, saliva, protects the upper-gastrointestinal tracts and the second, breast milk, contains immunoglobulins that provide passive immunity to neonates against respiratory and gut pathogens.

Physiology of CCL28

CCL28, a β-chemokine, is expressed by epithelial cells that line the mucosa, induces calcium mobilization in both $CD4^+$ and $CD8^+$ T cells, and has a high level of homology with a previously reported chemokine CCL27 [16]. This explains their shared a receptor, CCR10, and suggests that these two genes share a common ancestral gene and their existence is the result of a gene duplication event. Interestingly, CCL27 is mainly expressed in the skin and directs T cell homing

to cutaneous sites; therefore the gene duplication event led to selective chemokine differentiation and specialized tissue/cell type expression [17] Note that both mucosa and skin are barrier tissues, which suggests that specialization of function may explain their expression patterns.

The CCL28/CCR10 axis strongly correlates with CCL28 function, namely the recruitment of IgA^+ plasma cells to mucosal sites [18-20]. The mammary gland, the second highest site of expression for CCL28, is an exocrine gland that is part of the MALT. Unlike other immune organs it develops in a series of steps [21]. Upon reaching the final differentiated state, (after the onset of lactation) the ductal epithelia within the gland begin to express CCL28, thus CCL28 production in the mammary gland is a consequence of lactation and a sign that the gland has fully matured. In fact, the CCL28 promoter contains an estrogen-responsive motif [22]. Importantly, expression of CCL28 in the mammary gland parallels the accumulation of IgA plasma cells in the gland, a process that can be inhibited by anti-CCL28 antibodies [18]. Taken together, these results indicate that the CCL28/CCR10 axis regulates the recruitment of IgA secreting plasma cell to the mammary gland.

CCL28 has also been reported to bind CCR3 [17]. However, CCR3 is not the primary physiological receptor under healthy conditions [17, 23]. During pathological conditions, the ability of CCL28 may become relevant. For example, levels of CCL28 increase in patients with atopic asthma, this leads to the accumulation of IgE producing plasma cells, an effect likely due to the action of the CCL28/CCR3 axis [24].

As previously mentioned the highest site of human CCL28 expression is the salivary gland. Other mucosal sites include the small intestine and colon which can also express CCL28 [25]. Deregulated levels of CCL28 have been correlated to various pathologies including salivary gland tumors, Sjörgren's syndrome, and Hodgkin's disease (HD) [25-27]. Patients with salivary

gland tumors or Sjörgren's syndrome exhibit a reduction in CCL28 levels [25, 26]. Whereas HD patients show elevated levels of CCL28 which leads to accumulation of plasma cells and eosinophils in the cellular infiltrates observed [27].

Besides its chemotactic properties, CCL28 also exhibits broad spectrum antimicrobial activity against both gram positive/negative bacteria, and fungi [28]. The observed antimicrobial activity is a consequence of c-terminal region of CCL28, which has some homology with an antimicrobial peptide, Histadin5 [28]. CCL28 exerts its antimicrobial activity by the same mechanism described for antimicrobial peptides, the positively charged portion of the peptide is inserted into the negatively charged microbial wall which results in cell death. This mechanism is highly dependent on electrostatic interactions and is abolished in high salt concentrations [29]. Not surprisingly, CCL28 is present in secretions with low salt concentrations.

Materials and Methods

Body Index of Gene Expression [30] Database

The construction of the BIGE database has been previously described [1, 2]. Briefly, 105 different tissues and cells were collected from 8 healthy donors (4 male, 4 female) within 5 hours post-mortem. RNA was extracted from the tissues and cells and used to synthesize cDNA for hybridization to Affymetrix U133 2.0 gene arrays (Affymetrix, Santa Clara, CA). A probe set corresponding to *CCL28* was used to show the expression of this chemokine within the entire body panel of tissues and cells.

q-PCR

Quantitative real-time PCR (q-PCR) data was generated with a Roche Lightcycler 480 system using the Universal Probe Library (UPL) based system (Roche, Indianapolis IN). Total RNA was extracted from mouse tissues or cells using the Qiagen RNeasy® kit according to manufactures instructions (Qiagen, Valencia, CA). Equal concentrations of RNA were used for each sample in a reverse transcription reaction to synthesize cDNA. qPCR results were processed in Excel and analyzed using GraphPad Prism (http://www.graphpad.com).

Mice

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC), of the University of California, Irvine. C57BL/6J (stock number 000664) were obtained from the Jackson Laboratory (Bar Harbor, ME). *Ccl-28*$^{-/-}$ mice were obtained from Deltagen (Deltagen, San Mateo, CA). Germ free (GF) mice and specific pathogen free (SPF) mice were generous gifts from Mauro Teixeira (Universidade Federal de Minas Gerais, Brazil). Tissue samples from each mouse were stored in RNALater (Qiagen) prior to RNA extraction. The RNA extraction and q-PCR were performed as described above. Serum samples were

obtained through sub-mandibular cheek bleed as described [31]. Fecal samples were collected as described [32]. Breast milk samples were obtained by collecting the stomach contents of neonate mice. Briefly, stomach contents were centrifuged at max speed for 15 minutes and supernatants were collected.

ELISA

ELISA was performed according to manufacturer's protocol (Ebioscience, San Diego CA). Plates were read at 450nm. For fecal IgA ELISA plates (Sigma Aldrich, St Louis, MO) were coated with anti-IgA mAb (clone RMA-1) (Biolegend, San Diego CA) at 2ug/ml. 5% milk in PBS-T was used as blocking buffer. Plates were washed 3 times with PBS-T after each step. AP conjugated anti-mouse IgA (Southern Bio, Birmingham, AL) used as secondary antibodies for 1 hr at room temperature and washed 3 times with PBS-T. SureBlue substrate (KPLGaithersburg, MD) was added, reaction was stopped with 2N H_2SO_4. Plates were read at 630nm.

Salmonella Infection

Mice, WT and *Ccl28*$^{-/-}$, were pretreated with streptomycin (0.1 ml of a 200 mg/ml solution in sterile water) intragastrically prior to mock-infection or inoculation with a mixture of *S. Typhimurium* strain ($5x10^8$ cfu/animal) cfu (colony forming units). At 96 hr after infection, mice were euthanized and spleen, mesenteric lymph nodes, bone marrow, Peyer patches, terminal ileum and cecum was collected for analysis. Tissues were collected, and serial 10-fold dilutions were plated for enumerating bacterial cfu on agar plates containing the appropriate antibiotics.

Results

CCL28 is involved in shaping the immune microenvironment of the Mammary gland.

An extensive literature review confirmed CCL28's role in shaping the mucosal immune system. Its expression profile in our BIGE database also supports a mucosal role for CCL28 (Figure 3.3 and Table 3.2). My interest in CCL28 was its regulation in the mammary gland. Unlike its other sites of expression, it is specifically induced at the onset of lactation. I confirmed this using WT female mice at different stages of mammary gland development (Figure 3.4a). The observed up-regulation of CCL28, is also accompanied by an influx of $CCR10^{+}$ cells (Figure 3.4b). I confirmed that these newly arrived cells were IgA secreting plasma cells (Figure 3.4c)

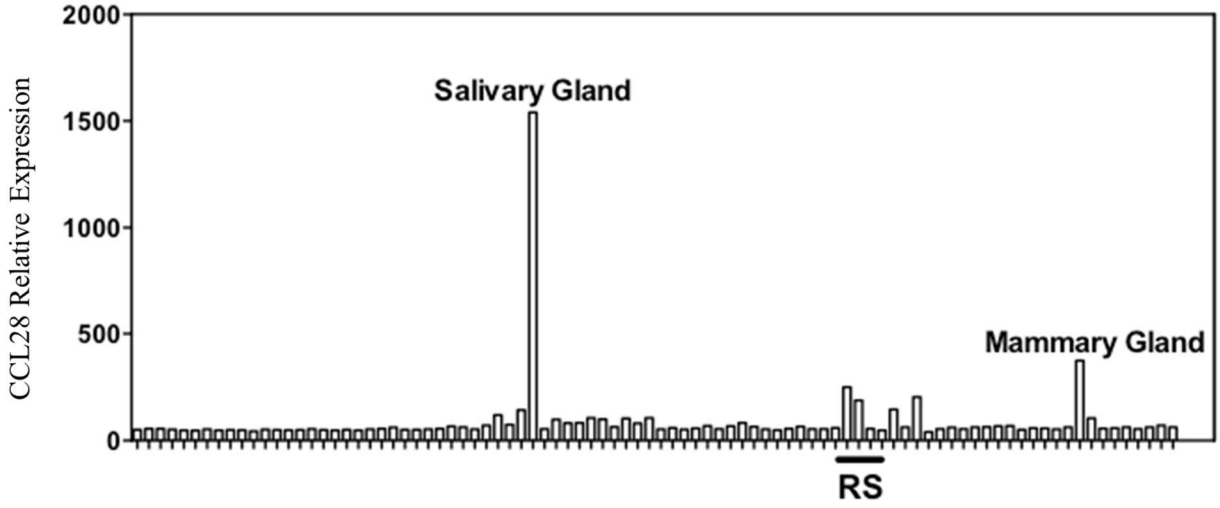

Figure 3.3. CCL28 is highly expressed by salivary and mammary glands. *CCL28* expression in normal human tissues and immune cells. Data from the BIGE showing the expression profile of *CCL28* mRNA. X-axis is organized by organ systems: CNS (central nervous system), Gut (gastrointestinal), Struct (structural), Vasc (vasculature), Resp (respiratory), Endo (endocrine), Ur (urinary), Rep (reproductive), Imm_T (immune tissue), Imm_C (immune cells), and Dev (developmental).

#	Tissue Type	Mean Expression	#	Tissue Type	Mean Expression
1	Salivary Gland	1540	11	Nipple (cross-section)	105
2	Mammary Gland	373	12	Small intestine	103
3	Trachea	249	13	Duodenum	99
4	Thyroid Gland	203	14	Stomach	99
5	Bronchus	188	15	Stomach fundus	83
6	Pancreas	145	16	$CD4^+$ T cells	83
7	Tongue Superior	143	17	Skin	82
8	Tongue	119	18	Placenta	82
9	Stomach Pyloric	106	19	Stomach cardiac	82
10	Colon	105	20	Colon caecum	80

Table 3.2. Top 20 of CCL28 expression from BIGE database. For a complete listing of tissues included in this database see [1, 2]

These data suggests that lactation promotes a change in the mammary gland cytokine milieu. This drastic metamorphosis induces CCL28 expression which results in a redefined microimmune environment of the mammary gland. Interestingly, the cells that express CCL28 at the onset of lactation, ductal epithelial cells, are the same cells that transform during the pathogenesis of breast cancer [33]. This suggests that the changes induced in the mammary gland at the onset of lactation promote protection against breast cancer and that the protection is occurs via a yet to be defined CCL28-mediated pathway.

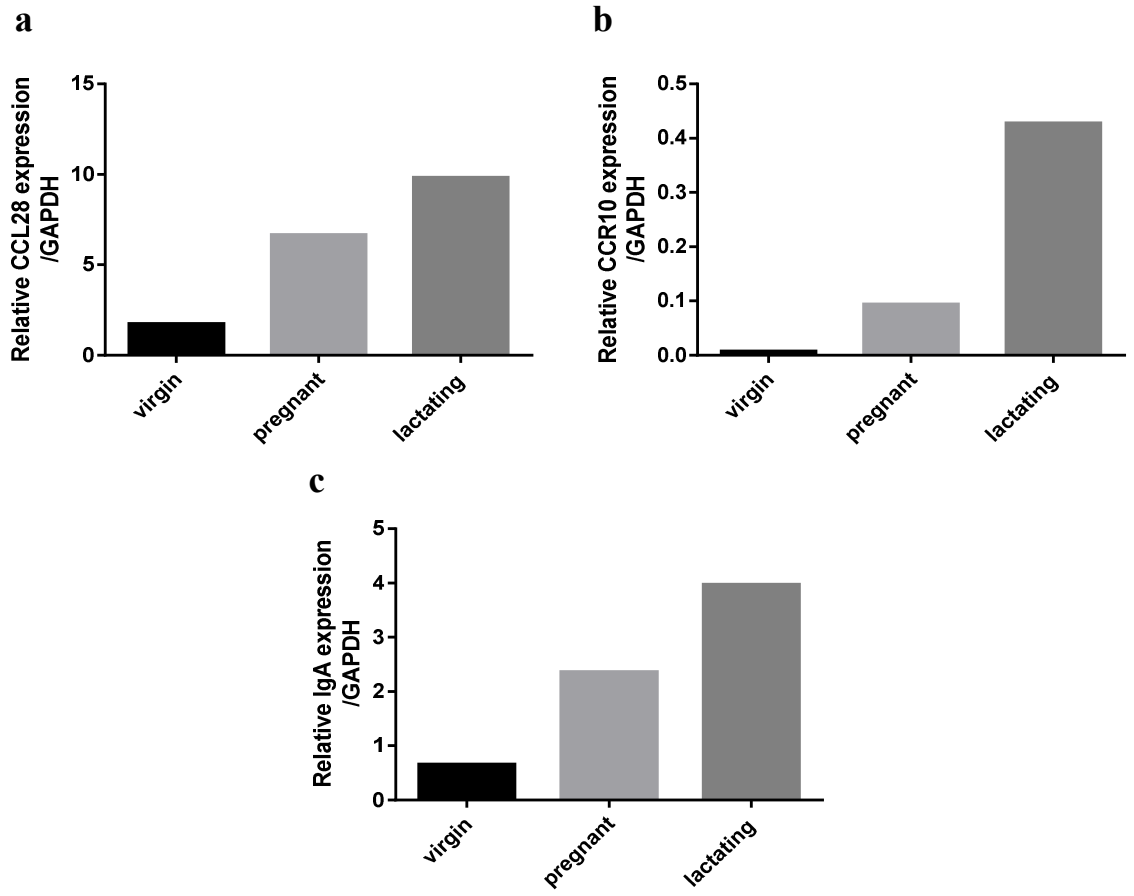

Figure 3.4. CCL28 is induced at the onset of lactation. (a) q-PCR WT female mammary gland tissue analyzing *Ccl28* expression (b) *Ccr10* expression (c) IgA

CCL28 is a dual chemokine expressed under homeostatic and inflammatory conditions.

Given the remarkable transformation that occurs in the mammary gland at the onset of lactation, it is not surprising to see signs of inflammation including swelling, redness, and warmth. Since CCL28 is the chemokine responsible for initiating these changes I wanted to investigate whether it is a homeostatic or inflammatory chemokine. To this end I analyzed its expression in tissues from germ free (GF) and specific pathogen free (SPF) mice (Figure 3.5). In addition to being expressed upon the onset of lactation, an inflammatory process [34] CCL28 expression is seen in the absence of inflammation, hence it is a dual chemokine. Unfortunately, I was not able to measure CCL28 expression in mammary gland tissue because the mice tissues received were all harvested from male mice.

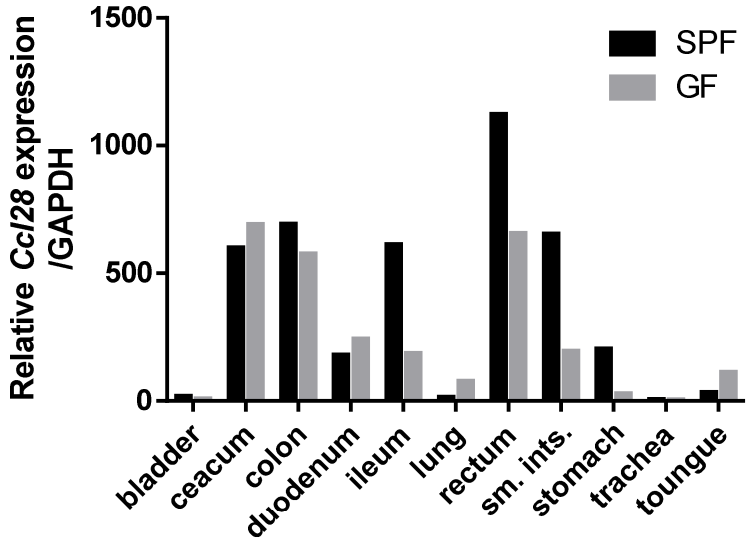

Figure 3.5.*Ccl28* is a dual chemokine. Quantitation of *Ccl28* in Germ free (GF) or Specific pathogen free (SPF) mouse tissues.

Functional characterization of CCL28 deficient mice (*Ccl28*$^{-/-}$)

To assess the *in vivo* consequences of CCL28 ablation we obtained a mouse deficient in CCL28, a *Ccl28*$^{-/-}$ mouse. This mouse was acquired from Deltagen as a heterozygote. To generate the mouse they utilized ES cells derived from the 129/OlaHsd mouse substrain. These were used to generate chimeric mice. The first filial (F1) generation male mice were subsequently bred with C57BL/6 females. Homozygous F2 mutant mice, *Ccl28*$^{-/-}$, obtained from the previous breeding cross, were produced by intercrossing F1 heterozygous males and females. The generated line was then backcrossed to C57BL/6 five times. We received the mouse at the heterozygous stage, *Ccl28*$^{+/-}$, similar to the F1 generation described above.

At the heterozygote stage this mouse breeds as efficiently as a WT. However once a complete knock-out, *Ccl28*$^{-/-}$, breeding efficiency is highly reduced both in number of litters and pups per litter. This has definitely stalled my studies of CCL28 *in vivo* activity. None the less I have been able to generate data by re-booting the colony though the use of *in vitro fertilization* (IVF), a service offered at our mouse transgenic facility.

Ccl28 deficient mice exhibit deregulated levels of IgA production

The primary function of CCL28 is the recruitment of IgA producing plasma cells to mucosal sites via its receptor CCR10. CCR10 is expressed by a variety of immune cells including IgA secreting plasma cells and some T cells. Its importance in the recruitment of immune cell population to mucosal sites, specifically IgA producing plasma cells, is well appreciated as illustrated by the generation of two independently constructed *Ccr10*$^{-/-}$ mice [19, 35]. Morteau et al., generated the first *Ccr10*$^{-/-}$ mouse. This mouse exhibits inappropriate accumulation of IgA producing plasma cells to the mammary gland, a direct consequence of CCR10 ablation. Accordingly, the levels of IgA in the breast milk and in the fecal contents of neonates are

severely reduced. Other sources of IgA remained unaffected including serum and fecal levels of IgA, in adult mice, an observation supported by the proper accumulation of IgA antibody secreting cells, ASC, accumulation to the gastrointestinal tract [19]. The second report, utilized an independently generated *Ccr10*$^{-/-}$ mouse, found that lack of CCR10 did not inhibit the accumulation of IgA ASC to the gastrointestinal tract under homeostatic conditions. However, upon antigenic challenge IgA antibodies had a severely restricted repertoire. This deficient response was exacerbated upon re-challenge as long lived plasma cells were not retained in the intestines [35]. Surprisingly, the second report does not include studies in the mammary gland. Another aspect of CCR10 that was overlooked by both of the studies above is the fact that CCR10 is a shared receptor, utilized by both CCL27 and CCL28. Neither publication explored any experimental venues regarding T cell homing to cutaneous sites.

These studies provide great insight into the physiological consequences of CCR10 ablation, but I wondered if abolishing CCL28 would have the same effect. After all CCL28 is expressed throughout the mucosa by the epithelial cells, and induces calcium flux in CD4^{+} and CD8^{+} T cells [16]. Furthermore, there are no previous reports utilizing a *Ccl28* deficient mouse; hence my studies have the potential to unveil previously unappreciated functions of CCL28's biology.

I began my analysis of the *Ccl28*$^{-/-}$ by quantifying IgA levels in serum, fecal contents, and breast milk. I found that IgA in the serum is unaltered (Figure 3.6a). This result was expected since because CCL28 is produced by epithelial cells of the mucosa, therefore should not have any consequences in IgA production in the serum. In addition, this result supports previous reports using the *Ccr10*$^{-/-}$ mouse, which also report no change in IgA levels in the serum [19]. The IgA levels in the fecal contents were surprising. The *Ccl28*$^{-/-}$ mouse has more IgA than its WT counterpart (Figure 3.6b). This may be a result of a compensatory mechanism, by which IgA is

secreted, but it may not be a high affinity antibody. This observation is reminiscent of the data reported in a CCR10-deficient mouse. In these studies the authors reported that IgA levels in the fecal contents were normal under homeostatic conditions [35]. However, upon closer analysis they report that the IgA produced at this site was a result of a compensatory mechanism and that upon antigenic challenge the Ccr10$^{-/-}$ mice had a severely reduced antibody repertoire [35]. In the breast milk IgA is drastically reduced compared to the *Il-40*$^{-/-}$ mice and WT mice (Figure 3.6 c and d).

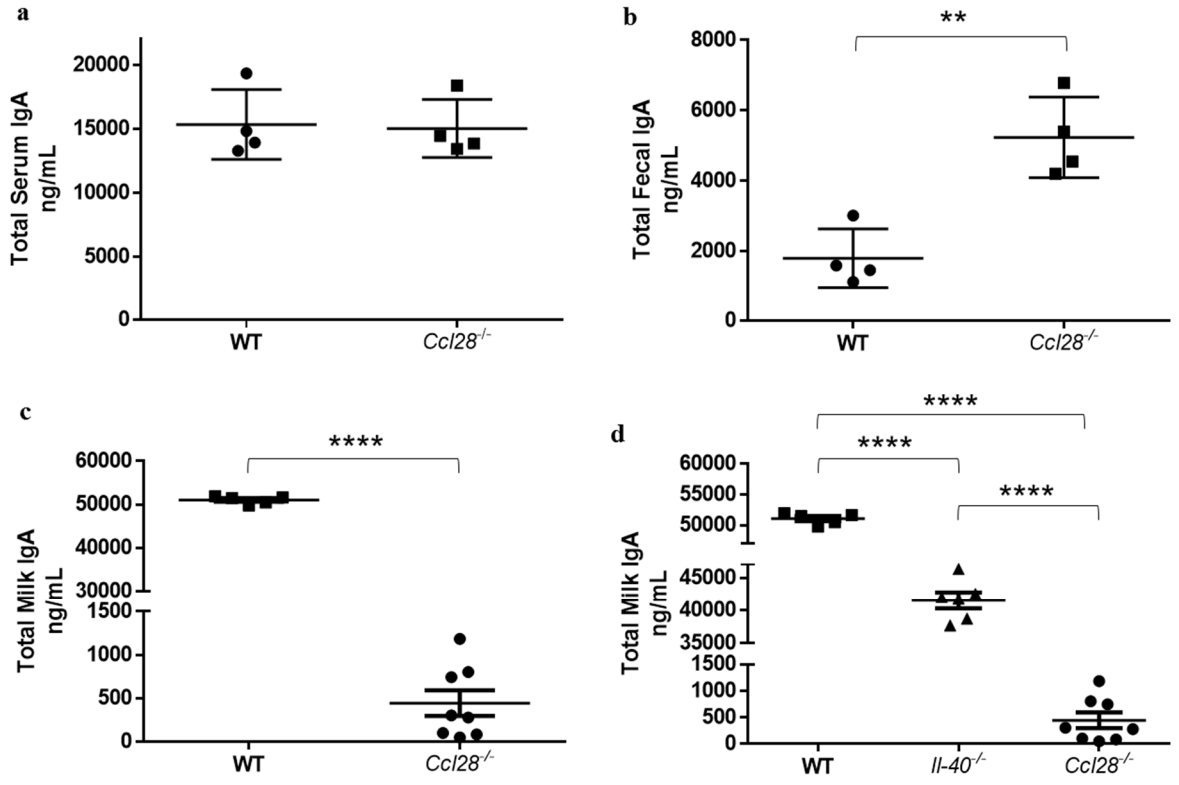

Figure 3.6. *Ccl28*$^{-/-}$ mouse exhibits deregulated levels of IgA. (a) Measurement of total IgA in serum by sandwich ELISA of WT vs *Ccl28*$^{-/-}$mice, (n= 4) (N.S.). (b) Measurements of total IgA in fecal pellets by sandwich ELISA of WT vs *Ccl28*$^{-/-}$mice (n=4)(**p<0.003) (c) Measurements of total IgA in breast milk by sandwich ELISA of WT vs *Ccl28*$^{-/-}$mice (WT n=5, *Ccl28*$^{-/-}$ n= 10)(****p<.0001.) (d) Measurements of total IgA in breast milk by sandwich ELISA of WT vs *Il-40*$^{-/-}$ mice vs *Ccl28*$^{-/-}$mice(WT n=5, *Il-40-/-* n= 6, *Ccl28*$^{-/-}$ n= 8) (****p<0.0001).

CCL28 and disease

Deregulation of CCL28 has already been linked to various pathologies including salivary gland tumors, Sjögren's syndrome, HD, and atopic asthma [24-27]. Given that CCL28 is also expressed throughout the small intestine and colon its absence in these sites may have pathological consequences that under normal conditions may not be relevant. Furthermore, CCL28 exhibits broad spectrum antimicrobial activity against both gram positive/negative bacteria, including Salmonella and Candida sp. [28, 36].

Accordingly, I decided that a salmonella infection model would prove to be an efficient tool to address the mucosal immune response of the *Ccl28*$^{-/-}$ mouse. Based on the expression profile of CCL28 and its antimicrobial properties I hypothesize that the *Ccl28*$^{-/-}$ mouse will more readily succumb to salmonella challenge compared to its WT counterpart. To this end we infected the mice with *S. typhimurium.* The protocol results in an acute infection that is taken out for 96 hours, however in the case of *Ccl28*$^{-/-}$ mice the experiment had to be stopped 72 hours post-infection because the *Ccl28*$^{-/-}$ mice became very ill. Within 72 hours the *Ccl28*$^{-/-}$ mice had lost more weight (Figure 3.7a) and had a higher salmonella burden, measured by CFU/mg, in the spleen, PP, terminal ileum, mesenteric lymph nodes, bone marrow, and caecum contents (Figures 3.7 b-g). This result makes it difficult to underscore the importance of CCL28 as an antibacterial peptide against *S. typhimurium.*

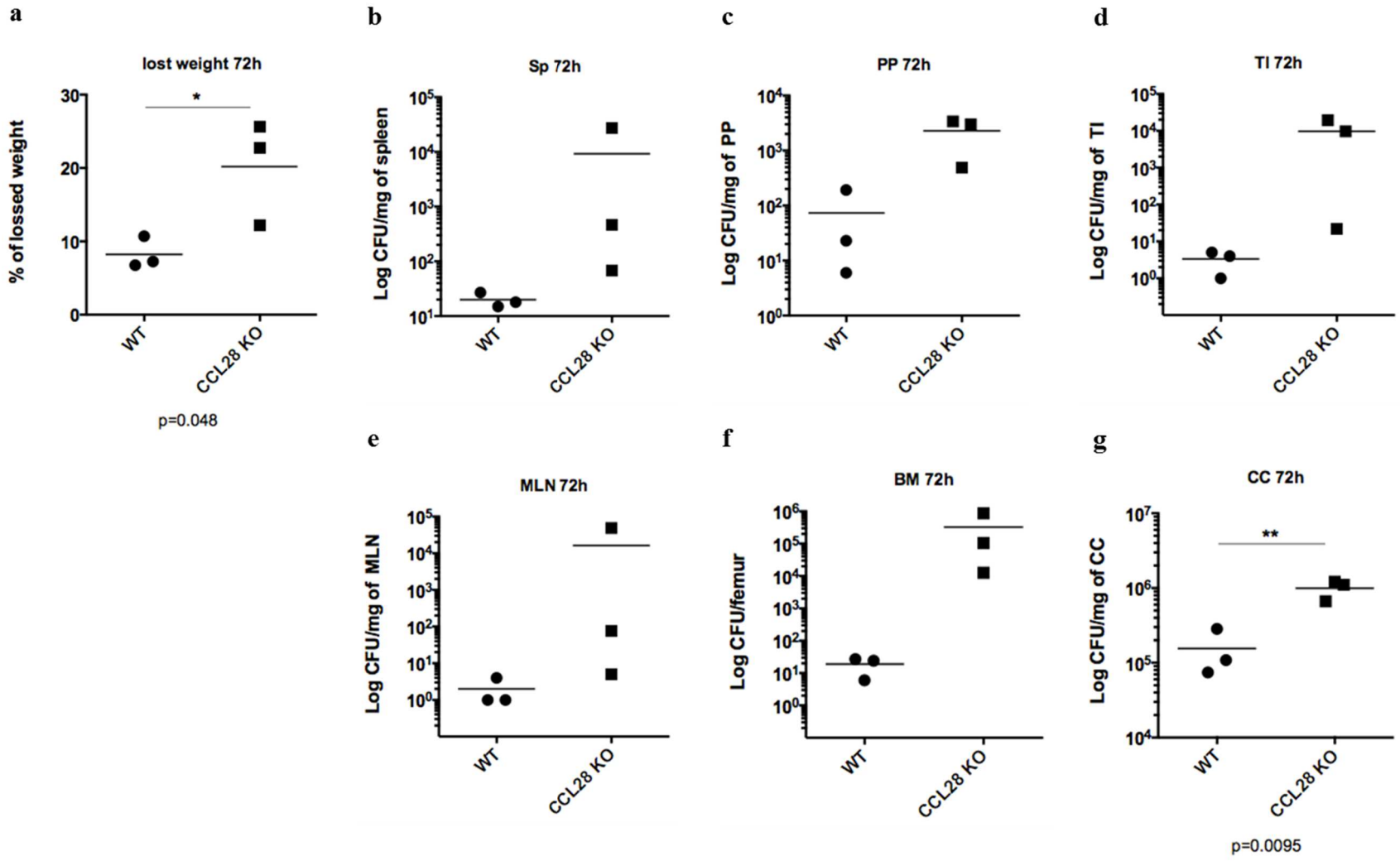

Figure 3.7. *Ccl28*$^{-/-}$ mice are highly susceptible to Salmonella infection. (a) Weight loss 72 hours post infection *Ccl28*$^{-/-}$ mice lost more weight than WT mice p<0.048*. (b-g) bacterial burden measured in CFU/mg, 72 hours post-infection. Sp – spleen, PP – Peyer's patches, TI – terminal ileum, MLN – mesenteric lymph node, BM – bone marrow, CC – caecum contents. CC in *Ccl28*$^{-/-}$ mice had a statistically significant higher bacterial burden than WT p<.0095**

Future Directions and Conclusions

Future Directions

The IgA levels observed in this mouse are deregulated, however another mucosal secretion that should be investigated is the saliva because the salivary glands are the primary site of CCL28 expression. I tried to collect saliva from the mice, but was unable to collect a sufficient amount to perform an ELISA. We are currently in the process of adding a protocol to our IACUC that will enable us to utilize pilocarpine to induce profuse salivation in the mice. Pilocarpine is a drug that induces the production of saliva and is clinically used for dry mouth. In the laboratory, it is used to study epilepsy [38].

In addition to analyzing IgA, it is important to measure other immunoglobulins such as IgM and total IgG. Furthermore, the study of CCL28 would be greatly enhanced by a complete immunophenotyping of immune compartments of this mouse including: the thymus, spleen, lymph nodes, peritoneal exudate cells, and bone marrow. However, at this point we are severely restricted by the colony output, hence we plan on performing another round of IVF to generate enough age-matched mice to use in these important studies.

The exacerbated response to Salmonella infection was extremely drastic. Although it cements CCL28 as an important antimicrobial peptide, the duration of the infection does not allow for an antibody response. However, I would be interested in performing a gut immunophenotyping before and after infection. This would reveal the immune cells present in the gut prior to *S. typhimurium* colonization and would elucidate their trajectories during the acute infection. Since there is not sufficient time to develop a humoral immune response, orchestrated by B cells, I hypothesize that the main participants of this mucosal symphony would be neutrophils. Nonetheless, since PP had elevated bacterial burden it would be interesting to perform a PP

staining, similar to what was done with the *Il-40*$^{-/-}$ mouse, to count germinal center B cells and IgA switched plasma cells within these immune compartments, before and after infection.

There are two possibilities to account for the increased susceptibility of the *Ccl28*$^{-/-}$ mouse to Salmonella infection. The first is that some immune cell populations may be absent from the gut of the *Ccl28*$^{-/-}$ mice that may be necessary to fight the infection. In a collaborative effort with the laboratory of Dr. Manuela Raffatellu, we have begun to study the first possibility. The *Ccl28*$^{-/-}$ mice seem to have inappropriate accumulation of T cells to the gut (Figure 3.8). This impaired ability to recruit T cells is restricted to $CD8^+$ T cells ($CD3^+CD8^{+)}$ and NKT cells ($CD3^+DN$) (Figure 3.8). Although this is a preliminary study it provides a starting point to the elucidation of behind the mechanism that leads to the increased susceptibility to Salmonella infection observed in the *Ccl28*$^{-/-}$ mouse. The second is that the inherent antimicrobial activity of CCL28 may be necessary to fight the Salmonella infection. One way to find out is to infect the *Ccr10*$^{-/-}$ mouse with *S. typhimurium* (which is now available from Jackson labs) and see if it is as susceptible as the *Ccl28*$^{-/-}$ mouse. These experiments are currently underway in a collaborative effort with the laboratory of Dr. Manuela Raffatellu.

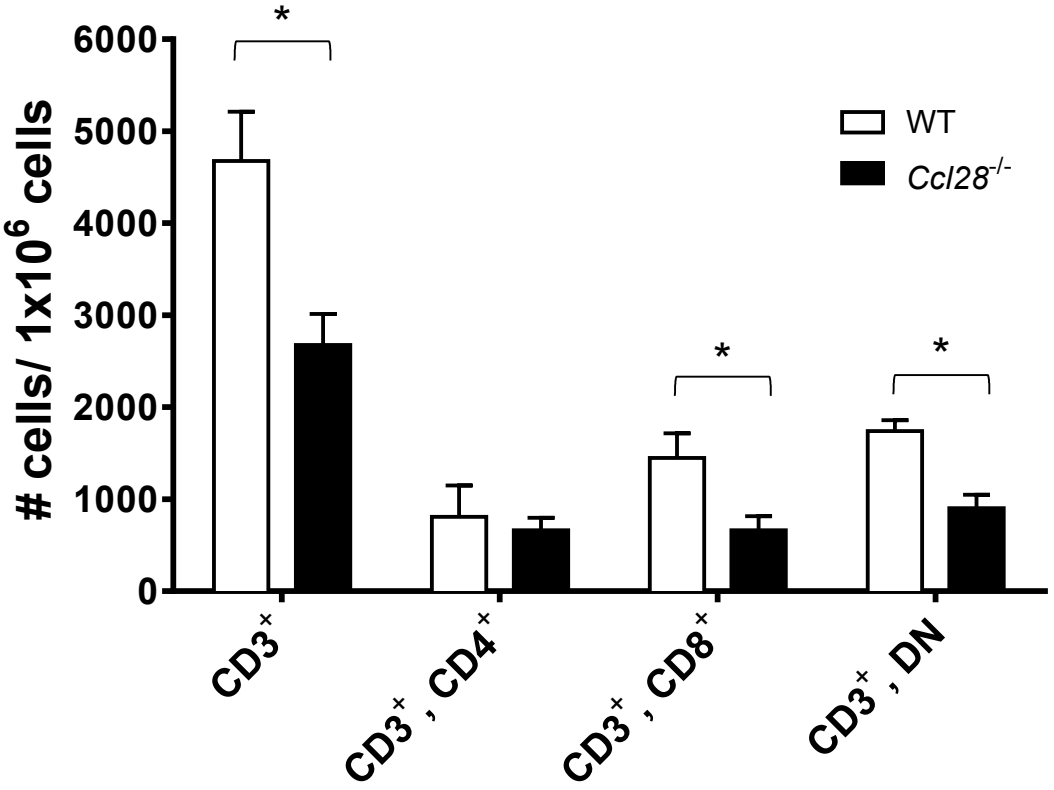

Figure 3.8. *Ccl28*$^{-/-}$ mice do not properly accumulate CD8^{+} T cells or NKT cells to the gut. Quantification of flow cytometry data from cells acquired from the gut of *Ccl28*$^{-/-}$ and WT mice. Differences in CD3+, CD3+CD8+, and CD3+DN are statistically significant, $p<0.03$*, $p<0.05$*, and $p<0.022$*, respectively.

Conclusions

Here I demonstrate the importance of CCL28 to the entire organism. First, I confirmed that CCL28 is important to the recruitment of $CCR10^+$ IgA producing plasma cells to the mammary gland. I also show that CCL28 is a dual chemokine expressed under both homeostatic and inflammatory conditions. Then, I confirmed deregulated levels of IgA in mucosal secretions. Finally, through the use of the Salmonella infection model, I have unequivocally proven the importance of CCL28 to the maintenance of homeostasis in the tissues of the GALT.

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Chapter 4

Future Directions and Conclusions

Future Directions

IL-40

The story of IL-40 is just beginning to unfold. I have already made a tremendous contribution to the fields of cytokine discovery and B cell biology with my initial characterization of IL-40. However, there are many more aspects of IL-40 biology to be elucidated. One that immediately stands out is the regulation of plasma cell differentiation in the absence of IL-40. The *Il-40*$^{-/-}$ mouse exhibits altered serum and mucosal IgA, a defect in the GALT, and a defective response to HI-VACV. Taken together these datum suggest a defect in plasma cell differentiation and/or plasma cell migration.

I have taken the first steps to begin to elucidate a possible defect in this aspect of B cell differentiation by performing a plasma cell induction assay. However, chemokines should be considered as well. For example, CXCR4/CXCL12 represents an interesting axis to further explore in the *Il-40*$^{-/-}$ mouse because CXCR4 participates in embryogenesis during fetal liver development, in the bone marrow in B cell differentiation, and in the recruitment of long-lived plasma cells back to the bone marrow [1]. It would be interesting to measure IL-40 induction in activated B cells from the *Cxcr4*$^{-/-}$ mouse, however a *Cxcr4*$^{-/-}$ is not viable therefore a transient knock-down of CXCR4 in a B cell cell line, such as A20-2J for mice and 2E2 for human, would provide a starting point to identifying a possible deregulation of the CXCR4/CXCL12 axis.

Model for IL-40 in the generation of humoral responses

The data that I have gathered throughout my dissertation indicates that IL-40 is involved in the differentiation and/or proliferation of plasma cells. Specifically, I have seen a notable defect in serum and mucosal levels of IgA production and a defect in production of IgG1 using heat inactivated vaccinia virus. B cells are not generally regarded as potent cytokine producers, but B cells can and do secrete cytokines given the proper differentiation and microimmune environment. For example, naïve B cells that are activated produce CCL17 and CCL22, these chemokines recruit Th2 T helper cells that are designed to provide B cells help to differentiate into plasma cells. These Th2 T helper cells secrete IL-4, which induces IL-40 production, thereby the B cell acquires the capacity to produce IL-40. This B cell has been activated under specific conditions that have induced its differentiation into a B40 cell. However, at this point we do not know if the B40 cells is the cell that gives rise to plasma cells that can go on to secrete antibodies of a given isotype of if the B40 cell is a helper B cell that provides necessary cues to differentiating plasma cells. Either way IL-40 is involved in the differentiation/proliferation of plasma cells.

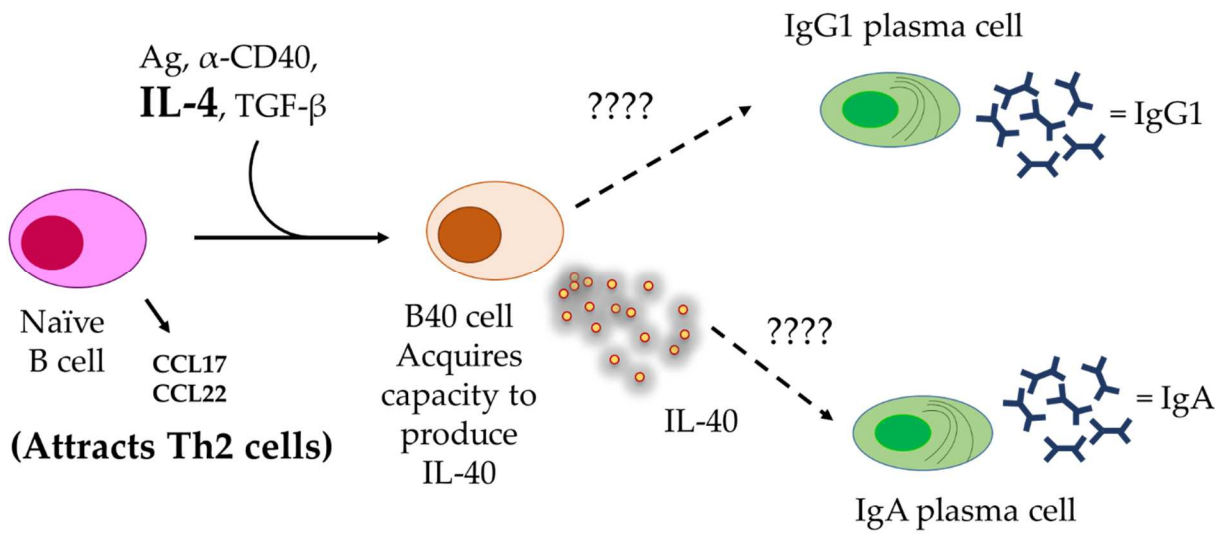

Figure 4.1. Model for IL-40 production and role in Ig responses. An activated naïve B cell produces CCL17 and CCL22 to recruit Th2 helper T cells. The helper T cell provides IL-4. This enables the B cell to become a B40 cell that can produce IL-40. The B40 cell could be the cell that differentiates into a plasma cell or a helper cell that assists in plasma cell differentiation and/or proliferation.

CCL28

CCL28 has been a fixture in the chemokine field for 15 years, however through the use of *Ccl28*$^{-/-}$ mouse I have found that CCL28 may participate in sensory development that ultimately affects behavior. Additionally, the IgA levels in the fecal samples are elevated, which suggests that this IgA may be produced via a compensatory mechanism and that it may be a low affinity antibody. If so, what is the compensatory mechanism? CCL25 is a well known mucosal chemokine that recruits T cell populations to the gut via its receptor CCR9 [2]. This chemokine along with mucosal integrins such as α4β7 are responsible for shaping the immune cell populations present in the gut [3]. Hence, a GALT immunophenotype would elucidate the both immune cell populations present in the gut and may clarify a possible compensatory mechanism. To investigate if the antibodies being produced are low or high affinity an assessment of the rate of SHM in the heavy chains of the immunoglobulin genes would be necessary [4].

The exacerbated response observed in the *Ccl28*$^{-/-}$ mouse post-infection with *S. typhimurium* was sufficiently severe that it required the experiment to be terminated at 72 hours post-infection instead the usual 96 hours. This confirms CCL28's antimicrobial properties, but what I am interested in elucidating is CCL28's role in maintaining gut homeostasis. The above experiment, a gut immunophenotyping, would also be beneficial to beginning to unravel the role of CCL28 in the maintenance of GALT homeostasis and pathogenesis of mucosal infections in its absence.

IL-40 and CCL28 and their role in IgA responses

Interestingly, IL-40 and CCL28 are both involved in humoral immunity specifically in IgA responses of the MALT. Both of these secreted proteins are induced upon the onset of lactation. IL-40 is expressed by the incoming B cells as the mammary gland matures and CCL28 is responsible for recruiting IgA plasma cells to the lactating mammary gland. Furthermore, the specific onset of expression of IL-40 and CCL28 in the mammary gland may play a role in changing the microimmune environment of the mammary gland. This may provide insight behind the observed protection against breast cancer following lactation. Their joint expression in the mammary gland reveals the role of each of these secreted proteins in the production of IgA (Figure 4.2). CCL28 is responsible for directing the migration of IgA plasma cells to the mammary gland, but does not play a role in the differentiation of these cells. Therefore the lack of IgA in the milk of the *Ccl28*$^{-/-}$ mouse is a direct result of a failure to recruit IgA plasma cells to the mammary gland. However, IL-40 plays a significant role in the differentiation or proliferation of IgA plasma cells as seen by reduced serum and mucosal levels of IgA.

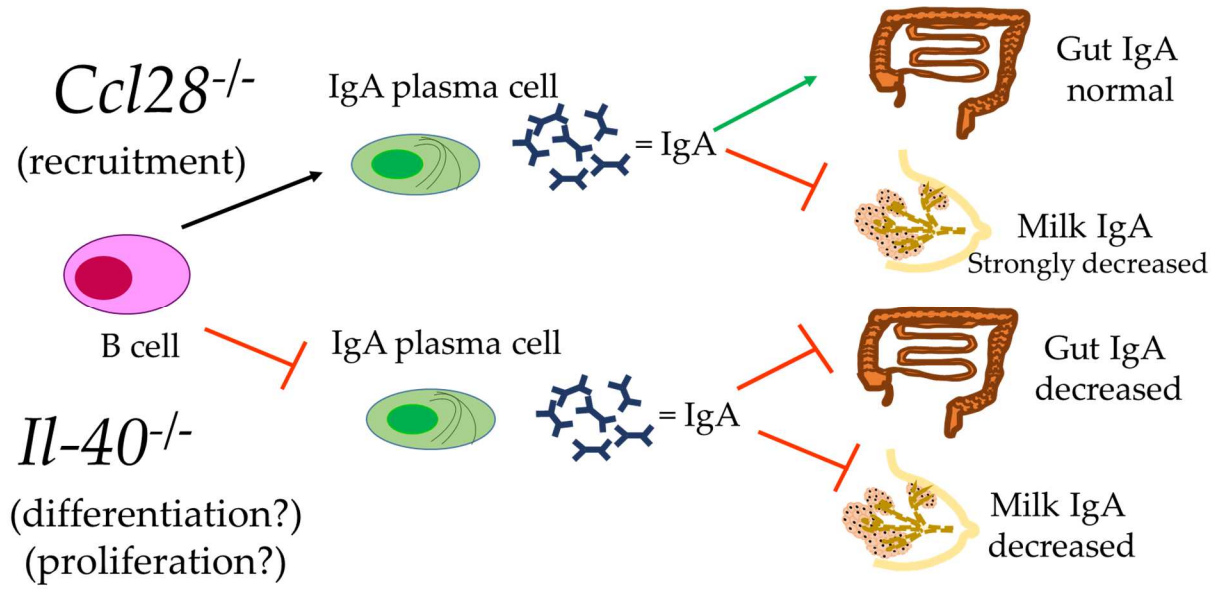

Figure 4.2. Different roles for IL-40 and CCL28 IgA responses. CCL28 is not involved in IgA production, rather the reduced IgA levels in the milk are a result of a failure to recruit IgA plasma cell to the mammary gland. On the other hand, IL-40 is involved in the generation of IgA responses as seen in reduction of IgA in both serum and mucosal secretions. This may be a result of either differentiation and/or proliferation of plasma cells.

Conclusions

The immune system is the definition of ordered chaos. Whether there is an active inflammatory response or not the different players of the immune system are constantly at work either mediating homoeostasis or performing immune surveillance. This intricate dance between the cells of the immune system, other tissues, and the various antigens encountered must be regulated in order to maintain the balance within the organism. This regulation is achieved primarily through the production of cytokines. As I have already discussed cytokines are pleiotropic in nature and exert their far reaching effects throughout the organism. Within the immune system, cytokines participate in nearly every facet of immune associated function, whether it be a homeostatic role, inflammatory response, or resolution of that same response. Hence, cytokines can be thought of as the orchestrators of the immune system.

Just as the important as the director of a symphony is to the success of a concert so are the instrument players that perform the actual concert. Here, the director of the symphony is represented by cytokines and the players of the various instruments represent different immune cell types. For example, my favorite immune cells, B cells, are responsible for generating a humoral response that is characterized by the production of antibodies. However, the magnitude of their response is driven by the secretion of cytokines. Hence, cytokines are ultimately responsible for the generation, modulation, and resolution of immune responses.

In the course of my thesis work I have characterized the functional significance of the genetic ablation of two different cytokines. The first is IL-40 is a novel cytokine produced by activated B cells. The second is a well-established mucosal chemokine CCL28. My studies have focused on identifying any possible defects within the immune system of model organism. Cytokines rarely work independently, rather the secretion of a group of cytokines is responsible for shaping an

immune response. I was very excited when I found a link between the cytokines that are the focus of my thesis, IL-40 and CCL28. These two work together in the immune remodeling of the mammary gland during lactation. Like CCL28, IL-40 is also upregulated in the mammary gland upon lactation, likely a consequence of incoming B cells. Furthermore, since CCL28 is not up-regulated in the lactating mammary gland the *Il-40*$^{-/-}$ mouse which results in a decrease of IgA in the breast milk. It is precisely the complex interplay between two very different cytokines that makes the immune system such an interesting field.

IL-40

The discovery of IL-40 was a processes driven by bioinformatics. IL-40 is encoded by a currently unannotated gene known as *C17orf99*. This gene's expression profile was immediately interesting because it is restricted to sites of hematopoiesis and activated B cells. Moreover, its sequence predicts a signal peptide which is a hallmark of secreted proteins. These two observations made it an extremely attractive candidate that merited further research. We continued our studies of this gene and found that its expression is only seen in mammalian genomes and confirmed that it is a secreted protein through Western blot analysis.

The human and mouse *IL-40* sequences are well conserved, hence we sought to obtain a mouse with a targeted deletion of the gene to characterize the biology of IL-40. The *Il-40*$^{-/-}$ mouse is viable and fertile and has no detectable defects in hematopoiesis. However, the *Il-40*$^{-/-}$ mouse exhibits altered B cell in the resting spleen, deregulated IgA in the serum and mucosal secretions, a defect in the GALT and a blunted response to HI-VACV immunization. This datum suggests that IL-40 may participate in homeostatic B cell processes and be important modulator of plasma cell differentiation and antibody secretion, specifically to IgA isotype. It is important to note that serum and mucosal immunoglobulin levels are deregulated even in the absence of inflammation,

hence IL-40 may be a dual cytokine that is expressed both under homeostatic and inflammatory conditions.

CCL28

Unlike IL-40, CCL28 is not a novel chemokine, but it was discovered in a previous laboratory by my mentor Dr. Zlotnik [5]. CCL28 is a mucosal associated chemokine expressed by epithelial cells that line mucosal surfaces. It can bind two different receptors, CCR10 and CCR3; however it seems that its ability to bind CCR3 is only relevant in pathological states [5-8]. The CCL28/CCR10 axis is well known for its recruitment of IgA secreting cells to mucosal sites [9]. In addition to its chemotactic properties CCL28 is also a potent antimicrobial peptide that can inhibit both gram positive and gram negative bacteria and *C. albicans* [10, 11].

Despite CCL28 being a well recognized mucosal associated chemokine, there are no reports of this chemokine using a mouse with a targeted deletion of CCL28. To properly evaluate the role of CCL28 in the mucosa I utilized a mouse deficient in Ccl28, a *Ccl28*$^{-/-}$ mouse. CCL28 ablation results in deregulated levels of IgA in mucosal secretions, and a high susceptibility to Salmonella infection. The observed deregulated IgA levels are a direct result of a breach in the CCL28/CCR10 axis. Without CCL28, CCR10 expressing cells will not migrate to mucosal sites; hence the reduction in IgA in the mammary gland. The IgA levels in fecal contents were actually higher than those observed in the WT counterpart. This data is reminiscent of the *Ccr10*$^{-/-}$ mouse which had normal levels of IgA in the fecal contents of adult mice as a result of a compensatory mechanism [12]. Finally, the *Ccl28*$^{-/-}$ mouse exhibited increased susceptibility to infection with *S. typhimurium*. The acute infection protocol is normally 96 hours in length, but we had to stop the experiment at 72 hours due to the condition of the mice. At 72 hours post infection the *Ccl28*$^{-/-}$ mouse had lost more weight and had a higher bacterial burden measured by CFU/mg. This data

unequivocally demonstrates that CCL28 is a powerful antimicrobial peptide that is involved in maintaining homeostasis of the GALT. Furthermore, this may suggests that lack of CCL28 negatively impacts the immune populations of the gut.

Summary

Overall, I have characterized a novel B cell associated cytokine, IL-40, and have identified CCL28 as an important homeostatic chemokine of the GALT. Importantly, I linked the two secreted proteins to the maturation of the mammary gland and the microimmune changes that occur in the lactating mammary gland. Furthermore, my work has laid out the foundation for the mechanism observed behind the protection against breast cancer following lactation. My dissertation work will undoubtedly open up new venues of research in B cell immunology, cancer research, and mucosal pathogenesis.

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