The requirement of Toll-like receptor signaling in the induction of humoral and cell-mediated immune responses to the orally administered Dukoral® vaccine

By

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ABSTRACT

Only a limited number of orally-administered vaccines have been licensed, such as the Dukoral® vaccine, comprised of killed whole-cell *Vibrio cholerae* and cholera toxin B subunit (CTB). Thus far, mechanistic details underlying the resulting protective immune generated by this vaccine, including the requirement of Toll-like receptor (TLR) signaling, remain largely unknown. Herein, I investigated the involvement of TLR signaling in the induction of humoral immune responses following oral and intramuscular immunization with Dukoral or its components by using TLR-2-, TLR-4-, MyD88- and Trif-deficient mice. I showed that wild type and all groups of TLR-deficient mice generated similar levels of *V. cholerae*- and CTB-specific IgG1 and IgG2c serum antibodies, and fecal IgA antibodies, following oral immunization with Dukoral, and with CTB alone. Additionally, the agglutinating activity of *V. cholerae*-specific antibodies was also found to occur independently of MyD88 signaling. However, intramuscular immunization with Dukoral, as well as with CTB alone, required MyD88 signaling for the induction of CTB-specific IgG1and IgG2c serum antibodies.

I also evaluated the involvement of TLR signaling in the induction of splenic cell-mediated immune responses following oral immunization with Dukoral. My results showed that CD4+ T-cell and CD19+ B cell proliferation occurred in a MyD88-dependent manner in response to stimulation by *V. cholerae* or CTB. In contrast, in response to CTB stimulation, Trif negatively regulated both CD4+ T-cell and CD19+ B-cell proliferation. Splenocytes from $MyD88^{-/-}$, $Trif^{/-}$, $TLR\text{-}2^{-/-}$, and $TLR\text{-}4^{-/-}$ mice were significantly inhibited in their ability to secrete IFN-γ in response to stimulation by *V. cholerae*. Furthermore, maturation of dendritic cells (DCs), as measured by increased cell-surface CD80, CD86, CD40, and MHCII expression and cytokine secretion was shown to occur in a MyD88-dependent manner in response to

stimulation with Dukoral vaccine components. However, despite the impaired ability of MyD88$^{-/-}$ DCs to mature, MyD88$^{-/-}$ animals, and indeed all TLR-deficient animals tested, showed serum and fecal antibody responses comparable to those seen in wild-type animals. Taken together, my results suggest that humoral responses (antibody production and agglutinating ability), following oral immunization with Dukoral, were able to occur independently of TLR signaling and DC maturation. This TLR-independence in the generation of humoral responses was lost when the vaccine was administered parenterally. Cell-mediated immune responses (cell proliferation, DC maturation, and cytokine secretion) were found to be TLR-dependent.

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LIST OF ABBREVIATIONS

WHO:	World health organization
IM:	Intramuscular (immunization)
SC:	Subcutaneous (immunization)
TLRs:	Toll-like receptors
DCs:	Dendritic cells
CD80:	Cluster of differentiation 80, or other
MHC II:	Major histocompatability complex class II
MALT:	Mucosa-associated lymphoid tissue
GALT:	Gut-associated lymphoid tissue
NALT:	Nasal-associated lymphoid tissue
MLN:	Mesenteric lymph nodes
M-cells:	Microfold-cells
MAdCAM-1:	Mucosal addressin cell adhesion molecule-1
L-selectin:	Lymphocyte-selectin
SHM:	Somatic hypermutation
CSR:	Class-switch recombination
ADCC:	Antibody dependent cell-mediated cytotoxicity
IFN-γ:	Interferon gamma
IL-:	Interleukin
TGF-β:	Transforming growth factor β
CCR9:	C-C chemokine receptor type 9
pIgR:	Polymeric immunoglobulin receptor
SIgA:	Secretory IgA
CTB:	Cholera toxin B subunit
PBMCs:	Peripheral blood mononuclear cells
TLR- :	Toll-like receptor (e.g. -2, -4, -5)
$TLR^{-/-}$:	Toll-like receptor deficient
MyD88:	Myeloid differentiation primary response gene (88)
ETEC:	Enterotoxigenic *E. coli*
HIV:	Human immunodeficiency virus
WT:	Wild-type
TNF-α:	Tumor necrosis factor alpha
PRR:	Pattern recognition receptor
TIR:	Toll/IL-1R homology domain
Trif:	TIR-domain-containing adapter-inducing interferon beta
mRNA:	Messenger ribonucleic acid

FAE:	Follicle-associated epithelium
IEL:	Intestinal epithelial lymphocytes
Tirap:	Toll-interleukin 1 receptor domain containing adaptor protein
Tram:	Trif-related adaptor molecule
LPS:	Lipopolysaccharide
IRF3:	Interferon regulatory factor 3
Mal:	MyD88-adaptor-like
IRAK:	Interleukin-1 receptor-associated kinase
TRAF6:	Tumor necrosis factor receptor-associated factor 6
OVA:	Ovalbumin
I.P:	Intra-peritoneal (injection)
PspA:	*Streptococcus pneumoniae* surface protein A
F-MLV:	Friend murine leukemia virus
HSV:	Herpes simplex virus
ELISA:	Enzyme-linked immunosorbent assay
PBS:	Phosphate-buffered saline
FCS:	Fetal calf serum
HRP:	Horseradish peroxidase
TMB:	3,3',5,5'-tetramethylbenzidine
RPMI:	Roswell Park Memorial Institute media
GM-CSF:	Granulocyte-macrophage colony stimulating factor
ELISPOT:	Enzyme-linked immunosorbent spot assay
BCIP/NBT:	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt / nitro-blue tetrazolium chloride substrate
CFSE:	Carboxyfluorescein succinimidyl ester
Th1:	T-helper 1
Th2:	T-helper 2

CHAPTER I: GENERAL INTRODUCTION

General aspects of vaccinology

Vaccination has been described as one of the most successful and efficacious medical procedures of modern human history [1-3]. The past 300 years have seen the development of a multitude of vaccines for the prevention of many infectious diseases. Beginning in the 1700's with Edward Jenner's inoculations of cowpox virus for the prevention of smallpox [4], Louis Pasteur followed in the late 1800's with the development of the first rudimentary vaccines against anthrax in cattle, and against rabies in humans [5,6]. The 1930's saw Max Theiler introduce a vaccine against yellow fever, [7]. This vaccine would later come to be known as one of the most effective vaccines in human history [8] . Indeed one of the crowning achievements of vaccinology came in 1980 when the world health organization (WHO) announced that smallpox had been eradicated [9]. Most modern vaccines are typically made up of two parts, the antigen (or active component of the vaccine) [10] to which the immune response is directed, in addition to an adjuvant, which is described as a substance that enhances the immune response to the accompanying antigen(s) [11]. Aluminum-based adjuvants have thus far been the only adjuvant approved for human use [12], although the search for novel adjuvants is ongoing. Several different types of vaccines exist today, such as inactivated or killed vaccines, protein subunit vaccines, live attenuated vaccines, toxoid vaccines, conjugate vaccines, as well as DNA vaccines [13].

Overall, successful vaccines work by inducing both a rapid and efficacious immune response comparable to that induced by infection, in addition to long-lived immunity capable of preventing subsequent infections or decreasing the severity of any subsequent infection by a

particular pathogen [14]. The induction of such long-lived immunity falls within the realm of the adaptive immune system, which is able to generate antigen-specific lymphocytes following immunization [15]. The generation of such antigen-specific immune responses to administered vaccines may occur through humoral immune responses or cell-mediated immune responses. Humoral responses rely on B-cells for the production of antibodies capable of binding to a particular pathogen or pathogen component [16]. A characteristic of B-cells is that they are able to take up and present certain antigens on their own, instead of having the antigen first processed by dendritic cells (DCs) and then being activated by a cognate CD4+ T-helper cell [17]. Such antigens are known as T-independent antigens and include large polymeric antigens comprised of repeating subunits, such as lipopolysaccharide (LPS) and bacterial flagellin [13]. On the other hand, cell-mediated immune responses are comprised of cytotoxic CD8+ T-cells capable of killing infected cells, and also CD4+ T-helper cells, which assist in the activation of both CD8+ T-cells and B-cells, thereby contributing to Th1-directed or Th2-directed responses, respectively [15].

Parenteral vaccination

The vast majority of vaccines currently licensed are administered parenterally by either intramuscular (IM) or subcutaneous (SC) injection [18]. For the efficient induction of an immune response following IM or SC immunization, the vaccine and adjuvant administered should provide relevant antigens capable of mediating a protective immune response, in addition to a minimum amount of pathogen-associated molecular patterns in order to induce an inflammatory reaction through the attraction of cells of the innate immune system (namely DCs, monocytes and neutrophils) [19]. Indeed, toll-like receptors (TLRs) and their associated

signaling pathways play an important role in detecting multiple pathogen components and inducing the production of proinflammatory cytokines [20]. Such proinflammatory cytokines work to further attract antigen presenting cells such as DCs and macrophages, and contribute to induce their maturation [21-23]. During this process of maturation, DCs and macrophages up-regulate the expression of various co-stimulatory markers such as CD80, CD83, CD86 and CD40, as well as MHCII [24,25] while migrating to draining lymph nodes (typically the axillary and inguinal lymph nodes, following injection into the deltoid or quadriceps muscles, respectively) [15]. Once in the lymph nodes or in the spleen, matured DCs or macrophages may begin activating antigen-specific T-cells as part of the initiation of the adaptive immune response [8], resulting in the induction of protective vaccine-induced immune responses. Indeed, many parenterally-administered vaccines exert their protective effects through the induction of protective antibody responses in the circulation [26], such as against tetanus [27], Lyme disease [28], and yellow fever [29], with only a limited number of parenterally-administered vaccines being more dependent upon cell-mediated immune responses for their protection, as has been described for tuberculosis [30] and varicella [31,32]. Overall, parenteral (IM or SC) immunization works to induce primarily systemic humoral and cellular immune responses [33]. Perhaps one of the exceptions to this rule are inactivated or subunit flu vaccines, whose serum antibodies have been cited as a correlate of protection in the control influenza infection within the respiratory tract, despite being administered by parenteral injection [26].

Mucosal vaccination

The mucosal immune system comprises all mucosal surfaces of the body, namely the lungs, digestive tract, uro-genital tract, as well as the eyes and inner ear surfaces [34]. The cells and tissues of the mucosal immune system are collectively known as mucosa-associated

lymphoid tissues (MALT), and comprise approximately 80% of the total immune cells in the entire body [34]. The mucosal immune system has been described to have three principal functions: i) to protect mucous membranes from colonization by pathogenic organisms, ii) to prevent the uptake of undegraded antigens (such as ingested food), and iii) to prevent the induction of harmful immune responses should such undegraded antigens reach inside the body [34]. One of the most important sub-groups of the MALT is the gut-associated lymphoid tissues (GALT), followed by the nasal-associated lymphoid tissues (NALT) [35]. Between mouse and man, the principal inductive sites of the GALT comprise the Peyer's patches, isolated lymphoid follicles, and even the appendix [35-40]. Closely related are the mesenteric lymph nodes (MLN), although they are not considered as being directly part of the MALT as they do not directly sample antigen [39].

Contrary to injected vaccines, there are far fewer licensed vaccines administered by mucosal routes [41]. Currently licensed orally administered vaccines include vaccines against poliomyelitis [42] , salmonella [43], cholera [43], and rotavirus [44]. As might be expected, the overall mechanistic details governing the induction of immune responses at mucosal sites differ from those governing the induction of systemic immune responses. Mucosal administration of vaccines has many potential benefits over injected vaccines, such as the induction of an immune response at mucosal sites of pathogen entry and increased ease of administration not requiring sterile needles [45]. However, the development and implementation of mucosally administered vaccines is not without its own challenges, and the development of mucosally administered vaccines has largely lagged behind that of injected vaccines as a result of these challenges. This appears to be partly due to the great difficulty associated with precisely measuring mucosal immune responses (particularly in humans), and to the difficulty in regulating the uptake of a

precise amount of vaccine antigen [46]. Oral vaccine delivery is also hindered by the acidic pH environment of the stomach and proteolytic enzymes found in the digestive tract, and may thus require the administration of neutralizing sodium bicarbonate to counteract such effects [47].

Early research showed that oral immunization was capable of inducing both a local immune response in the small intestine as well as in more distant mucosal sites [48-53], and this has resulted in the concept of a 'common mucosal immune system' [54,55], or as it has more recently been referred to, a 'compartmentalised' or 'integrated' mucosal immune system [56,57]. Such compartmentalization also limits the location where an immune response will be induced following mucosal immunization (reviewed in [34]). Namely, rectal and vaginal immunization has been described to induce antibody responses predominantly at the site of immunization [58,59], while nasal vaccination has been shown to induce antibody responses in vaginal and cervical secretions in humans [60]. In developing countries, oral vaccination may be particularly hampered by so-called "tropical barriers" to vaccination, which appear to involve disturbances of digestive and absorptive abilities [41], as well as deficiencies in zinc intake, which have been shown to affect immune responses to orally administered vaccines [61,62]. Taken together, such challenges will need to be overcome so that more effective mucosal vaccines can be developed in the future.

M-cell mediated uptake of antigens following oral immunization

Orally administered antigens are sampled from the gut lumen by specialized epithelial cells called microfold (M)-cells [63] . Such M-cells, capable of endocytosing antigens and even whole microorganisms, were originally identified in humans in 1974 by Owen and Jones [64] and were described to have 'microfolds' on their apical surface and to reside within the follicle-

associated epithelium of Peyer's patches. M-cells have also been identified within the rectal mucosa [65], as well as within nasal- and bronchus-associated lymphoid tissues [66]. M-cells differ from neighboring absorptive enterocytes by the fact that they contain short and irregular microvilli, compared to the uniform and densely packed microvilli present on enterocytes [67]. Another discerning feature is the presence of a 'pocket' on the basolateral side of M-cells [68], which has been found to contain T-cells, B-cells, DCs, and macrophages [69]. Thus, the polarized orientation and largely exposed state of M-cells makes them ideally suited for endocytosing antigens from the intestinal lumen, and they have been previously found to transport both soluble and particulate antigens [63], including *Vibrio cholerae* [67]. The precise mechanisms by which M-cells take up antigen have been shown to vary according to the size and physic-chemical characteristics of the antigen [70]. For example, uptake of larger particulate antigens and whole-cell bacteria has been shown to involve rearrangement of the M-cell cytoskeletal structure [71], whereas the uptake of viruses has been shown to involve clathrin-mediated endocytosis [72], and fluid-phase endocytosis has been described for soluble components [73]. A role for M-cells in the initiation of mucosal immune responses has been proposed by Pappo and Mahlman [74] , who demonstrated that rabbit M-cells were able to secrete pro-inflammatory IL-1 following stimulation with LPS in vitro. The targeting of M-cells for enhancing the potency of orally-administered vaccines has also been described [75].

Lymphocyte homing following vaccination

The ability of lymphocytes to traffic to various sites throughout the body is known (reviewed in [76]). Additionally, the homing potential of lymphocytes has been found to depend upon the site of antigen encounter [77]. The mucosal addressin cell adhesion molecule-1 (MAdCAM-1), expressed within Peyer's patch high endothelial venules (HEVs), has been found

to be the principal mucosal homing receptor ligand for lymphocytes within the GALT [78]. Circulating lymphocytes are able to bind MAdCAM-1 by way of their cell surface α4β7 integrins [79-81]. This is in contrast to lymphocytes which home to peripheral lymph nodes, which have been shown to preferentially express lymphocyte (L)-selectin after systemic immunization [82,83], including IgG-switched plasma cells [84]. Despite the use of differing adhesins by peripheral immunization compared to mucosal immunization, mucosal immunization is still capable of inducing systemic IgA and IgG antibodies due to a portion of activated B-cells expressing the peripheral homing receptors α4β1which binds the peripheral addressin L-selectin [85].

Induction of antibody isotypes following immunization

Successful immunization can result in the induction of various antibody isotypes. Such antibody responses occur as a result of antigen-specific germinal center reactions, where B-cells undergo somatic hypermutation (SHM) and class-switch recombination (CSR) in order to produce high affinity antibodies (reviewed in [86]). Repeated instances of antigen exposure or immunization may eventually result in the induction of immunological memory, defined as the induction of specific antibody for an extended period of time after vaccination or upon subsequent antigen re-encounter [87].

In mice, B-cells which undergo CSR may change their antibody isotype from IgM to either IgG1, IgG2a/b/c, IgG3, IgA, or IgE [88]. IgG antibodies are the predominant antibody isotype found in the serum [87]. With regards to the IgG2a isotype, it is important to note that Balb/C mice express the IgG2a isotype, whereas C57BL/6 mice carry a different gene, and thus express an alternative isotype named IgG2c, which displays a 16% difference in its amino acid sequence compared to IgG2a [89,90]. The IgG2a antibody isotype has been described to have

potent complement-activating activity [91] and antibody dependent cell-mediated cytotoxicity (ADCC) [92]. In contrast, the mouse IgG1 isotype has been shown to have a limited ability to carry out such functions [91,92]. The induction of various antibody isotypes has been previously shown to be affected by cytokines, with interferon (IFN)-γ and interleukin (IL)-4 having opposing effects in this matter (reviewed in [93]).

IgA antibodies are the predominant antibody isotype found in mucosal secretions (reviewed in [94,95]), and are indeed the most abundant antibody isotype produced in the entire human body [96]. As introduced above, the principal inductive sites for IgA production of the GALT comprise the Peyer's patches and isolated lymphoid follicles [35-40]. The environment within Peyer's patches has been found to be very conducive to the induction of IgA antibodies by the presence of particular cytokines such as transforming growth factor β (TGF-β) [97], in addition to IL-4, IL-6 and IL-10 which have been shown to aid in the differentiation and expansion of intestinal IgA-secreting B-cells [98-100]. Equally important in the induction of IgA-producing cells is retinoic acid, which has been shown to be critical for conferring gut-homing properties to intestinal IgA-producing B-cells, by up-regulating CCR9 and α4β7 mucosal homing receptor expression [101]. Taken together, GALT inductive sites, with their cytokine microenvironment skewed towards inducing IgA antibodies, induce predominantly IgA-secreting B-cells. Such IgA-secreting B-cells have been described to represent almost 70% of all germinal center B-cells present in the Peyer's patches [102].

IgA performs various functions in the intestinal tract, from regulating the commensal microbial population [95] to facilitating the uptake of IgA-coated pathogens by M-cells [103]. Indeed, the presence of commensal intestinal flora has been shown to be critical for proper IgA homeostasis, as germ-free animals have been observed to have impaired IgA production [104].

IgA secretion in mucosal tissues entails production of the IgA by mucosal/ intestinal B-cells, followed by its binding and extracellular transport via the polymeric Ig receptor (pIgR) [105]. Once translocated across the surface of intestinal epithelial cells, the secreted IgA is known as secretory IgA (SIgA), which is comprised of IgA antibodies bound together to form a dimeric molecule [106], which can mediate various protective effects [107]. Secretory IgA accomplishes part of its protective effects through a process known as immune exclusion, whereby pathogenic organisms are bound by IgA and become trapped within the mucous layer and are subsequently cleared and expelled via the peristaltic movement of the gut [105]. Additional findings have shown mucosal IgA to be capable of neutralizing pathogens within intestinal epithelial cells during their translocation process [108]. The dimeric SIgA is able to exert such effects in the harsh conditions of the intestinal tract by way of its increased resistance to degradation [109]. In addition, mucosal IgA has been previously shown to have anti-inflammatory effects, demonstrated by its inability to activate complement [110] and its ability to down-regulate proinflammatory TNF-α and IL-6 synthesis [111].

Toll-like receptors

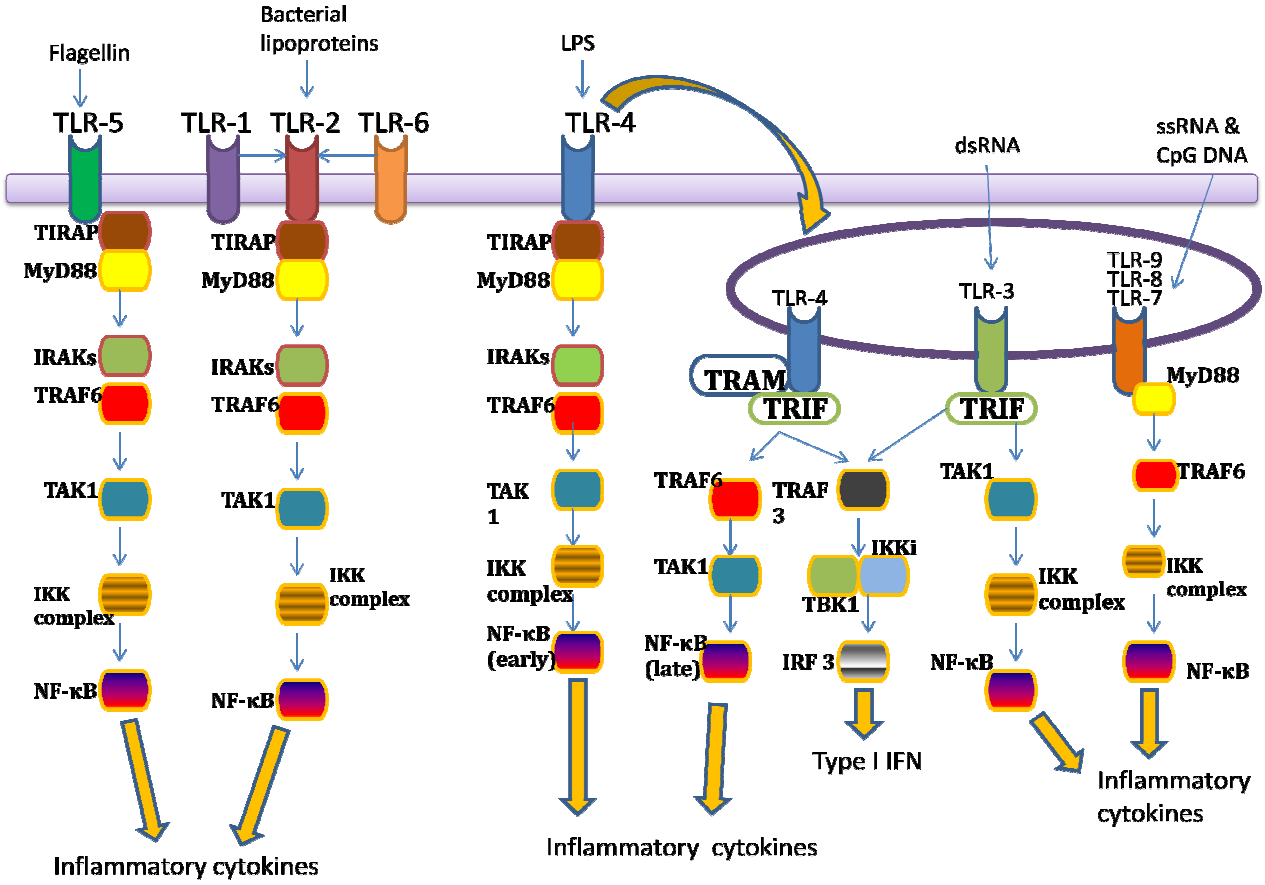
The earliest immune response against invading pathogens occurs via the innate immune system, which initiates an early, non-specific response [52,112]. This initial immune response is able to occur through the sensing of multiple microbial structures [113] by receptors known as pattern-recognition receptors (PRRs) [114], which include C-type lectin receptors, Nod-like receptors, and most importantly, Toll-like receptors (TLRs) [115]. TLRs were originally discovered in *Drosophila melanogaster*, and were found to play a critical role in the dorsal-ventral embryonic development of *Drosophila* [116]. Subsequent research found that TLRs were critical for anti-fungal immune responses in *Drosophila* [117], and later identified homologous

receptors in humans [118]. TLRs are integral trans-membrane glycoproteins which contain an extracellular domain with multiple leucine-rich repeat motifs, as well as a cytoplasmic signaling domain with homology to the cytoplasmic domain of the IL-1 receptor, referred to as the Toll/IL-1R homology (TIR) domain [119]. These receptors appear to be largely evolutionarily conserved from *Caenorhabditis elegans* through to mammals [120,121]. There are currently 12 TLRs identified in mammals (reviewed in [122]) , some of which are expressed on the cell surface (TLR-1, -2, -4, -5, -6) and some of which are expressed intracellularly (TLR-3, -7, -8, -9). TLR-11, related to TLR-5, has also been found intracellularly [123], in addition to TLR-13 [124]. Altogether, TLRs are able to sense various microbial components not normally present in mammalian hosts, such as Gram-negative bacterial lipopolysaccharide (LPS) detected by TLR-4 [125-127], bacterial lipoproteins detected by TLR-2 [127], flagellin proteins detected by TLR-5 [128,129], unmethylated CpG DNA from bacteria and viruses detected by TLR-9 [130], and double-stranded and single-stranded RNA, detected by TLR-3 [131] and TLR-7 [132], respectively. They are also able to sense various components of protozoan parasites as well as fungi [115,133] (Figure 1).

TLRs are expressed predominantly on cells such as monocytes/macrophages [134] and DCs [135,136], as well as on B-cells [137,138]. Recent data has also shown TLRs to be expressed on T-cells [139,140]. Indeed, a comprehensive study found that TLR mRNA was expressed in most tissues of the body, but predominantly in immunologically important sites such as the spleen and on peripheral blood lymphocytes, or on organs exposed to the external environment such as the lungs and gastrointestinal tract [141].

Figure 1. TLR signaling.
Schematic representation demonstrating the induction of inflammatory cytokines and type I interferons by TLR signaling and associated signaling mediators. TLRs may be found on the cell surface (TLR-1, 2, 4, 5, 6) or intracellularly (TLR-3, 7, 8, 9). Binding of a cognate TLR ligand results in the activation of the MyD88-dependent or Trif-dependent pathways, resulting in the production of proinflammatory cytokines and type I interferons via NF-κB and other transcription factors. TLR-2 signaling involves the dimerization of TLR-2 with either TLR-1 or TLR-6 to induce the production of proinflammatory cytokines via the MyD88-dependent pathway. TLR-4 signaling is capable of stimulating the production of both inflammatory cytokines and type I interferon through activation of both the MyD88-dependent and Trif-dependent pathways, respectively.

(Adapted from Kawai and Akira in *Immunity 34*, May 27, 2011).


Flagellin
Bacterial lipoproteins
LPS
TLR-5
TLR-1
TLR-2
TLR-6
TLR-4
dsRNA
ssRNA & CpG DNA
TIRAP
MyD88
IRAKs
TRAF6
TAK1
IKK complex
NF-κB
TLR-4
TLR-3
TLR-9
TLR-8
TLR-7
TRAM
TRIF
TRAF6
TRAF 3
IKKi
TBK1
IRF 3
Type I IFN
NF-κB (early)
NF-κB (late)
Inflammatory cytokines

Toll-like receptor expression in the intestine

Research with germ-free mice has suggested that commensal bacteria may play an important role in the maintenance and induction of intestinal TLR expression (particularly TLR-2, -3, -4 and -5) [142]. Work by Chabot et al. has shown TLR-2 to be expressed on both the follicle-associated epithelium (FAE, the region overlying Peyers patches or isolated lymphoid follicles) and on the intestinal epithelial cells (IECs) of mouse intestinal villi and crypts [143]. TLR-4 was expressed in these regions, although to a lesser extent. Stimulation with TLR-2 and TLR-4 ligands was shown to increase particle uptake by the FAE, suggesting that TLRs may play a role in regulating the function of the FAE [143]. Additional research has shown compartmentalized expression of TLR-4 in the mouse gut [144]. Subsequent work supported these findings, and demonstrated that both TLR-2 and TLR-4 expression was compartmentalized throughout the gut, and that this compartmentalization was directly affected by the local mucosa-associated microbiota [145]. Intestinal TLR expression in humans also appears to be tightly regulated, as increased expression of TLR-2 and TLR-4 has been observed in inflammatory bowel diseases such as ulcerative colitis and Crohn's disease [146].

TLR signaling: MyD88-dependent and -independent signaling pathways

Binding of TLRs by their cognate ligands results in the recruitment of multiple downstream signaling adaptors which ultimately result in the production of proinflammatory cytokines and type I interferons. These signaling adaptor molecules include myeloid differentiation primary response gene 88 (MyD88), TIR-associated protein (Tirap), TIR-containing adapter-inducing interferon-β (Trif) (also known as TIR-domain-containing molecule 1 (Ticam-1) [147]), and Trif-related adaptor molecule (Tram) [20].

All TLRs, excluding TLR-3, utilize the MyD88 signaling adaptor [115,122,148] to induce the production of proinflammatory cytokines by way of the nuclear factor kappa-light-chain enhancer of activated B-cells (NF-κB) and other transcription factors. Alternatively, TLR-4 has been shown to utilize both the MyD88 and Trif signaling adaptors, in order to induce the production of both proinflammatory cytokines and type I interferons by way of NF-κB and interferon regulatory factor 3 (IRF-3) [122,148] (Figure 1). MyD88 has been shown to be critical for the production of TLR-induced inflammatory cytokines through work with MyD88-deficient mice, and particularly MyD88-deficient macrophages [131,149,150]. Further evidence has also shown that LPS is able to stimulate NF-κB activation in MyD88- deficient macrophages [151], suggesting the existence of a MyD88-independant pathway. Subsequent research demonstrated similar findings, whereby MyD88-deficient DCs were still able to express co-stimulatory molecules in response to LPS stimulation [152]. Taken together, these findings indicated the existence of both MyD88-dependant and MyD88-independant signaling pathways. Additional findings have suggested that the TIR domain-containing adaptor protein (Tirap)/MyD88-adaptor-like (Mal) as well as IRF-3 may be adaptor proteins involved in the MyD88-independant signaling pathway (reviewed in [153]). Trif has also been described as a signaling adaptor molecule in the MyD88-independent signaling pathway, stimulated by TLR-3 and TLR-4 [154,155]. Thus, most TLRs (including TLR-2, -4, -5, -7, -8, and -9) signal via the MyD88 adaptor protein, whereas TLR-4 has been shown to stimulate the MyD88-dependant pathway as well as the MyD88-independant pathway, via the signaling adaptor Trif.

As introduced above, TLR-2 is involved in sensing bacterial lipoproteins detected by TLR-2 [127]. TLR-2 has been recently found to detect multiple ligands such as lipoproteins and peptidoglycans from Gram-negative as well as Gram-positive bacteria, in addition to lipoteichoic

acid from gram-positive bacteria [115]. In order to signal, TLR-2 forms heterodimers with either TLR-1or TLR-6, with TLR-2-TLR-1 recognizing triacylated lipopeptides from Gram-negative bacteria, while TLR-2-TLR6 recognize diacylated lipopeptides from Gram-positive bacteria [115]. Ligand binding results in the recruitment of the MyD88 and adaptor protein TIRAP, which recruits additional signaling adaptors such as interleukin-1 receptor-associated kinases (IRAKs) and tumor necrosis factor receptor-associated factor 6 (TRAF6). This ultimately results in the activation of NF-κB and the production of inflammatory cytokines (Figure 1).

Distinct from the TLR-2 signaling pathway, the TLR-4 pathway induces signaling via a MyD88-dependent as well as MyD88-independent pathway [122,148], as it recruits both MyD88 and Trif signaling adaptors to induce the production of both inflammatory cytokines as well as type I interferons [122]. While extensively reviewed elsewhere [148], briefly, TLR-4 signaling results in the recruitment of MyD88, which requires the cofactor Tirap [156,157], as MyD88 has been described to not bind the cytoplasmic TIR region of TLR-4 (and TLR-2) efficiently, and this is thought to be due to weak electrostatic attractions with MyD88 [158]. An additional adaptor molecule, Tram, is required for TLR-4 signaling in order to recruit the signaling adaptor Trif [159], and this process appears to require the endosomal internalization of TLR-4 so that TLR-4 may interact with Tram [160]. MyD88 and Trif result in the further recruitment of TRAF6 and TRAF3 cofactors, respectively, to ultimately induce MyD88-dependent NF-κB -directed inflammatory cytokine production, and Trif-dependent IRF3-directed type I interferon production [122,148] (Figure 1).

Involvement of TLR signaling in the induction of immune responses to licensed vaccines: Yellow fever vaccine YF-17D

Many existing vaccines have been developed by empirical methods, and despite their success, little is known about how they induce protective immune responses [161,162]. Querec et al. have undertaken such mechanistic research with regards to the yellow fever vaccine YF-17D and determined that it activated multiple DC subsets through the stimulation of TLR-2, -7, -8, and -9, which induced proinflammatory cytokine secretion of IL-12p40, IL-6 and interferon-α [8]. Their results also showed that MyD88 positively regulated the production of Th1 cytokines, while TLR-2 negatively regulated the production of such cytokines [8]. Overall, their findings indicated that the YF-17D vaccine activated multiple TLR pathways on specific DC subsets, and the authors hypothesized whether synthetic vaccines with a similar TLR-stimulatory profile may be able to mimic the potent immunogenicity of the YF-17D vaccine [161]. Querec et al. have further built upon their findings with YF-17D by adopting a 'systems biology' approach to determining gene signatures in humans capable of predicting vaccine efficacy prior to immunization [163]. While further work remains to be done to elucidate mechanistic details underlying the protective immune responses from additional licensed vaccines, the above findings certainly represent a new beginning for the field of vaccinology.

TLR signaling in the induction of humoral and cellular immune responses

The requirement of TLR signaling for antibody production appears incompletely understood at this time. Pasare and Medzhitov have previously found that optimal antigen-specific antibody production to a T-dependent antigen, injected parenterally, required TLR signaling on B-cells [164]. They also demonstrated that TLR signaling was particularly important for the induction of IgM, IgG1 and IgG2c antibody responses, but was dispensable for

the induction of IgE and serum IgA antibody responses, in response to injection of ovalbumin (OVA)-LPS. Conversely, Nemazee and Beutler subsequently showed that mice double-deficient in both MyD88 and Trif signaling molecules were still able to induce robust antibody responses against the T-dependent antigen trinitrophenol-hemocyanin, in Freund's complete adjuvant [165], and alum [166] , comparable to those seen in WT control animals. Such findings suggested that antibody responses were still capable of being generated, following intra-peritoneal (IP) injection, despite the complete absence of TLR signaling.

Humoral and cellular immune responses to additional pathogens appear to show a differential requirement of TLR signaling for the induction of immune responses. Induction of LPS-specific serum antibodies to another Gram-negative bacterium, *Brucella abortus*, was shown to occur independently of TLR signaling, while MyD88 was critical for the clearance of *Brucella* in vivo [167]. In a mouse model of Legionnaires' disease, TLR-2-induced MyD88 signaling was found to be critical for the clearance of *Legionella* bacteria in vivo [168]. The induction of serum IgM responses, and survival following challenge with the pathogen *Borrelia hermsii*, were found to be dependent upon TLR-2 signaling [169]. Regarding the regulation of immune responses to viral pathogens, Heer et al. previously showed that TLR signaling did not play a critical role in the induction of cellular immune responses to the influenza B virus, however MyD88 signaling was found to be important in regulating antibody isotype switching to the IgG2a & IgG2c isotypes [170]. Against the Friend murine leukemia virus (F-MLV) retrovirus, Browne and Littman demonstrated that MyD88 signaling was critical to the induction of F-MLV-specific humoral responses, as MyD88$^{-/-}$ mice infected with F-MLV were unable to induce any virus-specific IgG antibodies [171]. Furthermore, MyD88$^{-/-}$ animals did not generate any F-MLV-neutralizing antibody titers. Conversely, cell-mediated immune responses were only

partially reduced in MyD88$^{-/-}$ animals. In a mouse model of genital herpes infection, MyD88 signaling was found to be critical for the early control of herpes simplex virus (HSV) replication and disease, whereas it was dispensable for the generation of HSV-specific serum IgG antibodies, as well as for surviving HSV challenge following immunization [172].

A study by Shang et al. has reported that transgenic mice expressing constitutively active TLR-4 within intestinal epithelial cells demonstrated increased intestinal B-cell recruitment, B-cell-tropic cytokine production, and increased IgA class switching, resulting in increased fecal IgA production [173]. Their results also showed that TLR-4-deficient mice displayed normal (steady-state) fecal IgA levels, while MyD88-deficient mice displayed significantly decreased fecal IgA levels, compared to WT control animals [173].

Previous research by Park et al. showed that *Streptococcus pneumoniae* surface protein A (PspA)-specific systemic and mucosal antibody responses, following oral or intra-nasal vaccination by an attenuated *Salmonella* vector, were induced in a MyD88-independent manner, while PspA-specific CD4+ T-cell proliferation was significantly decreased in MyD88$^{-/-}$ animals [174]. Interestingly, MyD88 signaling was critical for survival from challenge by virulent *S. pneumoniae*. Seibert et al. showed that both *Salmonella*-specific humoral and cellular immune responses occurred independently of TLR signaling, following infection by *Salmonella* [175]. Recently, Isaac et al. showed that MyD88$^{-/-}$ mice demonstrated significantly impaired recruitment of inflammatory cells into the peritoneal cavity as well as the spleen, following infection with *Salmonella* [176].

The requirement of TLR signaling in the induction of humoral and cellular immune responses to licensed oral vaccines against polio, cholera, and rotavirus (with the exception of Salmonella) does not appear to have been thoroughly investigated at this time. In addition, given

the differential requirement of TLR signaling for the induction of humoral and cellular immune responses to the various bacterial and viral pathogens described above, it is quite likely that the requirement of TLR signaling for the induction of immune responses, as well as for the generation of protective immune responses, will be pathogen-specific, and also depend upon factors such as the characteristics of the antigen/ adjuvant used, as well as the route of immunization.

The Dukoral vaccine

As introduced above, orally administered vaccines are significantly fewer than parenterally injected vaccines. One such licensed vaccine is the orally administered vaccine against *Vibrio cholerae* of the 01 serogroup, and is available as a formulation of inactivated whole-cell *Vibrio cholerae* 01 with 1 mg of recombinant cholera toxin B-subunit (CTB) [177] under the trade name Dukoral [43]. A second oral vaccine also exists (under the trade name Orochol), and is an attenuated strain of *V. cholerae*, CVD 103-HgR, administered as a single dose [178].

The Dukoral vaccine is described as an oral, inactivated travellers' diarrhea and cholera vaccine, containing a total of $1x10^{11}$ *V. cholerae* (comprised of heat-inactivated *V. cholerae* 01 Inaba classic strain and Ogawa classic strain, and formalin-inactivated *V. cholerae* 01 El Tor strain and Ogawa classic strain) along with 1 mg of recombinant CTB [179]. It is indicated for the prevention of travellers' diarrhea caused by enterotoxigenic (ETEC) *Escherichia coli*, as well as for the prevention of cholera, caused by *V. cholerae* [179]. This vaccine was manufactured by SBL Vaccin AB (Stockholm, Sweden) and was licensed in Canada by Sanofi Pasteur Limited (Toronto, Canada) at the time this project was undertaken. Included with the vaccine is a bicarbonate buffer, ingested together with the vaccine at the time of immunization, for the

purpose of neutralizing residual stomach acid in order to protect the integrity of the vaccine antigens [179]. Mechanistic details on precise immune pathways involved in the induction of an immune response to this vaccine are largely lacking from the product monograph. Briefly, it is mentioned that "*No established immunological correlates of protection against cholera after oral vaccination have been identified. There is a poor correlation between serum antibody responses, including vibriocidal antibody response and protection. Locally produced secretory IgA antibodies in the intestine probably mediate protective immunity."* [179]. Since precise mechanistic details are currently lacking, research into determining the critical signaling pathways involved in the induction of humoral and cellular immune response to this vaccine would be beneficial.

Animal models of *V. cholerae* infection

While natural infection of animals with *V. cholerae* does not typically occur [180], some animal models have been developed to facilitate *V. cholerae*-related research. One of the earliest animal models created to study *V. cholerae* infection was the ileal loop model in rabbits [181,182]. Later, infant mouse models became more widely used [183,184], and results with the infant mouse model were found to parallel findings seen in human trials [183]. A limitation of this model is that only passive immunity may be evaluated [183]. Germ-free mouse models of *V. cholera* infection also exist [102], however such models have been described as being difficult to maintain due to their cost, and findings with such models may be limited due to their immunological immaturity [185]. To overcome these limitations, Nygren et al. have recently developed an adult mouse challenge model of *V. cholerae*, whereby adult mice are rendered permissive to infection by treatment with streptomycin antibiotics, and their findings

demonstrated that their model was effective at evaluating the protective efficacy of cholera vaccines in adult mice [185].

TLR activation by *V. cholerae*

Little appears known regarding the specific TLR signaling pathways activated by *Vibrio cholerae*, apart from the known TLR ligands described for Gram-negative bacteria (described further, below). There are currently no published reports describing the requirement of specific TLR signaling pathways for the induction of a protective immune response to the Dukoral vaccine. A study published in 2005 by Dehus et al. comparing the immune-stimulating activities of LPS from 11 different bacteria demonstrated that LPS from *V. cholerae* was dependent upon both TLR-2 and TLR-4 signaling for its ability to induce IL-6 production in LPS-stimulated human peripheral blood mononuclear cells (PMBCs) [186], while Zughaier et al. demonstrated that purified *V. cholerae* LPS appeared to signal predominantly via the MyD88-dependent pathway [187]. A study published in 2009 by Nandakumar et al. indicated that stimulation with live *V. cholerae* was able to increase TLR-4 expression, and decrease TLR-5 expression, in human intestinal epithelial cell lines [188]. Another study identified the *V. cholerae* phosphatidylserine decarboxylase protein as a novel MyD88- and TLR-4-dependent agonist [189]. Apart from these findings, there are no published reports describing the requirement of specific TLR signaling in the induction of protective immune responses to the Dukoral vaccine.

CTB: Adjuvant and tolerizing effects

Adjuvant effects of CTB

As described above, the Dukoral vaccine also contains cholera toxin B subunit (CTB) as one of its components. According to the Dukoral product monograph, enterotoxigenic *E. coli*

(ETEC) bacterial strains produce an enterotoxin structurally similar to the cholera toxin, which is also neutralized by antibodies against CTB [190]. CTB constitutes the homopentameric non-toxic component of cholera toxin, of which the toxic A subunit is responsible for the intense diarrhea accompanying cholera infection [191], which can result in fluid losses up to 1 L/hour [192]. The cognate receptor for the CTB component has been found to be the ganglioside GM1 [193], found on most tissues [194] including the intestines [195,196]. The ability of enterotoxins such as cholera toxin to act as an adjuvant in animal models to improve immune responses to co-administered antigens has been well described [197-204]. In humans, the use of such compounds is limited to the non-toxic B subunit, due to the severe toxicity associated with the A subunit [191]. CTB has also been previously employed as an experimental adjuvant with various antigens administered by various routes [205-208], as well as in various experimental vaccines such as for HIV [209] and influenza [210-212]. It is believed to exert its adjuvant effect by increasing antigen presentation of linked or co-administered antigens [213].

Tolerizing effects of CTB

Conversely, there is also evidence for the enhanced tolerizing ability of CTB [214], and this has also been described in the literature for suppressing the development of autoimmune diseases [215,216], including IgE-mediated allergies [217] and even graft rejection reactions [34]. A phase I/II human clinical trial involving patients with Behcet's disease (an autoimmune disease of the eye) evaluated the oral administration of a Behcet's disease-specific peptide linked to CTB. The results of the study suggested that patients who received the disease-specific peptide linked to CTB demonstrated an improvement of their autoimmune symptoms, as manifested by decreased relapse of uveitis (swelling of the eye) and a lack of disease-specific

CD4+ T-cell proliferation as well as IFN-γ and TNF-α production [218].

Recent findings in mice suggest that B-cells treated with antigen conjugated to CTB induced the expansion of antigen-specific regulatory T-cells [219]. Additional findings with human cells suggested that CTB-linked antigen was able to inhibit DC maturation, resulting in decreased production of pro-inflammatory cytokines, with a concomitant increase in immunosuppressive IL-10 [220]. Thus, CTB may be capable of acting both as an adjuvant to improve immune responses to co-administered antigens, and also as a tolerizing agent during immunotherapy for autoimmune or allergic conditions.

TLR-stimulating ability of enterotoxins

Toxins of *V. cholerae* and *E. coli* are grouped into two main families of structurally-related toxins known as type I and type II toxins (reviewed in [221]). The type I family includes cholera toxin (including CTB), as well as the *E. coli* LT-I toxin, while the type II family is comprised of the *E. coli* LT-IIa and LT-IIb toxins. In the type I family, the B subunits have been found to be structurally similar, exhibiting approximately 80% amino acid identity, while the B subunits from the type II family have been shown to have less homology (<14%) with the B subunits from the type I family [221]. Previous findings by Hajishengallis et al. showed that CTB was able to induce IL-8 production in THP-1 cells, and this production was significantly reduced when THP-1 cells were treated with TLR-2 neutralizing antibodies [222]. The B subunits of the LT-IIa and LT-IIb toxins were also found to induce cytokine secretion by human THP-1 cells and mouse macrophages in a TLR-2-dependent manner [222]. Subsequent findings showed that the A subunit of LT-IIb was able to negatively regulate the ability of the LT-IIb B

subunit to interact with TLR-2 [223], whereas the ganglioside GD1a receptor was found to act as a co-receptor for LT-IIb B subunit-mediated TLR-2 signaling [224]. Recent findings also demonstrated that TLR-2 signaling was important for enhanced DC antigen uptake and increased expression of CD80, CD86, CD40 and MHCII molecules following stimulation with the B subunit of LT-IIb [225]. Thus, evidence has shown that the B subunits of LT toxins, as well as CTB, have the ability to stimulate TLR signaling. Further work is required to more completely elucidate the ability of CTB to stimulate TLR signaling.

Rationale

In light of the continued absence of effective prophylactic vaccines for major infectious diseases such as HIV, it is clear that a better understanding of the mechanistic details underlying protective vaccine-induced immune responses is required so that more effective vaccines may be designed in the future. Many of the currently licensed vaccines have been largely developed by empirical methods, and without an understanding of precise mechanistic and signaling details underlying their protective effects [15,226]. Furthermore, as most infections start at mucosal surfaces [227-229], and since specific mechanistic details regarding the signaling pathways critical to the induction of protective immune responses of the orally administered Dukoral vaccine are currently unknown, a better understanding of the mechanisms responsible for the induction of protective immune responses at mucosal sites would also be beneficial [75]. As doctors Czerkinsky and Holmgren have stated, "*To date, arguably, vaccines have done more for immunologists than immunologists have done for vaccines*" [41]. This underscores the need for research to determine the precise mechanistic details responsible for the induction of protective immune responses from licensed vaccines. The focus of this research was to begin uncovering such mechanistic details, by examining the requirement of TLR signaling (specifically, the

requirement of MyD88, Trif, TLR-2 and TLR-4 signaling) in the induction of humoral and cell-mediated immune responses, in mice, following oral immunization with the Dukoral vaccine.

Hypothesis

This project evaluated the hypothesis that TLR signaling is critical for the immune response resulting from oral immunization with the Dukoral vaccine.

Objectives

The specific objectives of this project were to:

1. Research the requirement of TLR signaling in the induction of i) humoral, and ii) cellular immune responses in mice following oral immunization with the Dukoral vaccine;

2. Evaluate the ability of Dukoral vaccine components to induce maturation of bone marrow-derived dendritic cells;

3. Evaluate the ability of serum from orally vaccinated animals to agglutinate live *V. cholerae*.

CHAPTER II: MATERIALS AND METHODS

Vaccine

The orally-administered, inactivated travellers' diarrhea and cholera vaccine Dukoral® (Sanofi Pasteur, Toronto, Canada) was used throughout this project. The vaccine consists of heat and formalin-inactivated *Vibrio cholerae* of the 01 Ogawa classic strain, the 01 Inaba El Tor strain, and the 01 Inaba classic strain, for a total of $1x10^{11}$ *V. cholerae*, supplied along with 1 mg of recombinant cholera toxin B subunit (CTB), in a 3 mL volume. Included with the vaccine was a sachet of sodium hydrogen carbonate. The included sachet was reconstituted in distilled water as per the manufacturer's instructions, and then sterile-filtered through a 0.2 μm syringe filter (Pall Corporation, Ann Arbor, MI, USA). Reconstituted sodium hydrogen carbonate buffer was administered to mice in order to neutralize residual stomach acid prior to vaccination. For in-vitro experiments, whole-cell *V. cholerae* from 1 vial of Dukoral were centrifuged at 14,000 rpm and 4°C, and pelleted *V. cholerae* were re-suspended in 2 mL of PBS by vortexing. Whole-cell *V. cholerae* were then washed three times in PBS before being reconstituted in 3 mL of PBS.

Experimental animals

Animal experiments were approved by the University of Ottawa Animal Care and Veterinary Service, under the protocol CHEO-102. Animals were housed in micro-isolator cages under specific-pathogen free conditions, with food and water provided *ad libitum*. All mice being orally immunized were fasted for at least 4 hours prior to immunization and first received a 100 μL dose of sodium hydrogen carbonate buffer to neutralize residual stomach acid. All

neutralizing buffer and vaccine doses were administered intragastrically via a gavage needle. All oral immunizations were administered in a 100 μL volume.

Pre- and post-vaccination serum, feces, and saliva was collected. Blood was collected via saphenous vein puncture, centrifuged to obtain serum, and stored at -20^{o}C until used. For saliva collection, 75 μL of 1 mg/mL pilocarpine (Sigma-Aldrich, Oakville, ON, Canada) was administered by intra-peritoneal (IP) injection and resulting saliva was collected via pipette into an eppedorf tube and frozen at -80^{o}C until used. Fecal pellets were collected and stored at -80^{o}C prior to use. To extract fecal antibody, 100 mg of feces per mouse was weighted out, then dissolved in 1 mL PBS containing 2.5% non-fat milk with complete mini EDTA-free protease inhibitors (Roche Applied Science, Laval, QC, Canada). Fecal pellets were broken up using a pipette tip, vortexed, then incubated on ice for 1 hr with intermittent vortexing. Next, samples were centrifuged for 15 minutes at 14,000 rpm and 4^{o}C to pellet debris. The supernatant was then collected and stored at -80^{o}C until analyzed.

Oral and intra-peritoneal vaccination of mice with Dukoral

To optimize the oral vaccine dose, 10-week old female Balb/C and C57BL/6 mice (Charles River Laboratories) were divided into four total groups. Three groups received the vaccine orally (n = 4 mice per group), while one group serving as the control group received the vaccine via IP injection (n = 6 mice). The three orally immunized groups received the vaccine at varying dosages, in a 100 μL volume, as follows:

Group 1: $3x10^8$ *V. cholerae* + 10 μg CTB;

Group 2: $3x10^9$ *V. cholerae* + 10 μg CTB;

Group 3: $3x10^{10}$ *V. cholerae* + 10 μg CTB.

The IP control group received $1x10^7$ *V. cholerae* with 10 μg CTB, in a 200 μL volume. Mice were immunized orally on days 0, 10, 20 and 30, while IP-immunized mice were immunized on days 0 and 14.

All subsequent animal studies utilized female mice of the C57BL/6 background, between 7 – 10 weeks of age (Jackson Laboratory). TLR mutant mice were MyD88 knockout (stock # 009088), Trif mutant (stock # 005037), TLR-4 mutant (stock # 007227) and TLR-2 knockout (stock # 004650) mice were immunized in order to evaluate the requirement of TLR signaling on the induction of immune responses following oral immunization with Dukoral. Mutant strains were described as having significant deletions that eliminated expression of both mRNA and protein of the specified TLR. These knockout and mutant strains are henceforth referred to as $MyD88^{-/-}$, $Trif^{-/-}$, $TLR\text{-}2^{-/-}$ and $TLR\text{-}4^{-/-}$ mice. TLR mutant mice (n = 5 mice per group) and wild-type (WT) controls (n = 5 mice) were orally immunized on days 0, 10, 20, and 30, with $3x10^9$ *V. cholerae* and 10 μg CTB.

Intramuscular vaccination of mice with Dukoral

In order to evaluate the requirement of TLR signaling on the induction of an immune response following parenteral immunization with Dukoral, WT and $MyD88^{-/-}$ mice (n= 5 mice per group) were vaccinated intramuscularly on days 0, 10, 20, and 30 with the complete Dukoral vaccine ($1x10^7$ *V. cholerae* with 10 μg CTB). The vaccine was prepared in a 50 μL volume, with 25 μL injected into each hind limb.

Oral and intramuscular vaccination with CTB

In order to confirm findings observed with the administration of the Dukoral vaccine, WT and MyD88$^{-/-}$ mice were vaccinated intramuscularly or orally (n = 4 mice per group) with CTB alone, in order to evaluate the requirement of TLR signaling on the induction of an immune response to the CTB protein alone. Purified CTB was purchased (Enzo Life Sciences, Ann Arbor, MI, USA) and mice were immunized intramuscularly or orally with 10 μg of the CTB protein. Mice immunized intramuscularly received a 50 μL injection (25 μL into each hind limb) given on days 0 and 10. Mice immunized orally were vaccinated on days 0, 10, 20 and 30. Pre- and post-vaccination serum was collected for intramuscularly vaccinated mice, while serum and feces was collected for orally immunized mice.

Analysis of antibody responses by ELISA

For measuring *V. cholerae*- and CTB-specific serum IgG antibody responses, 50 μL of serum diluted 1:100 was applied to *V. cholerae* or CTB-coated plates. All serum, fecal and saliva samples were diluted in blocking buffer comprised of 2% vol/vol fetal calf serum (FCS) (PAA Cell Culture Company, Etobicoke, ON, Canada) diluted in PBS. For measuring fecal IgA antibody responses, fecal pellets was processed as described above, and 50 μL of clarified fecal supernatant at an undiluted (neat), 1:10 and 1:100 dilution was applied to *V. cholerae*- or CTB-coated plates. For measuring saliva IgA antibody responses, saliva was collected as described above, and 50 μL of saliva, diluted 1:5 – 1:625, was applied to *V. cholerae*- or CTB-coated plates.

V. cholerae-specific responses were measured as previously described [230], with the following modifications: Briefly, high-binding Costar ELISA plates (Corning Inc., Tewksbury, MA, USA) were coated overnight at 4^{o}C with 50 μL of 1.2x10^{7} *V. cholerae*/mL, diluted in PBS,

or with 50 µL of 0.5 µg/mL CTB. Plates were spun at 1600 rpm for 5 minutes to help distribute the coating buffer across the bottom of the 96-well plates. The remaining steps were identical for all ELISAs: The next morning, coating buffer was decanted and plates were washed once in PBS. Plates were then blocked for 2 hours at 37^{o}C with 2% FCS-PBS. After blocking, plates were decanted and washed once in PBS. The diluted sample (50 µL of serum, fecal supernatant, or saliva) was then added to duplicate wells. Blocking buffer alone was added to the blank (uncoated) wells. After incubating for 1 hour at 37^{o}C, plates were washed 4 times with PBS and then incubated for 1 hour at 37^{o}C with 50 µL of secondary horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) in 2% FCS-PBS (goat anti-mouse IgG-Fc, IgG1, IgG2a, IgG2c) (Bethyl Laboratories Inc., Montgomery, TX, USA) or secondary HRP-conjugated goat anti- mouse IgA antibody (1:2000) in 2% FCS-PBS (Southern Biotech, Birmingham, AL, USA). Plates were then washed 5 times in PBS and developed for 12 minutes with 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) one component HRP microwell substrate (Surmodics IVD, Eden Prairie, MN, USA) for evaluating serum antibody responses, or developed for 6 minutes for evaluating fecal or saliva antibody responses. The reaction was stopped by the addition of 100 µL of 450 nm liquid stop solution (Surmodics IVD). Plates were then read on a microplate reader (Bio-Rad iMark) at 450 nm.

Preparation, maturation and characterization of bone marrow-derived DCs

Bone marrow-derived DCs were prepared as described previously [231]. Briefly, mice were euthanized with an IP injection of euthansol. The tibia and femur were extracted from the carcass, cleaned of all muscle and connective tissue, and then soaked in 70% ethanol for 5 minutes followed by a rinse in PBS. The end of each bone was cut and bone marrow was flushed with PBS from the tibia and femur using a syringe mounted to a 27 gauge needle. Each bone was

flushed from each side with 10 mL of PBS. The bone marrow was then treated for 10 minutes with ammonium chloride buffer to lyse residual red blood cells. Cells were then washed twice in complete RPMI media containing 10% heat-inactivated FCS, 500 U/mL penicillin (Pharmaceutical Partners of Canada, Richmond Hill, ON, Canada), 24 μg/mL gentamicin (Sandoz, Boucherville, QC, Canada), and 50 μM 2-mercaptoethanol (Sigma-Aldrich). Cells were then counted, and seeded at a density of $2x10^6$ cells/mL for 24 hours in 6-well plates (Beckton Dickinson, Mississauga, ON, Canada). The next day, non-adherent cells were collected, counted and re-seeded at a density of $1x10^6$ cells/mL in complete RPMI containing 10 ng/mL recombinant granulocyte-macrophage colony stimulating factor (GM-CSF) (Invitrogen, Carlsbad, CA, USA) and 10 ng/mL recombinant interleukin IL-4 (Invitrogen, Carlsbad, CA, USA). The cells were then plated in 12-well tissue culture plates over 6 days. Two-thirds of the cell culture medium was replaced on day 3 with fresh medium containing GM-CSF and IL-4. On day 5 two-thirds of the cell culture medium was replaced with fresh medium containing GM-CSF and IL-4, and cultures were stimulated with indicated doses of Dukoral vaccine components. After 24 hours, 700 μL of the cell culture supernatant was collected for cytokine analysis and frozen at -80^{o}C until analysed by cytokine array. Loosely adherent cells were then harvested, washed in 2% FCS-PBS, and 100,000 cells were counted and prepared for phenotypic analysis. The cells were blocked with 100 μL of 10 μg/mL of Mouse BD Fc block (BD Pharmingen, San Diego, CA, USA) for 20 minutes at room temperature (RT), in the dark. The cells were then washed again and stained at RT in the dark for 25 minutes with anti-mouse CD11c (eBioscience, San Diego, CA, USA), CD80, CD86, CD40, and MHC II antibodies (BD Pharmingen). After staining, cells were washed twice with 3 mL of 2% FCS-PBS and then fluorescent staining was evaluated by flow cytometry on a BD FACSCanto through the

collection of 10,000 forward scatter/ side-scatter (FSC/SSC) events. Results were analyzed using Cyflogic flow cytometry analysis software (www.cyflogic.com). Surface marker expression was evaluated as the mean fluorescent intensity (MFI) of $CD11c^+$ cells within the FSC/SSC gate.

IFN-γ and IL-4 ELISPOT assay

IFN-γ and IL-4 ELISPOT assays were performed by using an ELISPOT assay kit according to the manufacturer's instruction (Mabtech, Cincinnati, OH, USA). Briefly, 96-well $MultiScreen_{HTS}$ 0.45 μM filter plates (EMD Millipore, Toronto, ON, Canada) were treated with 15 μL of 35% ethanol for 1 minute to activate the PVDF membrane, under sterile conditions. The plates were washed 5 times with sterile PBS, 200 μL/well, following which 100 μL of 10 μg/mL coating antibody was added to the plates. The plates were incubated overnight at 4^oC. The next day, under sterile conditions, excess coating antibody was removed and the plates were washed 5 times with sterile PBS. The plates were then blocked for 30-60 minutes at room temperature with 200 μL/well of complete RPMI media. After blocking, the plates were decanted and mouse splenocytes were seeded in triplicate at 250,000 cells/well in a 200 μL final volume. Cells were stimulated with either a 2000:1 ratio of *V. cholerae*, 10 μg CTB, or 5 μg/mL of concanavalin A as the positive control (Sigma). The plates were then incubated for 72 hours at 37^oC and 5% CO_2 in a humidified chamber. After 72 hours, the plates were decanted to remove cells, washed 5 times with PBS, and incubated for 2 hours at room temperature with 100 μL/well of 1 μg/mL biotin detection antibody, diluted in 0.5% FCS-PBS. After washing the plates 5 times with PBS, plates were incubated for 1 hour at room temperature with streptavidin-alkaline phosphatise (ALP) antibody (diluted 1:1000). The plates were again washed 5 times with PBS and developed for 30 minutes with 100 μL/well of 5-bromo-4-chloro-3'-indolyphosphate p-

toluidine salt/nitro-blue tetrazolium chloride (BCIP/NBT) substrate. Colour development was stopped by extensively washing with distilled water. Spots were counted by eye using a dissection microscope.

CFSE proliferation assay

Proliferation of mouse splenocytes was evaluated by a carboxyfluorescein succinimidyl ester (CFSE) dilution assay. Splenocytes from orally-immunized mice were labelled with 5 μM CFSE (Sigma) as previously described [232], washed 3 times with 5% FCS-PBS, and re-suspended in complete RPMI media at 250,000 cells/mL followed by stimulation with vaccine components for 5 days. 10 μg/mL of concanavalin A (Sigma) was used as a positive control for inducing splenocyte proliferation. After 5 days, cells were washed in 2% FCS-PBS, stained for CD4 or CD19 markers (BD Pharmingen) and evaluated for CFSE dilution by flow cytometry.

Agglutination assay

The agglutination assay was performed by Dr Helen Tabor and Morganne Jerome at the National Microbiology Laboratory (Winnipeg, MB, Canada) as per the following protocol: *Vibrio cholerae* strains were pulled from frozen stocks, plated to nutrient agar and incubated at 37^{o}C overnight. A small amount of culture was then emulsified in one drop of PBS on a glass slide. One drop of serum (or PBS for negative control) was then added to the drop of live culture and the slide rocked gentle for 1 minute. Agglutination (or lack thereof) was observed by eye and graded from 1-4+. Negative (-): no agglutination, fluid is cloudy. 1+: approximately 25% agglutination, fluid is still cloudy. 2+: approximately 50% agglutination, fluid is moderately cloudy. 3+: approximately 75% agglutination, fluid is slightly cloudy. 4+: approximately 100%

agglutination, fluid is clear. Strains were tested with antisera on a slide prior to use and also tested with PBS alone to evaluate for any autoagglutination.

Cytokine array

Cell culture supernatants from DC cultures were assayed for cytokine production using a 13plex mouse Th1/Th2/Th17/Th22 FlowCytomix kit (eBioscience) according to the manufacturer's instructions. Standard curves for each cytokine (IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-10, IL-13, IL-17, IL-21, IL-22, IL-27 and TNF-α) were generated by using the reference cytokines supplied by the manufacturer. Data was collected by acquiring 3900 events, gating on the smallest of two bead populations. Data from the cytokine array was analyzed according to the supplied instructions using FlowCytomixPro3.0 software.

Statistical analysis

Statistical analysis was performed with unpaired, 2-tailed t-tests utilizing GraphPad Prism v5.0. P-values of $p \leq 0.05$ were considered as significant.

CHAPTER III: RESULTS

3.1 Optimization of the Dukoral vaccine dose for oral immunization

To determine the optimal oral dose of Dukoral vaccine, Balb/C and C57BL/6 WT mice were orally immunized with $3x10^8$ (Group 1), $3x10^9$ (Group 2), or $3x10^{10}$ (Group 3) *V. cholerae* along with 10 μg CTB, on days 0, 10, 20 and 30. Serum was collected pre-immunization and 9 days after each immunization.

Mice were tested for the generation of *V. cholerae*- and CTB-specific antibodies in serum, feces, and saliva, by ELISA. Low levels of *V. cholerae*-specific IgG1 and IgG2c/IgG2a antibodies were detected after one immunization (9 days after the first vaccination), in both C57BL/6 and Balb/C mice. However, the levels of *V. cholerae*-specific antibodies gradually increased after subsequent immunizations. After four oral immunizations, C57BL/6 and Balb/C mice receiving $3x10^9$ and $3x10^{10}$ *V. cholerae* with 10 μg CTB showed the highest post-vaccination *V. cholerae*-specific serum IgG1 and IgG2c/IgG2a antibodies (Figure 2 and 3). Similar results were seen for CTB-specific serum IgG1 and IgG2c/IgG2a antibody responses (Figure 4 and 5).

V. cholerae and CTB-specific IgA antibodies in feces were measured pre-immunization and in feces collected after the 4^{th} oral immunization with Dukoral. Pre-immunization fecal IgA ELISA signal was subtracted the from the post-4^{th} vaccination fecal IgA ELISA signal. C57BL/6 mice receiving $3x10^9$ *V. cholerae* and 10 μg CTB (Group 2) showed the highest post-vaccination *V. cholerae*-specific and CTB-specific fecal IgA responses (Figure 6), while Balb/C mice receiving $3x10^9$ and $3x10^{10}$ *V. cholerae* and 10 μg CTB (Group 3) showed the highest *V. cholerae*-specific and CTB-specific fecal IgA antibodies (Figure 7).

Figure 2. Optimization of Dukoral vaccine dose following oral immunization: Generation of *V. cholerae*-specific IgG1 antibodies. C57BL/6 mice (**A**), and Balb/C mice (**B**) were orally immunized with Dukoral on days 0 (P1V), 10 (P2V), 20 (P3V) and 30 (P4V) at the indicated doses, along with 10 μg CTB. Post-vaccination serum was collected 9 days after each vaccination, and *V. cholerae*-specific serum IgG1 was measured by ELISA. Results are shown as the mean O.D 450nm ± SEM. P0V indicates pre-immunization time point. P1V – P4V denotes post-first vaccination through post-fourth vaccination time points.

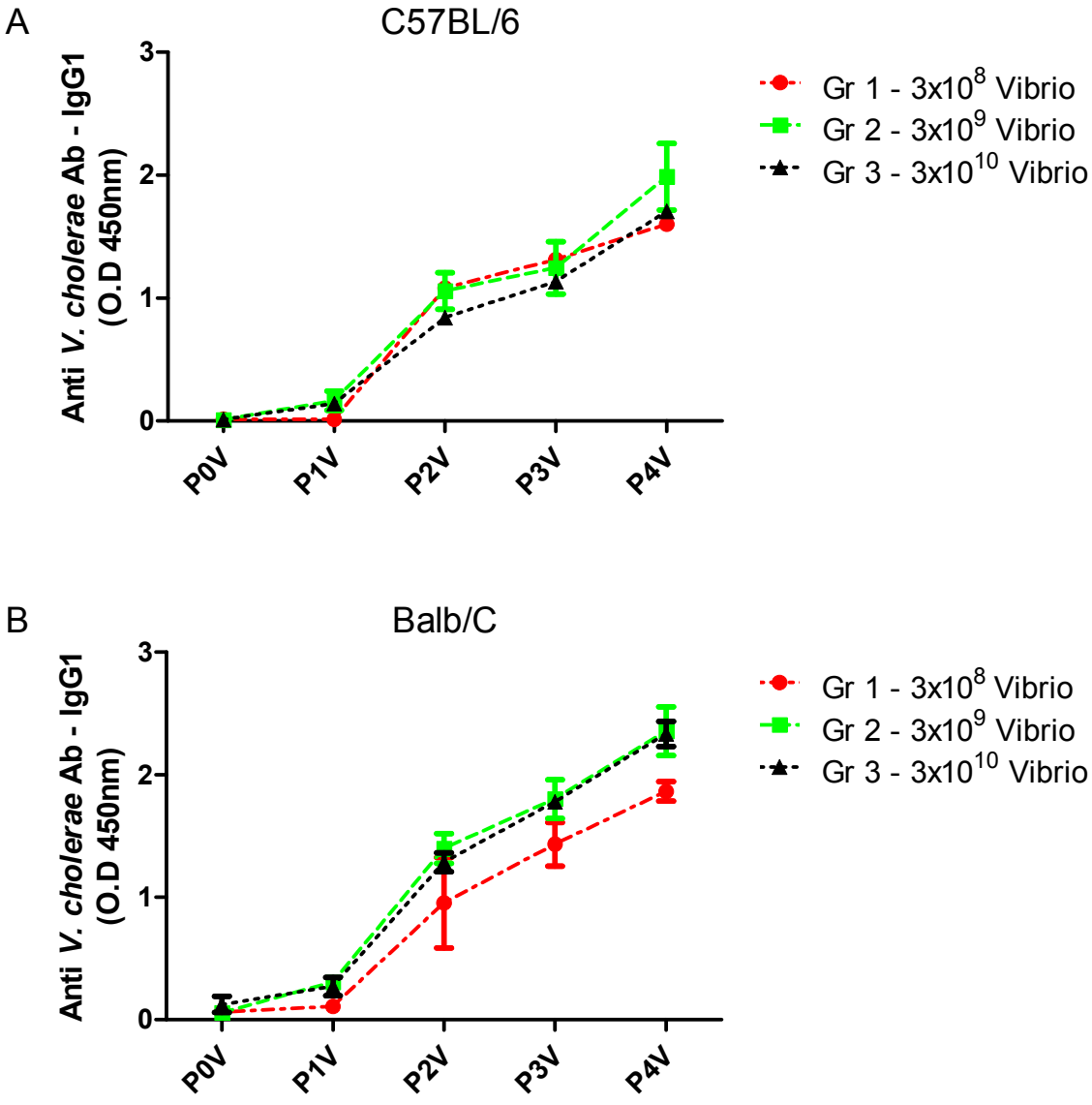

A
C57BL/6
Anti V. cholerae Ab - IgG1 (O.D 450nm)
3
2
1
0
P0V
P1V
P2V
P3V
P4V
Gr 1 - 3x10^8 Vibrio
Gr 2 - 3x10^9 Vibrio
Gr 3 - 3x10^10 Vibrio
B
Balb/C
Anti V. cholerae Ab - IgG1 (O.D 450nm)
3
2
1
0
P0V
P1V
P2V
P3V
P4V
Gr 1 - 3x10^8 Vibrio
Gr 2 - 3x10^9 Vibrio
Gr 3 - 3x10^10 Vibrio

Figure 3. Optimization of Dukoral vaccine dose following oral immunization: Generation of *V. cholerae*-specific IgG2a/c antibodies. C57BL/6 mice (A), and Balb/C mice (B) were orally immunized with Dukoral on days 0, 10, 20 and 30 at the indicated doses, along with 10 μg CTB. Post-vaccination serum was collected 9 days after each vaccination and *V. cholerae*-specific serum IgG2c (A) and IgG2a (B) was measured by ELISA. Results are shown as the mean O.D 450 nm ± SEM. P0V – P4V are as defined in the legend to Figure 2.

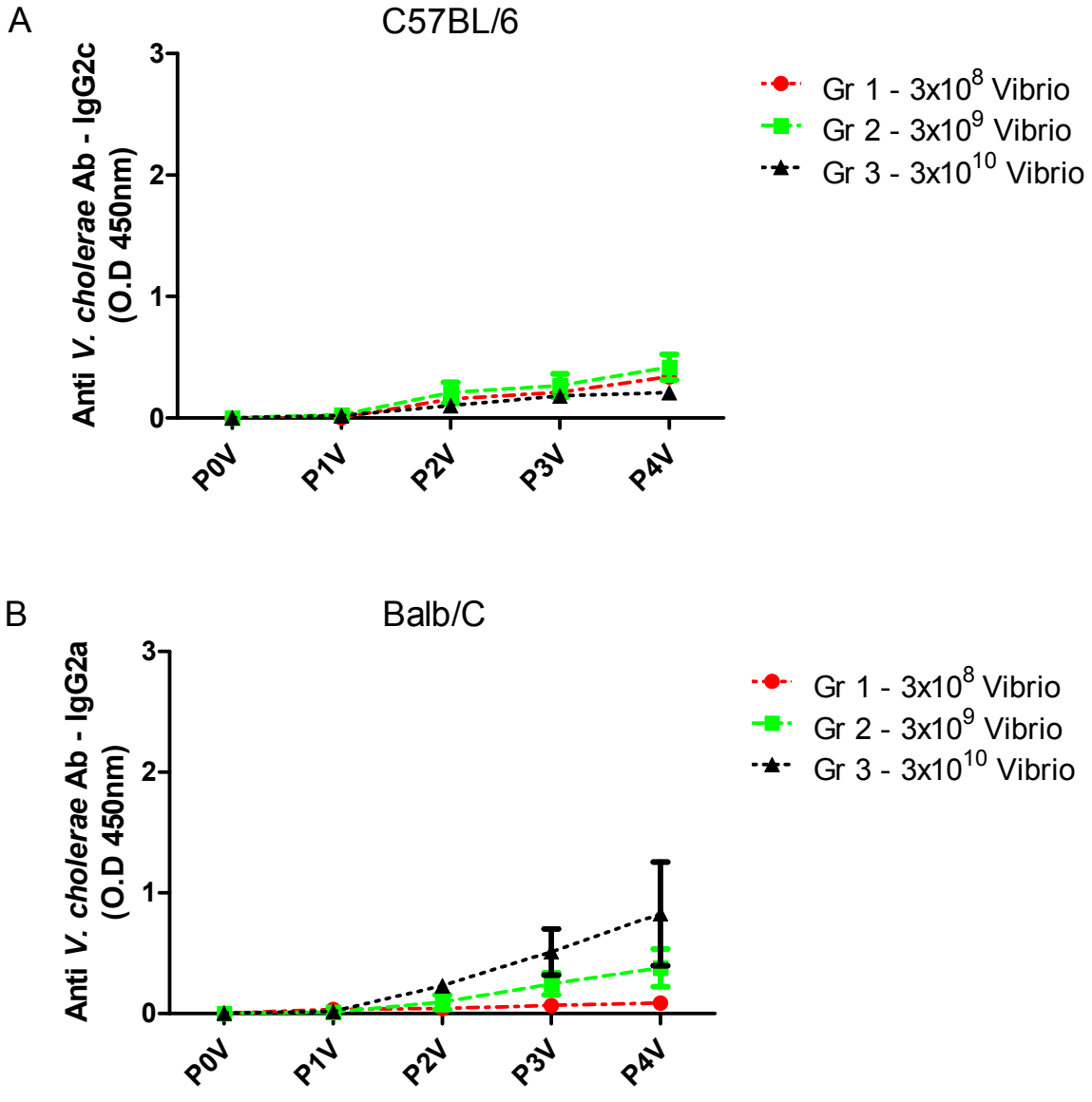
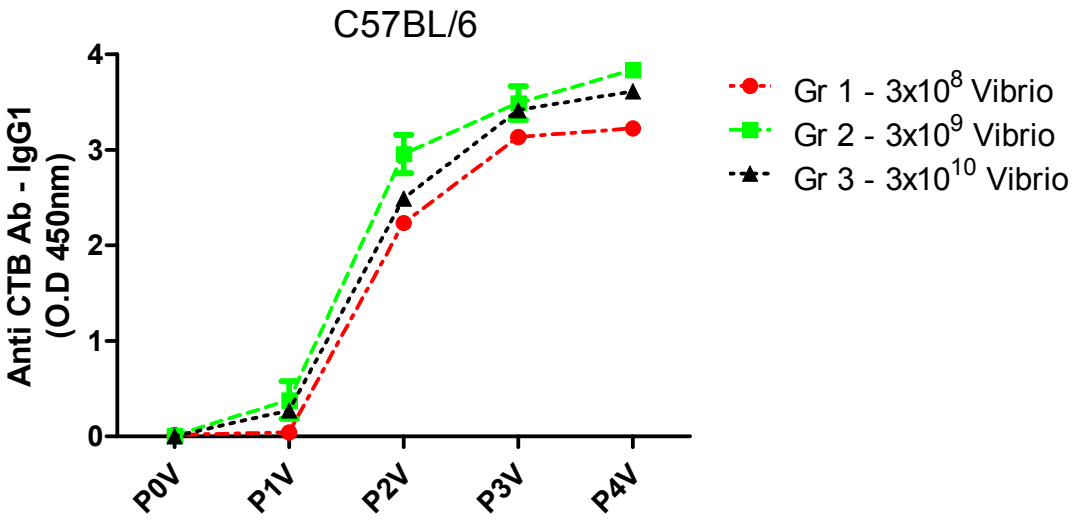

A
C57BL/6
Anti V. cholerae Ab - IgG2c (O.D 450nm)
3
2
1
0
P0V
P1V
P2V
P3V
P4V
Gr 1 - 3x10^8 Vibrio
Gr 2 - 3x10^9 Vibrio
Gr 3 - 3x10^10 Vibrio
B
Balb/C
Anti V. cholerae Ab - IgG2a (O.D 450nm)
3
2
1
0
P0V
P1V
P2V
P3V
P4V
Gr 1 - 3x10^8 Vibrio
Gr 2 - 3x10^9 Vibrio
Gr 3 - 3x10^10 Vibrio

Figure 4. Optimization of Dukoral vaccine dose following oral immunization: Generation of CTB-specific IgG1 antibodies. C57BL/6 mice (A), and Balb/C mice (B) were orally immunized with Dukoral on days 0, 10, 20 and 30 at the indicated doses, along with 10 μg CTB. Post-vaccination serum was collected 9 days after each vaccination, and CTB-specific serum IgG1 was measured by ELISA. Results are shown as the mean O.D 450 nm ± SEM. P0V – P4V are as defined in the legend to Figure 2.

A


C57BL/6
Anti CTB Ab - IgG1 (O.D 450nm)
0
1
2
3
4
P0V
P1V
P2V
P3V
P4V
Gr 1 - 3x10^8 Vibrio
Gr 2 - 3x10^9 Vibrio
Gr 3 - 3x10^10 Vibrio

B

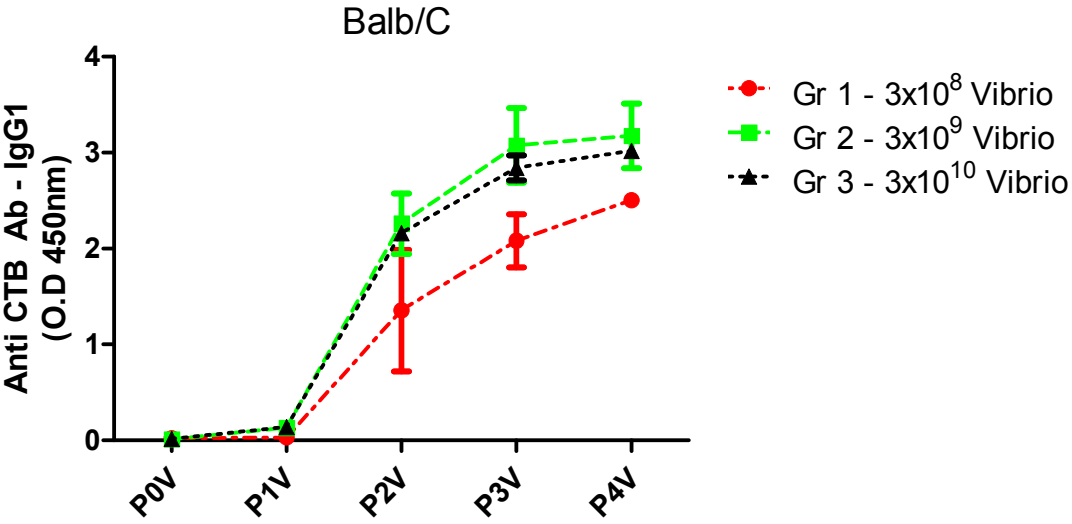

Balb/C
Anti CTB Ab - IgG1 (O.D 450nm)
0
1
2
3
4
P0V
P1V
P2V
P3V
P4V
Gr 1 - 3x10^8 Vibrio
Gr 2 - 3x10^9 Vibrio
Gr 3 - 3x10^10 Vibrio

Figure 5. Optimization of Dukoral vaccine dose following oral immunization: Generation of CTB-specific IgG2a/c antibodies. C57BL/6 mice (A), and Balb/C mice (B) were orally immunized with Dukoral on days 0, 10, 20 and 30 at the indicated doses, along with 10 μg CTB. Post-vaccination serum was collected 9 days after each vaccination and CTB-specific serum IgG2c (A) and IgG2a (B) was measured by ELISA. Results are shown as the mean O.D 450 nm ± SEM. P0V – P4V are as defined in the legend to Figure 2.

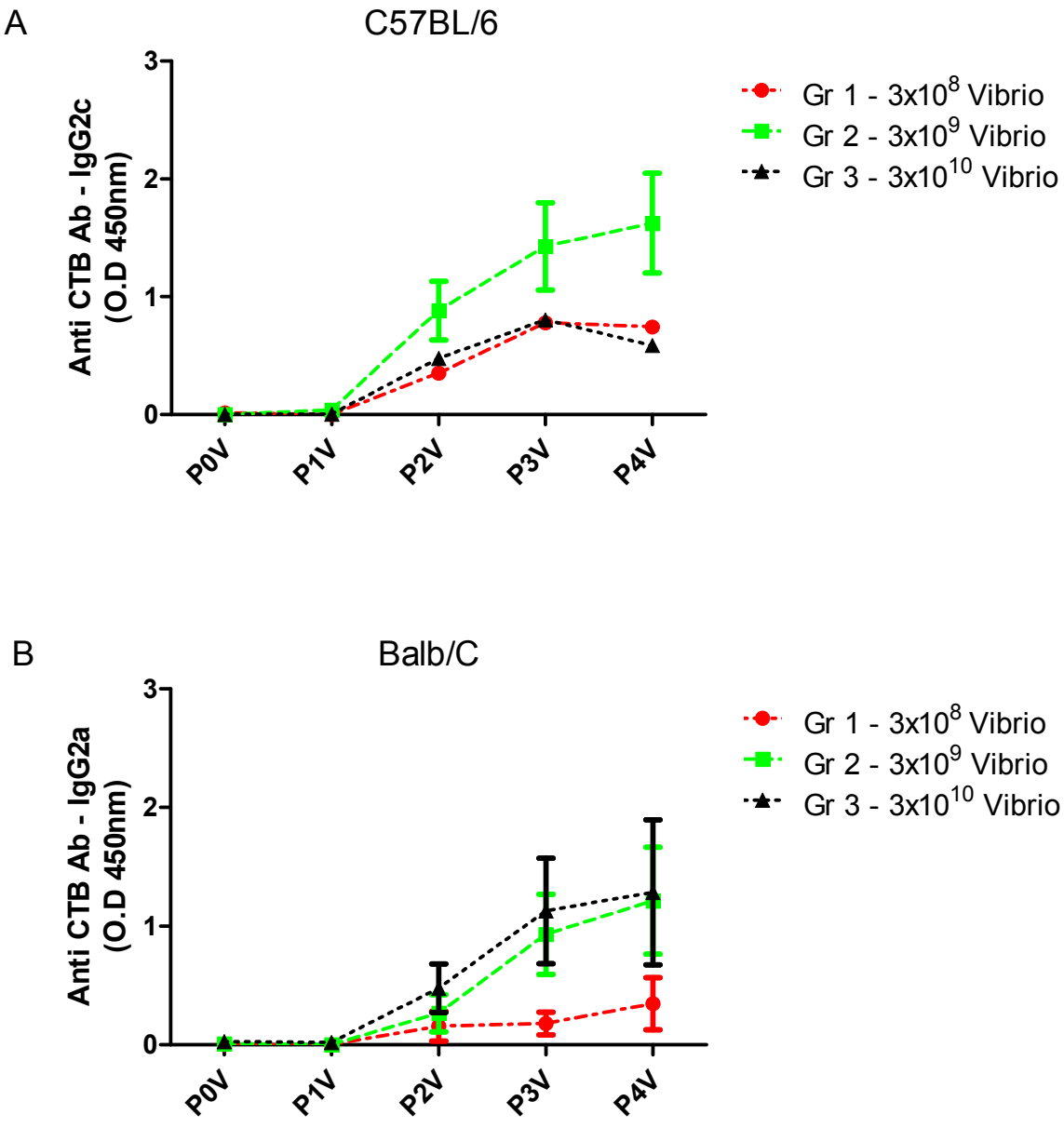
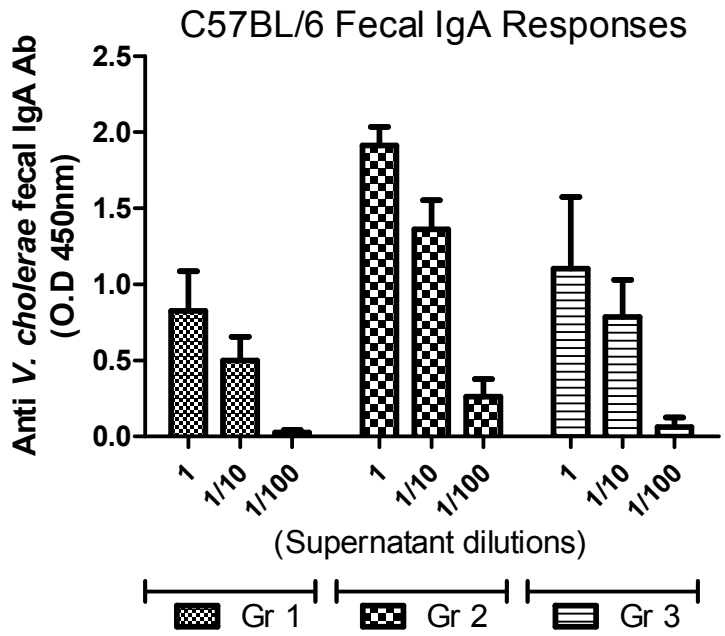

A
C57BL/6
Anti CTB Ab - IgG2c (O.D 450nm)
3
2
1
0
P0V
P1V
P2V
P3V
P4V
Gr 1 - 3x10^8 Vibrio
Gr 2 - 3x10^9 Vibrio
Gr 3 - 3x10^10 Vibrio
B
Balb/C
Anti CTB Ab - IgG2a (O.D 450nm)
3
2
1
0
P0V
P1V
P2V
P3V
P4V
Gr 1 - 3x10^8 Vibrio
Gr 2 - 3x10^9 Vibrio
Gr 3 - 3x10^10 Vibrio

Figure 6. Optimization of Dukoral vaccine dose folowing oral immunization: Generation of *V. cholerae*-specific (A) and CTB-specific (B) fecal IgA antibodies. C57BL/6 mice were orally immunized with $3x10^8$ (Group 1), $3x10^9$ (Group 2), or $3x10^{10}$ (Group 3) *V. cholerae* and 10 μg CTB, on days 0, 10, 20 and 30. Fecal pellets were collected pre-immunization and 9 days after the last immunization. Fecal supernatants were extracted as described in Materials and Methods, and *V. cholerae*-specific fecal IgA (A) and CTB-specific fecal IgA (B) antibodies were measured by ELISA at the indicated dilutions. Results shown are the post-4^{th} vaccination mean O.D 450 nm ± SEM, corrected for pre-vaccination background signal.

A


C57BL/6 Fecal IgA Responses
Anti V. cholerae fecal IgA Ab (O.D 450nm)
2.5
2.0
1.5
1.0
0.5
0.0
1
1/10
1/100
(Supernatant dilutions)
Gr 1
Gr 2
Gr 3

B

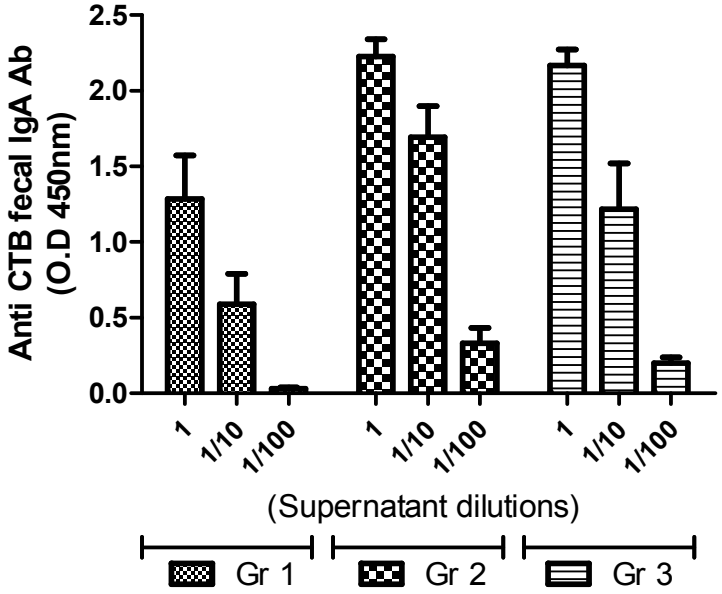
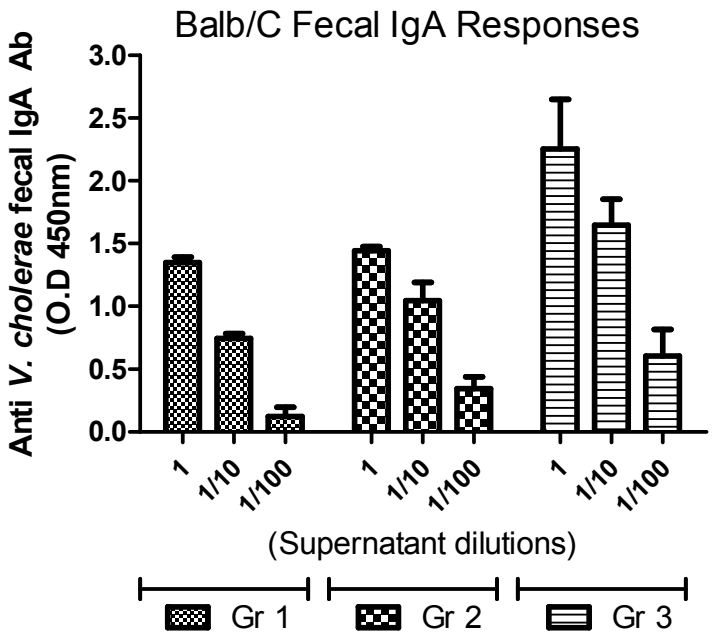
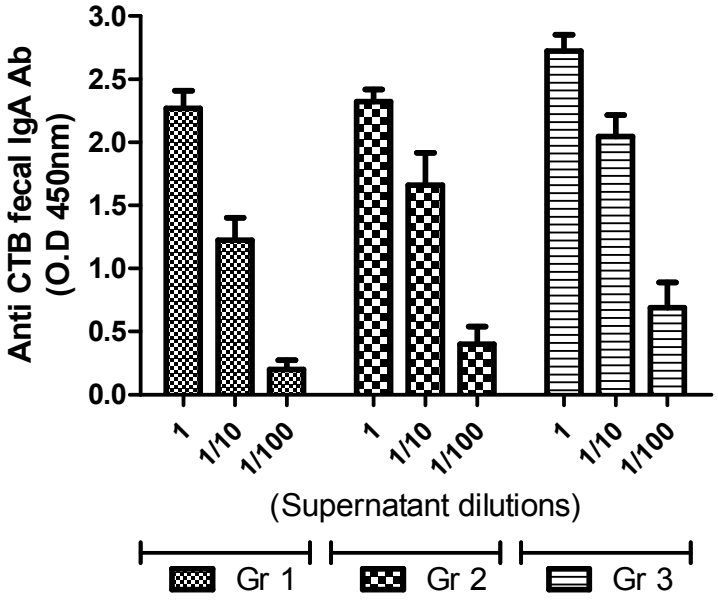

Anti CTB fecal IgA Ab (O.D 450nm)
2.5
2.0
1.5
1.0
0.5
0.0
1
1/10
1/100
(Supernatant dilutions)
Gr 1
Gr 2
Gr 3

Figure 7. Optimization of Dukoral vaccine dose following oral immunization: Generation of *V. cholerae*-specific (A) and CTB-specific (B) fecal IgA antibody production. Balb/C mice were orally immunized with $3x10^8$ (Group 1), $3x10^9$ (Group 2), or $3x10^{10}$ (Group 3) *V. cholerae* and 10 μg CTB, on days 0, 10, 20 and 30. Fecal pellets were collected pre-immunization and 9 days after the last immunization. Fecal supernatants were extracted as described in Materials and Methods, and *V. cholerae*-specific fecal IgA (A) and CTB-specific fecal IgA (B) antibodies were measured by ELISA at the indicated dilutions. Results shown are the post-4^{th} vaccination mean O.D 450 nm ± SEM, corrected for pre-vaccination background signal.

A


Balb/C Fecal IgA Responses
Anti V. cholerae fecal IgA Ab (O.D 450nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
1/10
1/100
(Supernatant dilutions)
Gr 1
Gr 2
Gr 3

B


Anti CTB fecal IgA Ab (O.D 450nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
1/10
1/100
(Supernatant dilutions)
Gr 1
Gr 2
Gr 3

Saliva was also tested for the presence of Dukoral vaccine-induced *V. cholerae*- and CTB-specific IgA antibodies. C57BL/6 and Balb/C mice orally immunized with different doses of Dukoral vaccine showed very weak induction of *V. cholerae* and CTB-specific saliva IgA (Figure 8 and 9). Since vaccine-specific saliva IgA antibodies were not generated to any significant extent, saliva was not collected in subsequent immunization studies.

In summary, C57BL/6 mice receiving $3x10^9$ *V. cholerae* with 10 μg CTB (Group 2 mice) showed the highest *V. cholerae*- and CTB-specific serum IgG and fecal IgA responses. Since this dose of vaccine resulted in the highest *V. cholerae*-specific serum and fecal antibody responses, four oral immunizations with $3x10^9$ *V. cholerae* and 10 μg CTB were found to be the optimal oral dose of Dukoral vaccine for C57BL/6 mice. Due to the fact that TLR mutant mice were available only on a C57BL/6 genetic background, Balb/C mice were not utilized for any further experiments.

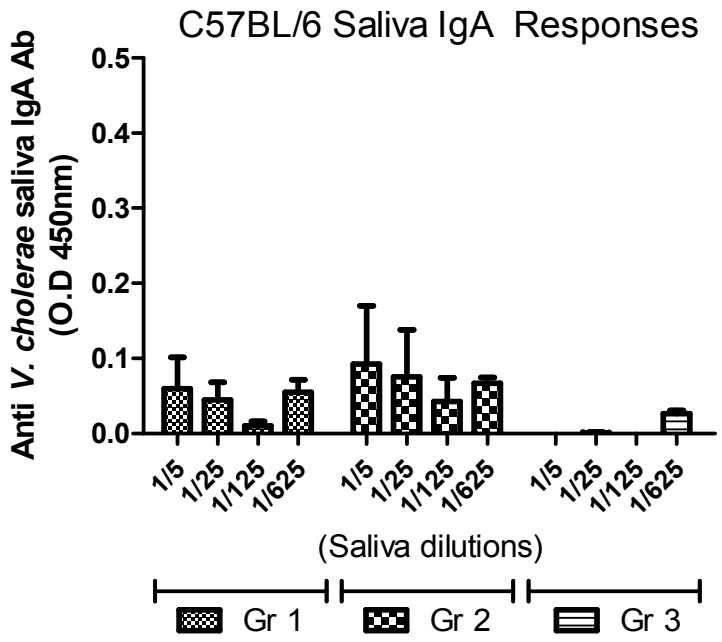
3.2 Immune responses against the *Vibrio cholerae* component of the Dukoral vaccine

3.2.1 CTB acts as an adjuvant for serum and fecal antibody responses following oral immunization with Dukoral

A study was undertaken to evaluate the ability of orally administered CTB to modulate *V. cholerae*-specific antibody responses in the serum and feces of mice immunized orally with the Dukoral vaccine, to determine if CTB acted as an adjuvant to enhance *V. cholerae*-specific responses. For this, mice were immunized orally with $3x10^9$ *V. cholerae* alone, or with the complete Dukoral vaccine ($3x10^9$ *V. cholerae* with 10 μg CTB). After vaccination on days 0, 10, 20 and 30, mice immunized with *V. cholerae* alone showed significantly reduced *V. cholerae*-specific serum total IgG ($p=0.0062$) compared to mice immunized with the complete Dukoral vaccine (Figure 10 A).

Figure 8. Optimization of Dukoral vaccine dose following oral immunization: Generation of *V. cholerae*-specific (A) and CTB-specific (B) saliva IgA antibodies. C57BL/6 mice were orally immunized with $3x10^8$ (Group 1), $3x10^9$ (Group 2), or $3x10^{10}$ (Group 3) *V. cholerae* and 10 μg CTB, on days 0, 10, 20 and 30. Saliva was collected pre-immunization and 9 days after the last immunization. Saliva production was induced by the administration of pilocarpine and saliva was collected by pipette. *V. cholerae*-specific saliva IgA (A) and CTB-specific saliva IgA (B) antibodies were measured by ELISA at the indicated dilutions. Results shown are the post-4^{th} vaccination mean O.D 450 nm ± SEM, corrected for pre-vaccination background signal.

A


C57BL/6 Saliva IgA Responses
Anti V. cholerae saliva IgA Ab (O.D 450nm)
0.5
0.4
0.3
0.2
0.1
0.0
1/5
1/25
1/125
1/625
(Saliva dilutions)
Gr 1
Gr 2
Gr 3

B

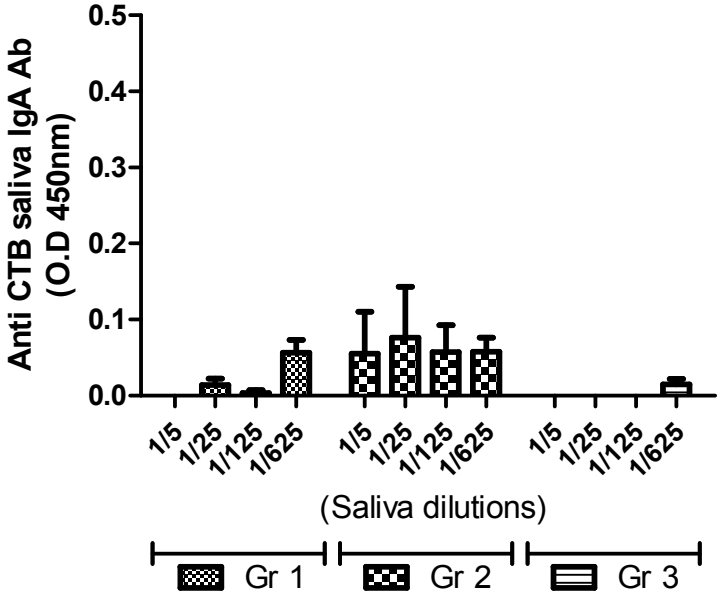
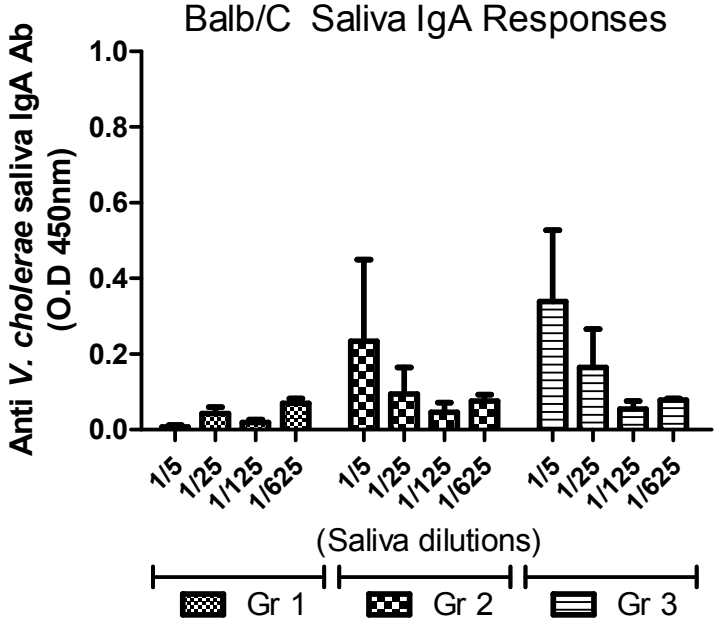

Anti CTB saliva IgA Ab (O.D 450nm)
0.5
0.4
0.3
0.2
0.1
0.0
1/5
1/25
1/125
1/625
(Saliva dilutions)
Gr 1
Gr 2
Gr 3

Figure 9. Optimization of Dukoral vaccine dose following oral immunization: Generation of *V. cholerae*-specific (A) and CTB-specific (B) saliva IgA antibodies. Balb/C mice were orally immunized with $3x10^8$ (Group 1), $3x10^9$ (Group 2), or $3x10^{10}$ (Group 3) *V. cholerae* and 10 μg CTB, on days 0, 10, 20 and 30. Saliva was collected pre-immunization and 9 days after the last immunization. Saliva production was induced by the administration of pilocarpine and saliva was collected by pipette. *V. cholerae*-specific saliva IgA (A) and CTB-specific saliva IgA (B) antibodies were measured by ELISA at the indicated dilutions Results shown are the post-4^{th} vaccination mean O.D 450 nm ± SEM, corrected for pre-vaccination background signal.

A


Balb/C Saliva IgA Responses
Anti V. cholerae saliva IgA Ab (O.D 450nm)
1.0
0.8
0.6
0.4
0.2
0.0
1/5
1/25
1/125
1/625
(Saliva dilutions)
Gr 1
Gr 2
Gr 3

B

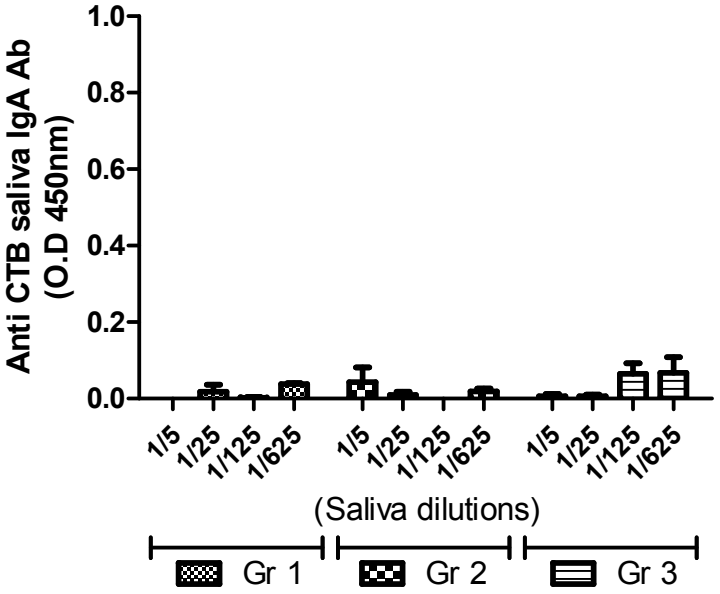
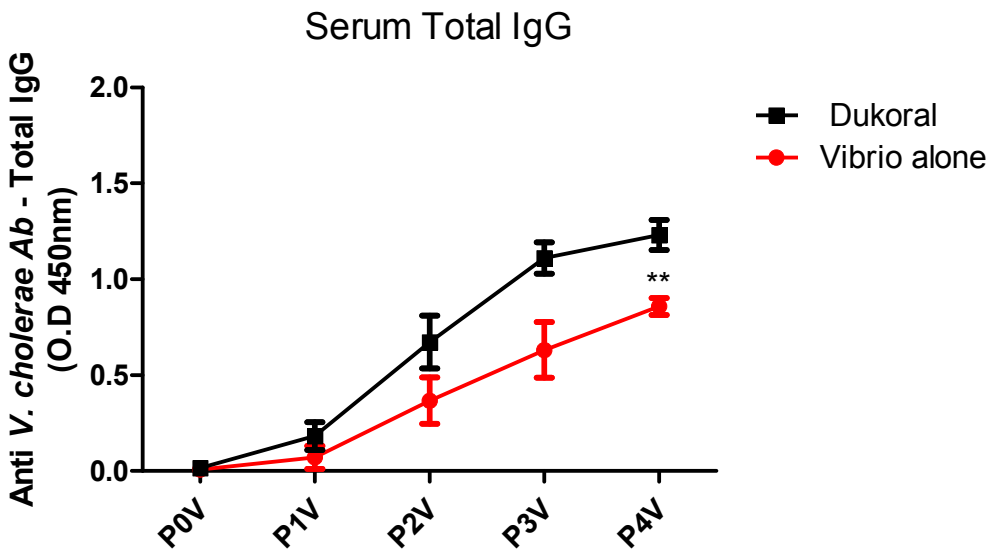
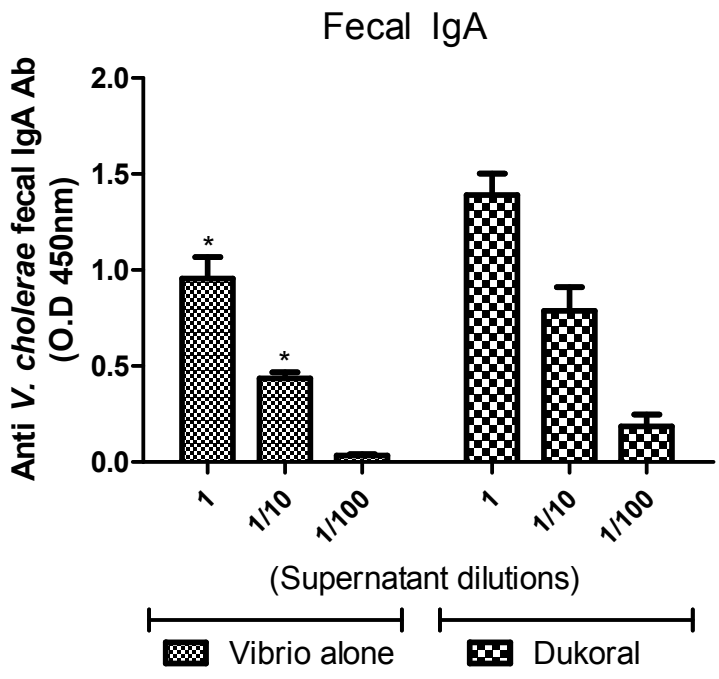

Anti CTB saliva IgA Ab (O.D 450nm)
1.0
0.8
0.6
0.4
0.2
0.0
1/5
1/25
1/125
1/625
(Saliva dilutions)
Gr 1
Gr 2
Gr 3

Figure 10. Orally administered CTB enhanced *V. cholerae*-specific serum IgG (A) and fecal IgA antibody responses (B) following oral immunization with Dukoral. C57BL/6 mice were immunized orally on days 0, 10, 20 and 30 with either $3x10^9$ *V. cholerae* alone or Dukoral ($3x10^9$ *V. cholerae* with 10 μg CTB). Serum was collected 9 days after each vaccination and *V. cholerae*-specific serum total IgG (A) was measured by ELISA. Results are shown as the mean O.D 450 nm ± SEM. Feces was collected pre-vaccination and 9 days after the last vaccination. Fecal supernatants were extracted as described in Materials and Methods and *V. cholerae*-specific fecal IgA (B) antibodies were measured by ELISA at the indicated dilutions. Fecal results shown are the post-4^{th} vaccination mean O.D 450 nm ± SEM, corrected for pre-vaccination background signal.* $p<0.05$, ** $p<0.01$.

A


Serum Total IgG
Anti V. cholerae Ab - Total IgG (O.D 450nm)
2.0
1.5
1.0
0.5
0.0
P0V
P1V
P2V
P3V
P4V
**
Dukoral
Vibrio alone

B


Fecal IgA
Anti V. cholerae fecal IgA Ab (O.D 450nm)
2.0
1.5
1.0
0.5
0.0
*
*
1
1/10
1/100
1
1/10
1/100
(Supernatant dilutions)
Vibrio alone
Dukoral

Similarly, mice immunized with *V. cholerae* alone showed significantly reduced *V. cholerae*-specific fecal IgA when tested undiluted (p=0.0304) and at a 1/10 dilution (p=0.0442), compared to mice immunized with the complete Dukoral vaccine (Figure 10 B). These results suggest that CTB exhibits adjuvant properties, as it enhanced the induction of *V. cholerae*-specific serum IgG as well as fecal IgA antibodies.

3.2.2 MyD88, Trif, TLR-2 and TLR-4 signaling was dispensable for the production of *V. cholerae*-specific serum IgG and fecal IgA antibodies following oral immunization with Dukoral

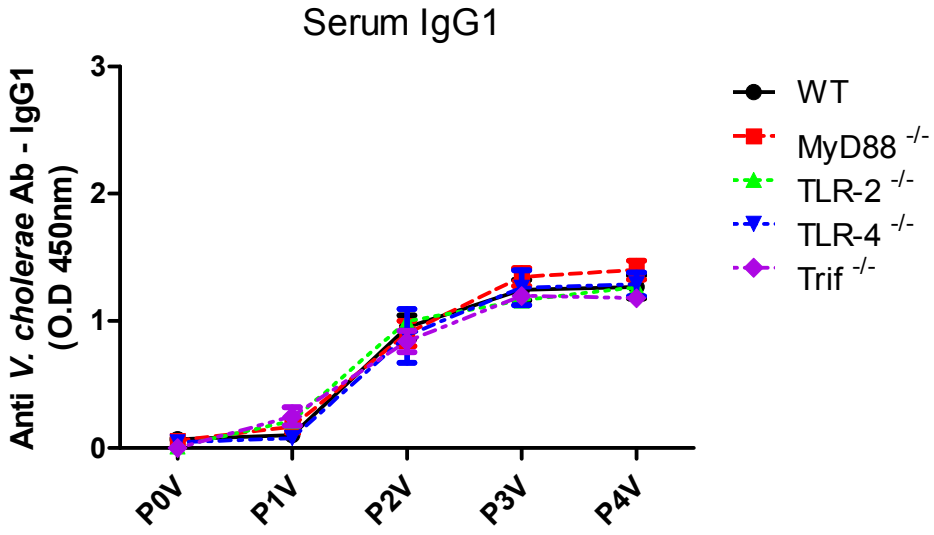
To determine the involvement of TLR signaling in the generation of humoral and cellular immune responses following oral immunization with Dukoral, MyD88$^{-/-}$, Trif$^{-/-}$, TLR-2$^{-/-}$, and TLR-4$^{-/-}$ animals were immunized and serum and mucosal humoral responses (serum IgG and fecal IgA) as well as peripheral cellular immune responses were evaluated.

After four oral immunizations, MyD88$^{-/-}$, Trif$^{-/-}$, TLR-2$^{-/-}$, and TLR-4$^{-/-}$ mice did not show any significant impairment in the generation of *V. cholerae*-specific serum IgG1 and IgG2c antibodies (Figure 11) compared to those generated in WT mice, at any time following immunization.

Similar to serum IgG antibody responses, MyD88$^{-/-}$, Trif$^{-/-}$, TLR-2$^{-/-}$, and TLR-4$^{-/-}$ mice did not show significant impairment of *V. cholerae*-specific fecal IgA antibody production (Figure 12). These findings suggest that TLR signaling is not critically required for the production of either *V. cholerae*- specific serum IgG1 and IgG2c antibodies, and *V. cholerae*-specific fecal IgA antibodies, at any time, following immunization with Dukoral.

Figure 11. TLR signaling was dispensable for *V. cholerae*-specific serum IgG1 (A) and IgG2c (B) antibody production following oral immunization with Dukoral. TLR mutant mice (TLR-2$^{-/-}$, TLR-4$^{-/-}$, MyD88$^{-/-}$ and Trif$^{-/-}$) were immunized orally on days 0, 10, 20 and 30 with Dukoral ($3x10^9$ *V. cholerae* with 10 μg CTB). Serum was collected 9 days after each vaccination and *V. cholerae*-specific serum IgG1 (A) and IgG2c (B) antibodies were measured by ELISA. Results are shown as the mean O.D 450 nm ± SEM.

A


Serum IgG1
Anti V. cholerae Ab - IgG1 (O.D 450nm)
3
2
1
0
P0V
P1V
P2V
P3V
P4V
WT
MyD88 -/-
TLR-2 -/-
TLR-4 -/-
Trif -/-

B

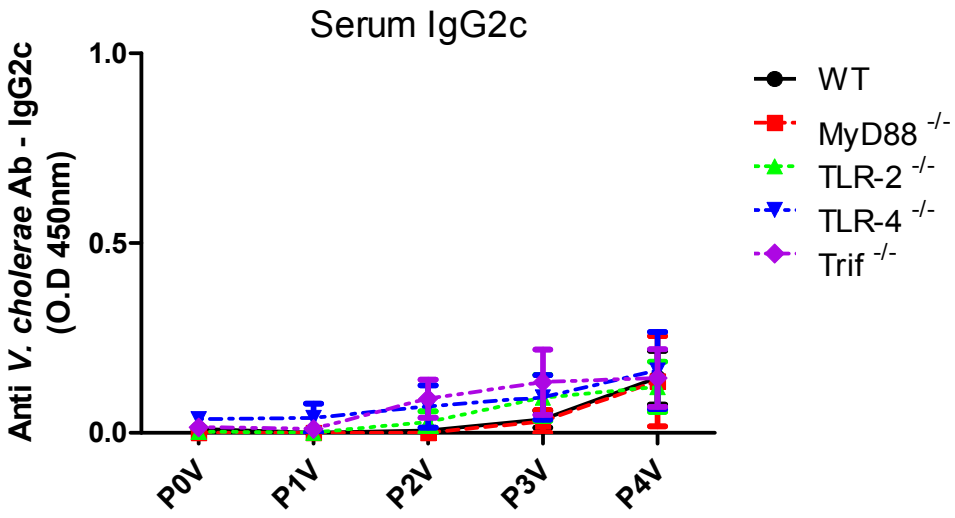
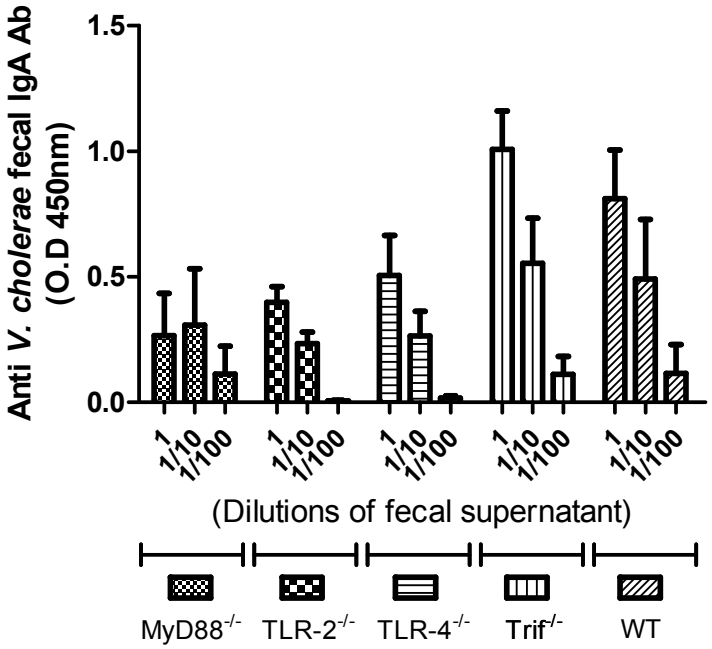

Serum IgG2c
Anti V. cholerae Ab - IgG2c (O.D 450nm)
1.0
0.5
0.0
P0V
P1V
P2V
P3V
P4V
WT
MyD88 -/-
TLR-2 -/-
TLR-4 -/-
Trif -/-

Figure 12. Generation of *V. cholerae*-specific fecal IgA antibodies did not involve TLR signaling following oral immunization with Dukoral. TLR mutant mice (TLR-2$^{-/-}$, TLR-4$^{-/-}$, MyD88$^{-/-}$ and Trif$^{-/-}$) were immunized orally on days 0, 10, 20 and 30 with Dukoral ($3x10^9$ *V. cholerae* with 10 μg CTB). Fecal pellets were collected pre-vaccination and 9 days after the last vaccination and fecal supernatants were extracted as described in Materials and Methods. *V. cholerae*-specific fecal IgA antibodies were measured by ELISA at the indicated dilutions (1, undiluted, 1/10, 10-fold diluted, 1/100, 100-fold diluted). Results shown are the post-4th vaccination mean O.D 450 nm ± SEM, corrected for pre-vaccination background signal.


Anti V. cholerae fecal IgA Ab
(O.D 450nm)
1.5
1.0
0.5
0.0
1
1/10
1/100
(Dilutions of fecal supernatant)
MyD88-/-
TLR-2-/-
TLR-4-/-
Trif-/-
WT

3.2.3 MyD88 signaling was dispensable for *V. cholerae*-specific antibody production following intramuscular immunization with Dukoral

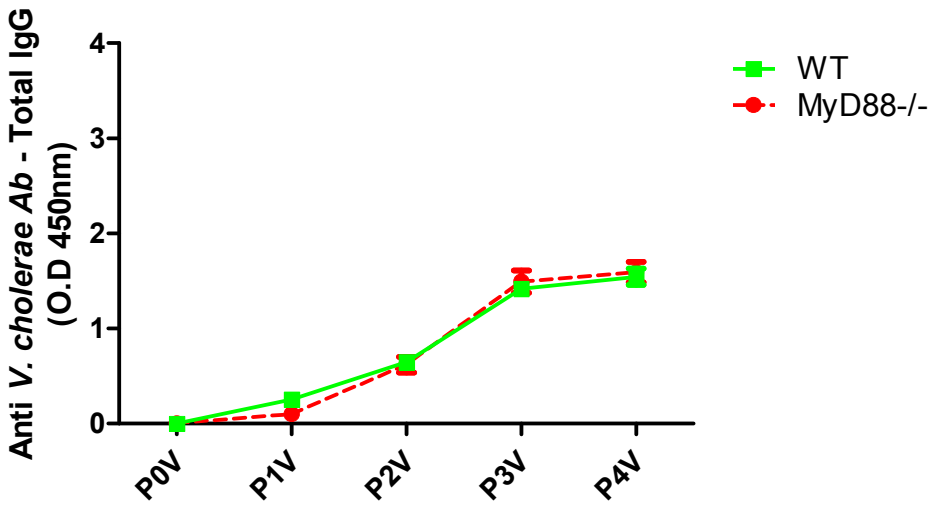
In light of the results which showed TLR signaling to be dispensable for the generation of *V. cholerae*-specific serum IgG and fecal IgA antibody production following oral immunization, I wished to extend these observations and determine if TLR signaling was important for serum antibody production following IM immunization with Dukoral. After four intramuscular immunizations, the results showed that MyD88$^{-/-}$ mice did not have significantly different *V. cholerae*-specific serum total IgG, IgG1, or IgG2c responses compared to WT mice (Figure 13). These results suggest that TLR signaling is dispensable for *V. cholerae*-specific serum antibody production following both oral and intramuscular immunization with the Dukoral vaccine.

3.2.4 Intramuscular immunization with Dukoral induced a higher titer of agglutinating antibodies compared to oral immunization

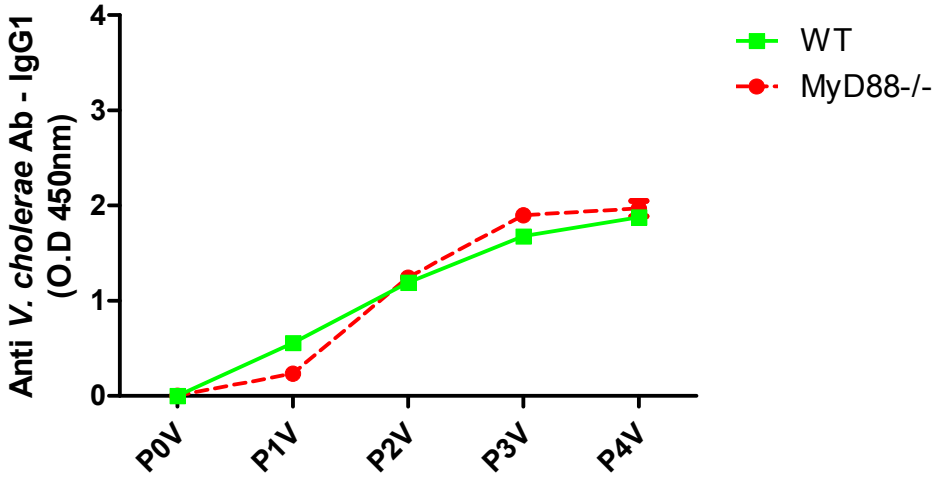
Serum from orally and intramuscularly immunized mice was subsequently tested for its ability to agglutinate live *V. cholerae* (Figure 14). The agglutination assay was performed at the Winnipeg National Microbiology Laboratory by Dr Helen Tabor and Morganne Jerome. Serum from WT and MyD88$^{-/-}$ mice intramuscularly immunized with Dukoral demonstrated agglutinating titers up to a 1/64 dilution of serum. Conversely, serum from WT and MyD88$^{-/-}$ mice orally immunized with Dukoral only demonstrated agglutinating titers up to a 1/4 dilution of serum. Taken together, these results suggest that intramuscular immunization induced

Figure 13. TLR signaling was dispensable for *V. cholerae*-specific serum antibody production following intramuscular immunization with Dukoral. MyD88$^{-/-}$ and WT mice were immunized intramuscularly on days 0, 10, 20 and 30 with Dukoral ($1x10^7$ *V. cholerae* with 10 μg CTB). Serum was collected 9 days after each vaccination and *V. cholerae*-specific total serum IgG (A) IgG1 (B) and IgG2c (C) responses were measured by ELISA. Results are shown as the mean O.D 450 nm ± SEM.

A


Anti V. cholerae Ab - Total IgG
(O.D 450nm)
4
3
2
1
0
P0V
P1V
P2V
P3V
P4V
WT
MyD88-/-

B


Anti V. cholerae Ab - IgG1
(O.D 450nm)
4
3
2
1
0
P0V
P1V
P2V
P3V
P4V
WT
MyD88-/-

C

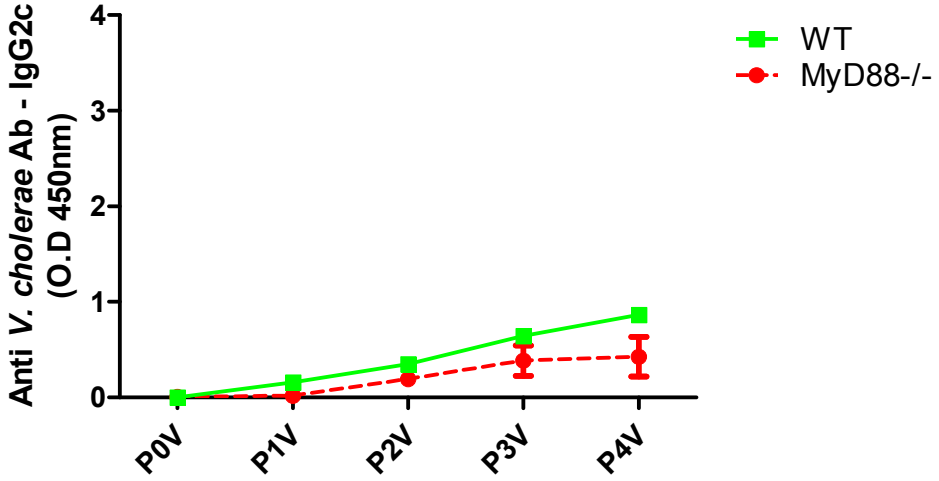
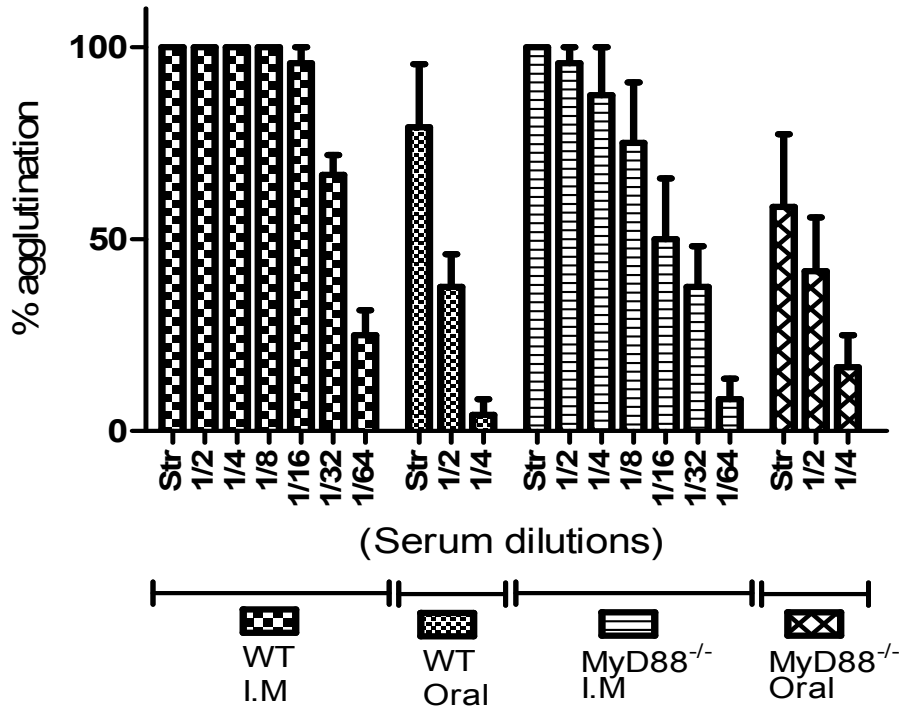

Anti V. cholerae Ab - IgG2c
(O.D 450nm)
4
3
2
1
0
P0V
P1V
P2V
P3V
P4V
WT
MyD88-/-

Figure 14. Oral immunization with Dukoral induced decreased titers of *V. cholerae*-agglutinating antibodies compared to intramuscular immunization. MyD88$^{-/-}$ and WT mice were immunized intramuscularly with Dukoral ($1x10^7$ *V. cholerae* with 10 μg CTB) or orally ($3x10^9$ *V. cholerae* with 10 μg CTB) on days 0, 10, 20, and 30, and blood was collected by cardiac puncture at the end of the study. Pooled serum was tested by a slide agglutination assay against a panel of six *V. cholerae* strains representing those found in the Dukoral vaccine (Inaba, Ogawa, and Ogawa El Tor trains), and agglutination was quantified by microscopic observation according to an established scale, as described in Materials and Methods. Results are shown as the mean ± SEM.

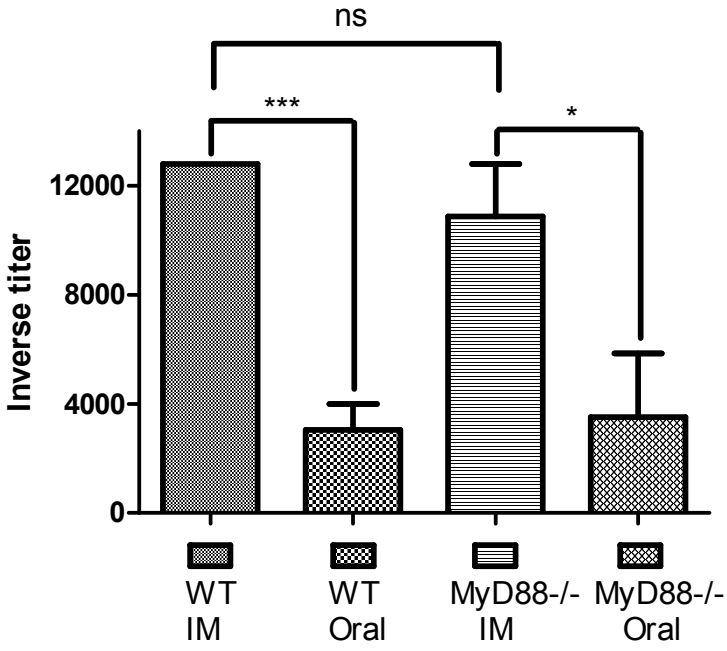

% agglutination
100
50
0
Str
1/2
1/4
1/8
1/16
1/32
1/64
(Serum dilutions)
WT
I.M
WT
Oral
MyD88-/-
I.M
MyD88-/-
Oral

significantly higher titers of *V. cholerae*-agglutinating antibodies compared to oral immunization, and that the agglutinating activity of *V. cholerae*-specific antibodies was able to occur independently of MyD88 signaling.

3.2.5 Intramuscular immunization with Dukoral generated a greater quantity of *V. cholerae*-specific serum total IgG antibodies compared to oral immunization

In order to find an explanation for why intramuscular immunization with Dukoral induced a higher titer of agglutinating antibodies compared to oral immunization, an endpoint titer ELISA was undertaken. The purpose of this *V. cholerae*-specific endpoint ELISA was to determine whether intramuscular immunization with Dukoral induced a greater quantity of *V. cholerae*-specific serum total IgG antibodies compared to oral immunization with Dukoral. To achieve this, serum from WT mice and MyD88$^{-/-}$ mice intramuscularly and orally immunized with Dukoral was collected at the end of the experiment and was serially diluted and assayed in a *V. cholera*e-specific ELISA. *V. cholerae*-specific serum total IgG antibody titers were taken as the highest dilution of serum resulting in an O.D 450 nm of ≥0.3 (twice the background signal of the assay). The results showed that WT mice orally immunized with Dukoral had a significantly decreased titer of *V. cholerae*-specific serum total IgG antibodies ($p<0.0001$), compared to WT mice intramuscularly immunized with Dukoral (Figure 15). Similarly, MyD88$^{-/-}$ mice orally immunized with Dukoral also had a significantly decreased titer of *V. cholerae*-specific serum total IgG antibodies ($p=0.0406$), compared to MyD88$^{-/-}$ mice intramuscularly immunized with Dukoral (Figure 15). MyD88$^{-/-}$ mice intramuscularly immunized with Dukoral did not have a significantly different *V. cholerae*-specific serum total IgG antibody titer than their WT counterparts.

Figure 15. Intramuscular immunization with Dukoral induced significantly higher titers of *V. cholerae*-specific serum total IgG antibodies than oral immunization with Dukoral. MyD88$^{-/-}$ and WT mice were immunized either intramuscularly with Dukoral ($1x10^7$ *V. cholerae* with 10 μg CTB) or orally ($3x10^9$ *V. cholerae* with 10 μg CTB) on days 0, 10, 20, and 30, and blood was collected by cardiac puncture at the end of the study. Serum was serially diluted from 1/100 to 1/12,800 and *V. cholerae*-specific serum total IgG was measured by ELISA. Antibody titer was taken as the highest dilution of serum resulting in an O.D 450 nm of ≥0.3. Results are shown as the inverse titer ± SEM. *$p<0.05$, ***$p<0.001$.


ns
***
*
12000
8000
4000
0
Inverse titer
WT
IM
WT
Oral
MyD88-/-
IM
MyD88-/-
Oral

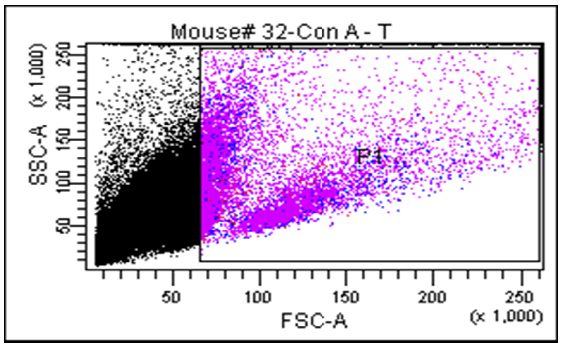
These results suggest that the decreased titer of agglutinating antibodies observed in WT and MyD88$^{-/-}$ mice orally immunized with Dukoral (Figure 14) may be due to the orally immunized mice having a decreased quantity of *V. cholerae*-specific serum total IgG antibodies compared to their intramuscularly immunized counterparts. Furthermore, these results suggest that oral immunization (with Dukoral) was less efficient than intramuscular immunization at inducing *V. cholerae*-specific serum total IgG antibodies.

3.2.6 CD4+ T-cells and CD19+ B-cells from TLR mutant mice orally immunized with Dukoral were impaired in their ability to proliferate in response to *V. cholerae* stimulation

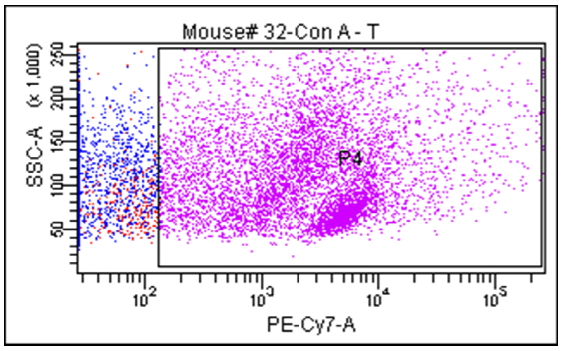
I next sought to examine the requirement of TLR signaling on the induction of cell-mediated immune responses following oral immunization with Dukoral. I first evaluated the ability of splenocytes from orally immunized WT and TLR mutant mice to proliferate in response to *V. cholerae* stimulation. In order to evaluate cell proliferation, a CFSE-dilution assay was utilized, and 10,000 FSC/SSC events were collected and results were analyzed on CD4^{+} T-cells or CD19^{+} B-cells within the gated region. Cells were considered to have proliferated if the intensity of their CFSE staining placed them within the histogram gate region falling between non-dividing cells and CFSE-unlabelled cells (Figure 16). The results showed that CD4+ T-cells from orally immunized TLR-2$^{-/-}$ ($p=0.0002$) and MyD88$^{-/-}$ ($p<0.0001$) mice were significantly inhibited in their ability to proliferate in response to stimulation with whole-cell *V. cholerae* (Figure 17 A), compared to WT control mice. Proliferation of CD4+ T-cells from TLR-4$^{-/-}$ mice was also decreased, but not significantly. Inhibited proliferation was not seen in CD4+ T-cells from Trif$^{-/-}$ mice, suggesting that CD4+ T-cell proliferation in response to *V. cholerae* stimulation occurred independently of Trif signaling. Similarly, CD19+ B-cells from orally immunized TLR-2$^{-/-}$ ($p<0.0001$), TLR-4$^{-/-}$ ($p=0.004$) and MyD88$^{-/-}$ ($p<0.0001$) mice were

Figure 16. Gating strategy for measuring the antigen-specific proliferation of CFSE-labelled splenocytes from mice orally immunized with the Dukoral vaccine. Splenocytes were collected 14 days after the last vaccination and cryo-preserved until used. Splenocytes were then thawed, labelled with 5 μM CFSE, and stimulated with vaccine components or concanavalin A for 5 days. At the end of the incubation period, cells were stained with anti-CD4 Pe-Cy7 or anti-CD19 PE-Cy7 antibodies and the extent of CFSE dilution was evaluated by flow cytometry by the following gating strategy: (A) 10,000 events were collected within the FSC/SSC region defined by gate by P1. CD4-Pe-Cy7-positive T-cells or CD19- Pe-Cy7-positive B-cells were then selected by gate P4 (B). CFSE-diluted cells were taken to be those within the histogram region gated by P5 (C). Histogram gate boundaries were established using unstained cells for the left-most border and non-dividing cells on the right-most border. A dot-plot representation showing CFSE dilution is shown in D.

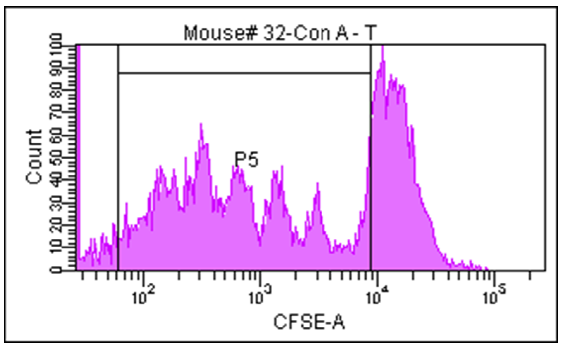
A


Mouse# 32-Con A - T
SSC-A (x 1,000)
FSC-A (x 1,000)
P1

B


Mouse# 32-Con A - T
SSC-A (x 1,000)
PE-Cy7-A
P4

C


Mouse# 32-Con A - T
Count
CFSE-A
P5

D

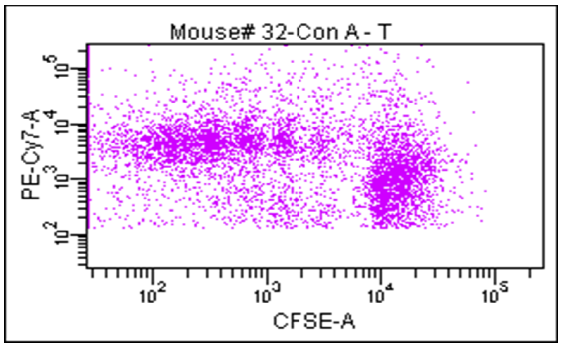
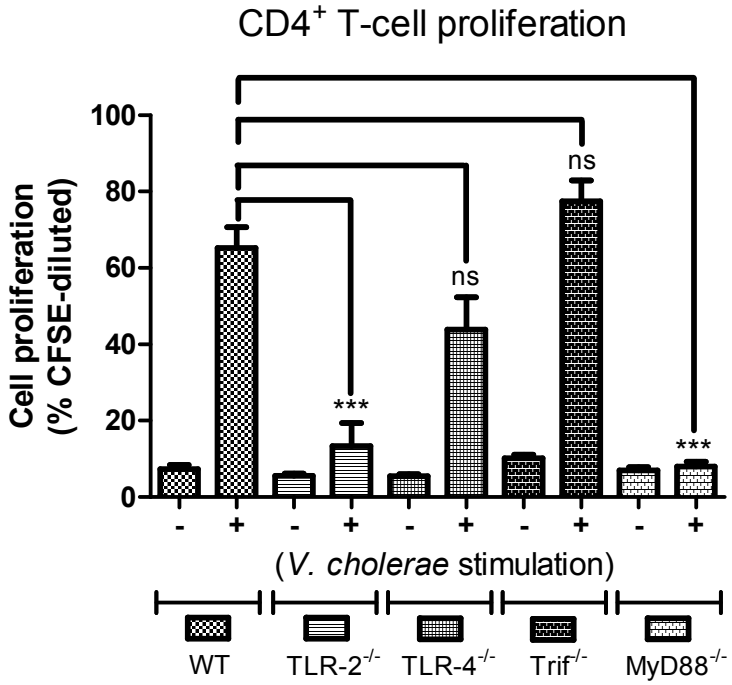
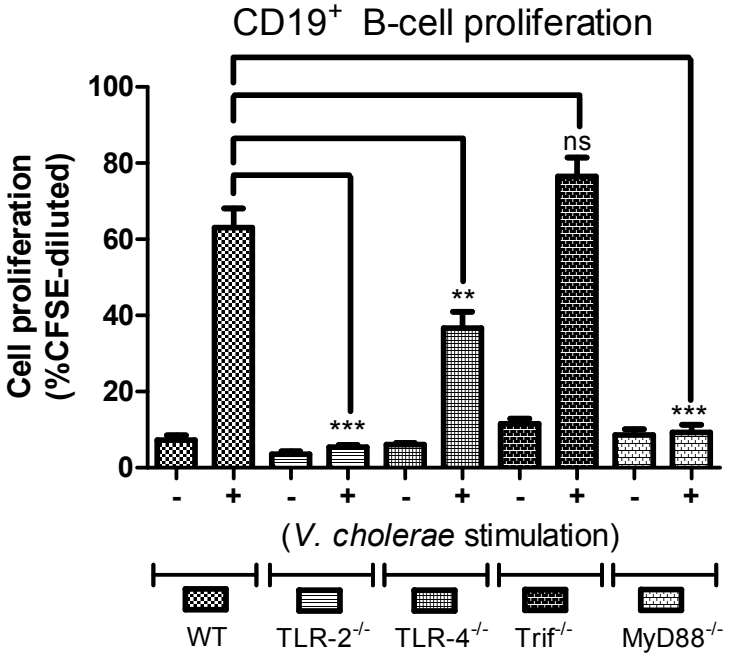

Mouse# 32-Con A - T
PE-Cy7-A
CFSE-A

Figure 17. CD4+ T-cell (A) and CD19+ B-cell (B) proliferation in response to stimulation by *V. cholerae* was regulated by MyD88-dependant pathways. TLR mutant mice (TLR-$2^{-/-}$, TLR-$4^{-/-}$, MyD$88^{-/-}$ and $Trif^{-/-}$) were immunized orally with Dukoral on days 0, 10, 20 and 30 ($3x10^9$ *V. cholerae* with 10 μg CTB). Spleens were collected 2 weeks after the last vaccination, and splenocytes were cryo-preserved until tested. Splenocytes were labelled with 5 μM CFSE and stimulated with *V. cholerae* for 5 days. Proliferation was evaluated by flow cytometry by the degree of CFSE-dilution on fluorescently-labelled CD4+ T-cells (A) or CD19+ B-cells (B). Cell proliferation is shown as the % of CFSE-diluted events from 10,000 gated events ± SEM.
-, unstimulated; +, stimulated with *V. cholerae* at a ratio of 1:2000.
** $p<0.01$, *** $p<0.001$.ns, not significant.

A


CD4+ T-cell proliferation
Cell proliferation (% CFSE-diluted)
100
80
60
40
20
0
ns
ns
***
***
- + - + - + - + - +
(V. cholerae stimulation)
WT
TLR-2-/-
TLR-4-/-
Trif-/-
MyD88-/-

B


CD19+ B-cell proliferation
Cell proliferation (%CFSE-diluted)
100
80
60
40
20
0
ns
**
***
***
- + - + - + - + - +
(V. cholerae stimulation)
WT
TLR-2-/-
TLR-4-/-
Trif-/-
MyD88-/-

significantly inhibited in their ability to proliferate in response to stimulation with whole-cell *V. cholerae* (Figure 17 B). However, CD19+ B-cells from orally immunized Trif$^{-/-}$ mice proliferated comparably to WT animals in response to stimulation with *V.* cholerae. These results suggest that proliferation of both CD4+ T-cells and CD19+ B-cells in response to *V. cholerae* was mediated via MyD88/TLR signaling, and independently of Trif signaling.

3.2.7 Splenocytes from TLR mutant mice orally immunized with Dukoral were significantly impaired in their ability to secrete cytokines in response to *V. cholerae* stimulation

To further determine the involvement of TLR signaling in the generation of cell-mediated immune responses against the *V. cholerae* component of the Dukoral vaccine, splenocytes from mice orally immunized with Dukoral were stimulated with whole-cell *V. cholerae* for 72 hours to evaluate their ability to secrete IFN-γ and IL-4, prototypical Th1/Th2 cytokines, by an ELISPOT assay. The results showed that splenocytes from TLR-2$^{-/-}$, TLR -4$^{-/-}$, MyD88$^{-/-}$ and Trif$^{-/-}$ mutant mice were significantly inhibited in their ability to secrete IFN-γ (TLR-2$^{-/-}$, $p<0.0001$; TLR-4$^{-/-}$, $p=0.0002$; MyD88$^{-/-}$, $p<0.0001$; Trif$^{-/-}$, $p=0.0063$) (Figure 18) in response to stimulation by *V. cholerae*, while only splenocytes from TLR-2$^{-/-}$ mice ($p=0.0002$) were significantly inhibited in their ability to secrete IL-4 (Figure 19).

3.2.8 *V. cholerae* stimulation induced maturation of bone marrow-derived DCs

I next determined the ability of *V. cholerae* stimulation to induce DC maturation. To accomplish this, DCs were induced from WT bone marrow cultures by stimulation with 10 ng/mL of both GM-CSF and IL-4. After 5 days, cell cultures were stimulated with varying

Figure 18. Splenocytes from TLR mutant mice orally immunized with Dukoral were impaired in their ability to secrete IFN-γ following stimulation with *V. cholerae*. TLR mutant mice (TLR-2$^{-/-}$, TLR-4$^{-/-}$, MyD88$^{-/-}$ and Trif$^{-/-}$) were immunized orally with Dukoral on days 0, 10, 20 and 30 ($3x10^9$ *V. cholerae* with 10 μg CTB). Spleens were collected 2 weeks after the last vaccination, and splenocytes were cryo-preserved until tested. Splenocytes were evaluated for their ability to secrete IFN-γ by ELISPOT. 250,000 splenocytes were seeded in triplicate wells and stimulated with whole-cell *V. cholerae* for 72 hrs. Results are shown as IFN-γ spot-forming cells (SFC) / $1x10^6$ cells ± SEM. -, unstimulated; +, stimulated with *V. cholerae* at a ratio of 1:2000. ** $p<0.01$, *** $p<0.001$.

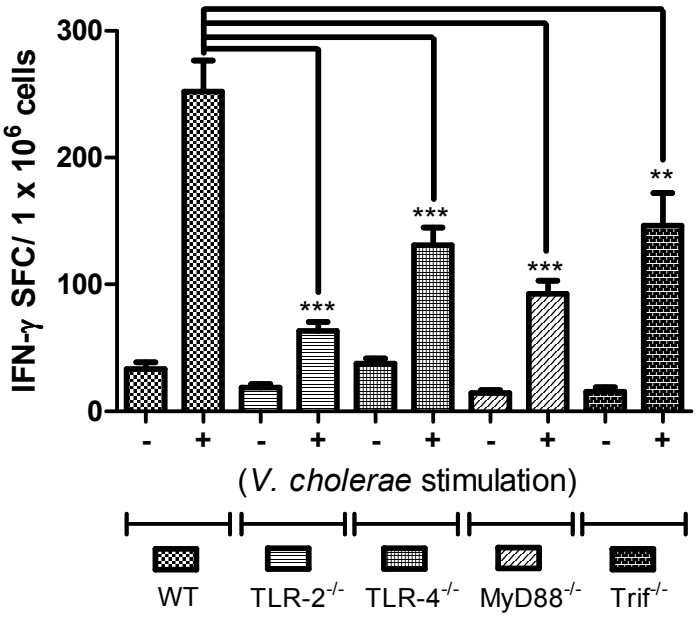
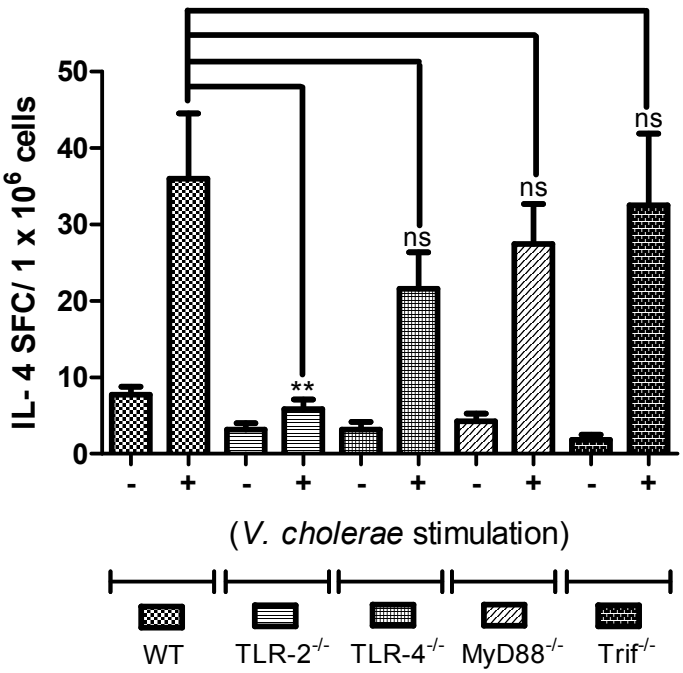

IFN-γ SFC/ 1 x 10^6 cells
300
200
100
0
***
***
***
**
- + - + - + - + - +
(V. cholerae stimulation)
WT
TLR-2-/-
TLR-4-/-
MyD88-/-
Trif-/-

Figure 19. Splenocytes from TLR-2$^{-/-}$ mice orally immunized with Dukoral were impaired in their ability to secrete IL-4 following stimulation with *V. cholerae*. TLR mutant mice (TLR-2$^{-/-}$, TLR-4$^{-/-}$, MyD88$^{-/-}$ and Trif$^{-/-}$) were immunized orally with Dukoral on days 0, 10, 20 and 30 ($3x10^9$ *V. cholerae* with 10 μg CTB). Spleens were collected 2 weeks after the last vaccination, and splenocytes were cryo-preserved until tested. Splenocytes were evaluated for their ability to secrete IL-4 by ELISPOT. 250,000 splenocytes were seeded in triplicate wells and stimulated with whole-cell *V. cholerae* for 72 hrs. Results are shown as IL-4 spot-forming cells (SFC) / $1x10^6$ cells ± SEM. -, unstimulated; +, stimulated with *V. cholerae* at a ratio of 1:2000. ** $p<0.01$. ns, not significant.


IL- 4 SFC/ 1 x 10^6 cells
50
40
30
20
10
0
**
ns
ns
ns
- + - + - + - + - +
(V. cholerae stimulation)
WT
TLR-2-/-
TLR-4-/-
MyD88-/-
Trif-/-

amounts of *V. cholerae* for 24 hours, and DC maturation was evaluated by the cell surface expression of co-stimulatory markers CD80 and CD86, as well as CD40 and MHCII. In addition, DC maturation was evaluated by measuring cytokine secretion in the culture supernatants by a 13-plex cytokine array (IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, IL-22, IL-27 and TNF-α). My results showed that the *V. cholerae* component of the Dukoral vaccine was able to induce DC maturation as shown by increased cell-surface expression of CD80, CD86, CD40, and MHCII molecules (Figure 20). A representative histogram of one independent experiment is shown in Figure 21. Stimulation of DC cultures by *V. cholerae* was also able to induce secretion of IL-13, IL-1α, IL-22, IL-27 and IFN-γ, in addition to high levels of IL-6 and TNF-α (Figure 22). IL-4 secretion was also detected, however since IL-4 was added to the cell culture media it has been omitted from this analysis. Thus, *V. cholerae* stimulation was able to induce maturation of DCs, as determined by the induction of multiple cell surface markers and cytokine production.

3.2.9 Dendritic cell maturation and cytokine secretion in response to *V. cholerae* stimulation occurred in a MyD88-dependant manner

Since the Dukoral vaccine was able to induce maturation of WT bone marrow-derived DCs (Figures 20 - 22), I determined whether DC maturation and cytokine secretion in response to stimulation with *V. cholerae* was mediated by TLR signaling. Bone marrow-derived DCs from WT and MyD88$^{-/-}$ mice were stimulated with *V. cholerae* and DC maturation was again evaluated by cell-surface CD80, CD86, CD40 and MHCII expression. Cell culture supernatants were also evaluated by a 13-plex cytokine array to evaluate the cytokines secreted during DC maturation. My results showed that DCs from MyD88$^{-/-}$ mice were significantly impaired in their ability to up-regulate expression of CD80 ($p=0.005$), CD86 ($p=0.05$) (Figure 23), as well as

Figure 20. Bone marrow-derived DCs matured in response to stimulation by whole-cell *V. cholerae*. Bone marrow progenitors were cultured with 10 ng/mL of both GM-CSF and IL-4 over 5 days. Cultures were then stimulated with the indicated ratios of *V. cholerae* for 24 hours. DCs were then evaluated for their ability to express CD80 (A), CD86 (B), CD40 (C) and MHCII (D) by flow cytometry. Results are shown as the MFI of three independent experiments ± SEM. Unstim denotes cells that received mock treatment comprised of media alone.

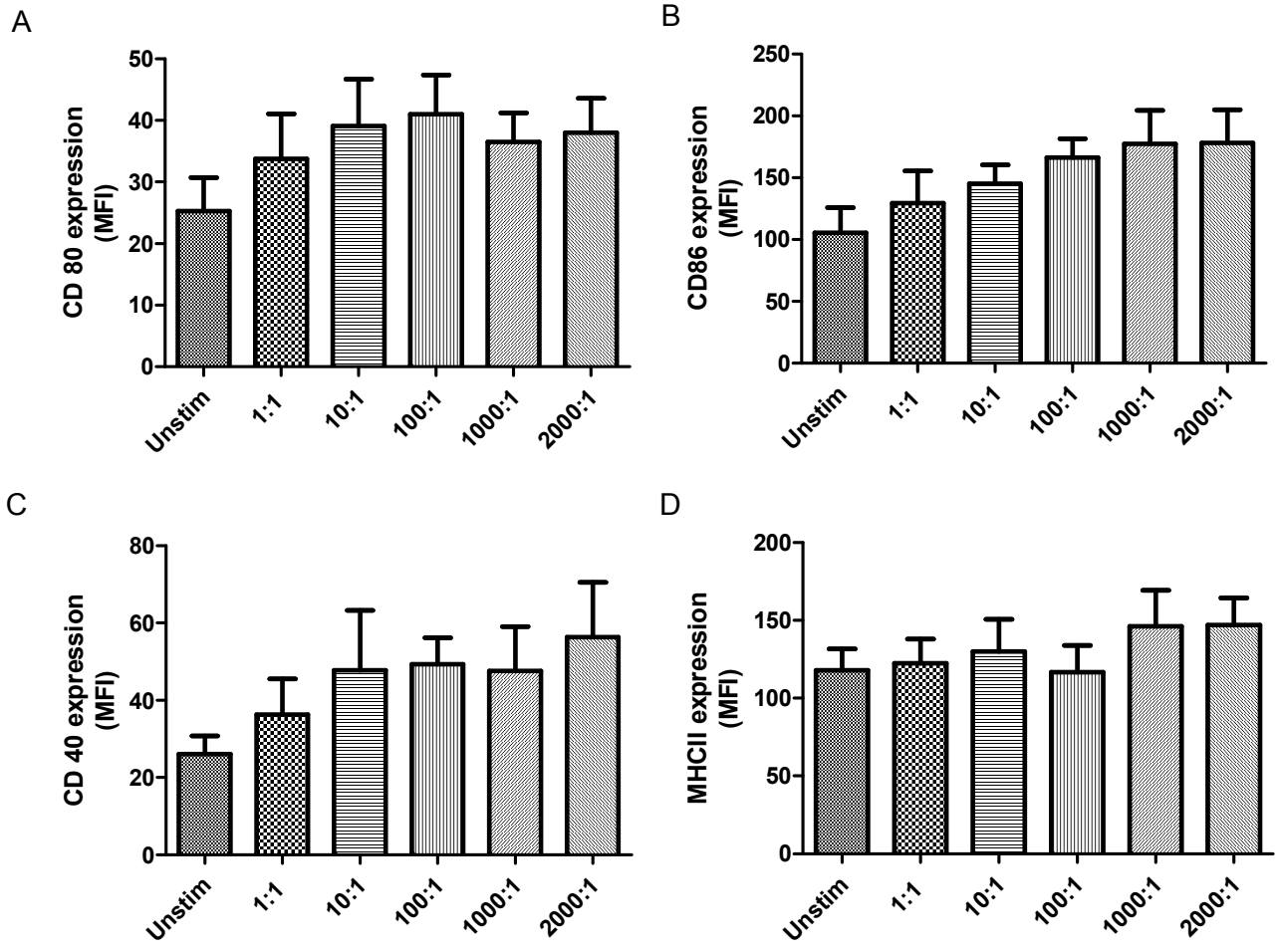

A
CD 80 expression (MFI)
0
10
20
30
40
50
Unstim
1:1
10:1
100:1
1000:1
2000:1
B
CD86 expression (MFI)
0
50
100
150
200
250
Unstim
1:1
10:1
100:1
1000:1
2000:1
C
CD 40 expression (MFI)
0
20
40
60
80
Unstim
1:1
10:1
100:1
1000:1
2000:1
D
MHCII expression (MFI)
0
50
100
150
200
Unstim
1:1
10:1
100:1
1000:1
2000:1

Figure 21. Bone marrow-derived DCs matured in response to stimulation by whole-cell *V. cholerae*. Bone marrow progenitors from C57BL/6 WT mice were cultured with 10 ng/mL of both GM-CSF and IL-4 over 5 days. Cultures were then either mock-stimulated (filled grey histograms) or stimulated with a 2000:1 ratio of whole-cell *V. cholerae* for 24 hours (red overlay) and cells were evaluated for their ability to express cell surface markers CD80 (A), CD86 (B), MHC II (C) and CD40 (D) by flow cytometry. Results shown are one representative histogram of three independent experiments.

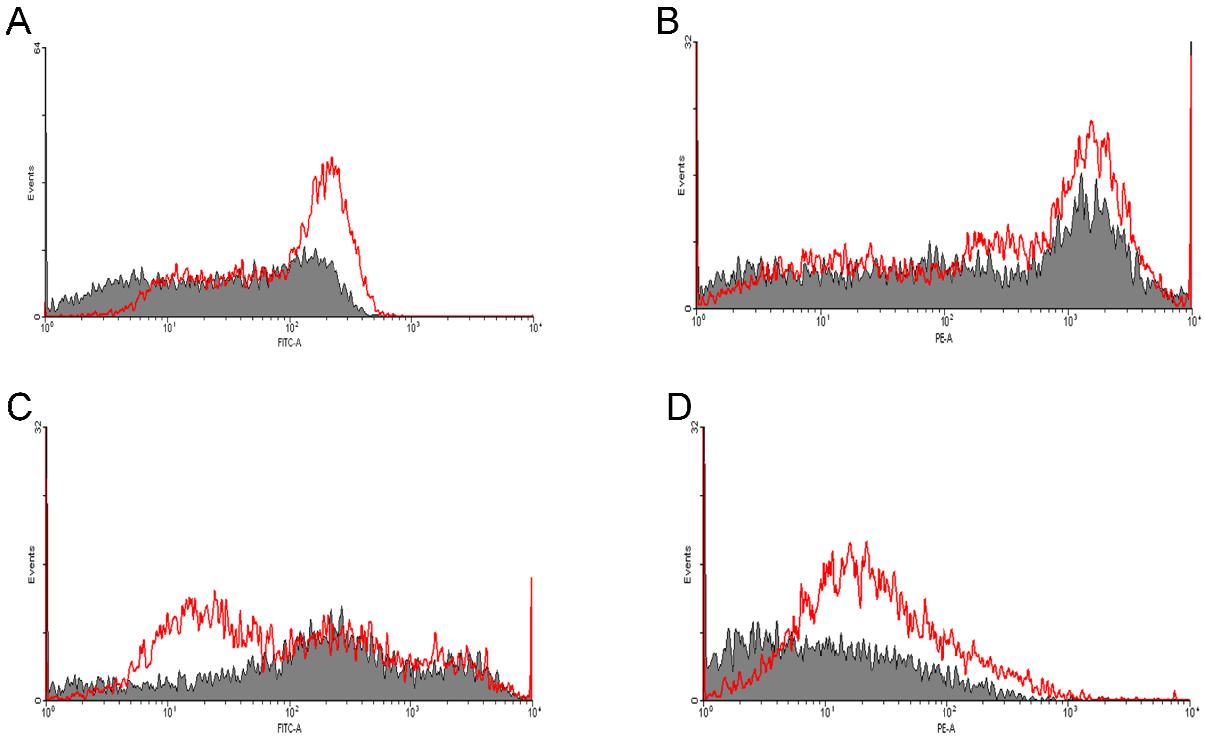

A
B
C
D
Events
FITC-A
PE-A

Figure 22. Bone marrow-derived DC cultures secreted multiple cytokines in response to *V. cholerae* stimulation. Bone marrow progenitors were cultured with 10 ng/ml of both GM-CSF and IL-4 over 5 days. Cultures were then stimulated with a 2000:1 ratio of *V. cholerae*-seeded cells for 24 hours and cell culture supernatants were collected and frozen until tested by cytokine array. Results are shown as the mean cytokine production of duplicate samples from three pooled independent experiments ± SEM.

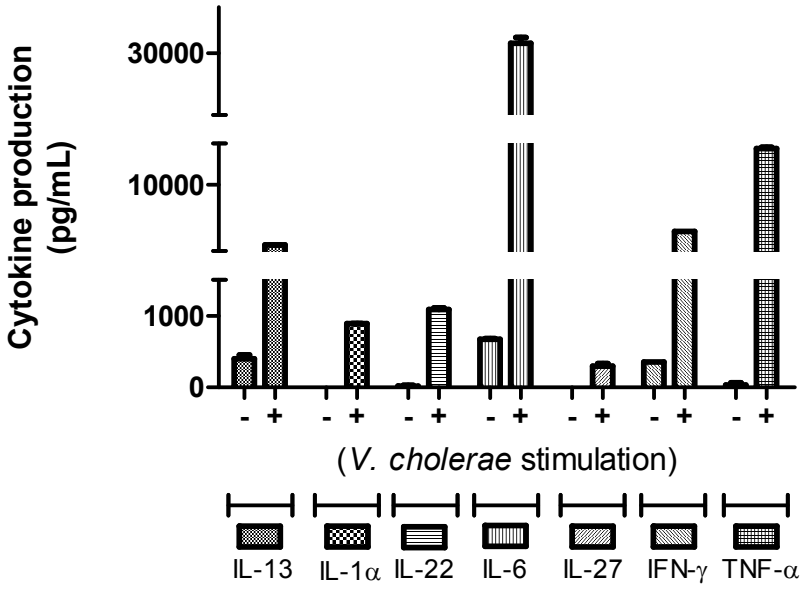
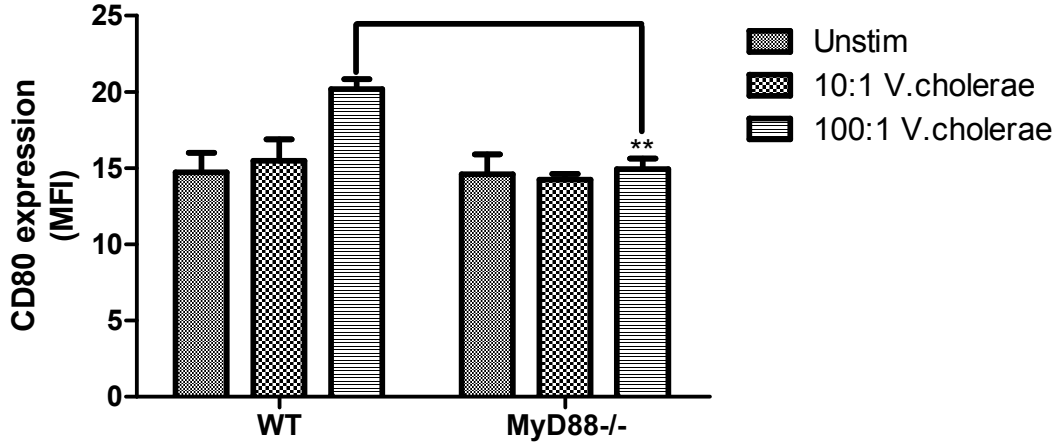
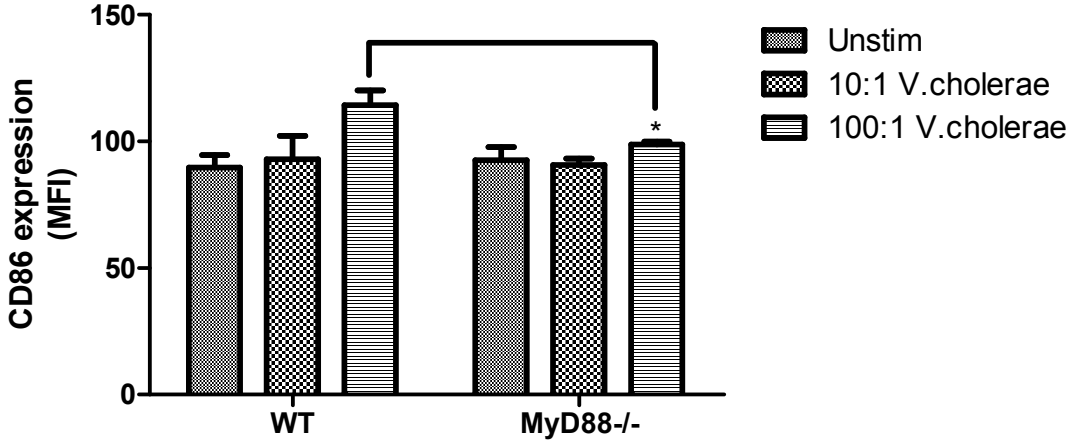

Cytokine production
(pg/mL)
30000
10000
1000
0
- + - + - + - + - + - + - +
(V. cholerae stimulation)
IL-13
IL-1α
IL-22
IL-6
IL-27
IFN-γ
TNF-α

Figure 23. Bone marrow-derived MyD88$^{-/-}$ DCs were impaired in their ability to express CD80 and CD86 co-stimulatory molecules in response to stimulation with *V. cholerae*. Bone marrow progenitors from WT and Myd88$^{-/-}$ mice were cultured with 10 ng/ml of both GM-CSF and IL-4 over 5 days. Cultures were then stimulated with the indicated ratios of *V. cholerae* for 24 hours and cells were evaluated for their ability to express co-stimulatory molecules CD80 (A) and CD86 (B) by flow cytometry. Results are shown as the MFI of three independent experiments ± SEM. *$p<0.05$, **$p<0.01$. Unstim denotes cells that received mock treatment comprised of media alone.

A


CD80 expression (MFI)
25
20
15
10
5
0
**
WT
MyD88-/-
Unstim
10:1 V.cholerae
100:1 V.cholerae

B

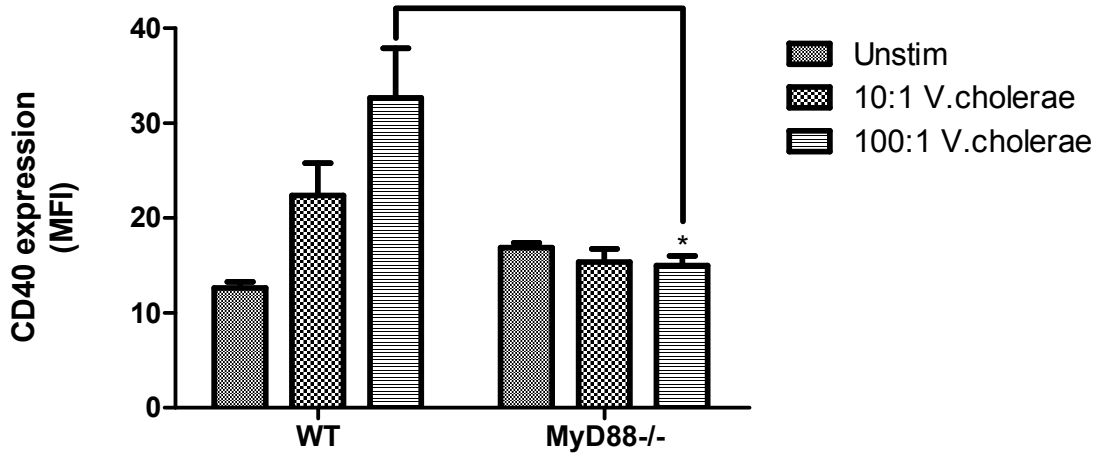

CD86 expression (MFI)
150
100
50
0
*
WT
MyD88-/-
Unstim
10:1 V.cholerae
100:1 V.cholerae

CD40 (p=0.02) and MHCII (p=0.05) (Figure 24) in response to stimulation with *V. cholerae*. A representative histogram is shown in Figure 25. The results of the cytokine array showed that DCs from MyD88$^{-/-}$ mice were unable to secrete IL-1α (p=0.0028) and IL-22 (p=0.0033), were significantly impaired in their ability to secrete IL-6 (p=0.0012), IFN-γ (p=0.0060), and TNF-α (p=0.0058), and were hindered in their ability to secrete IL-13 (Figure 26). IL-4 secretion was also detected, however since IL-4 was added to the growth media of the DC cultures it has been omitted from this analysis. The remaining cytokines were not found above the limit of detection of the cytokine array kit.

Taken together, these results indicate that DCs from MyD88$^{-/-}$ mice were unable to fully mature during vaccine stimulation by their inability to up-regulate cell surface expression of co-stimulatory molecules CD80, CD86 and CD40, as well as MHCII, and that these same cells were significantly impaired in their ability to secrete cytokines. These results suggest that the induction of a cell-mediated immune response to the Dukoral vaccine was critically dependent upon TLR signaling. In contrast, the induction of a humoral immune response is not critically dependent upon TLR signaling or DC maturation, as MyD88$^{-/-}$ mice elicited *V. cholerae*-specific humoral immune responses comparable to those seen in WT mice, despite the fact that MyD88$^{-/-}$ DCs were unable to mature in-vitro in response to *V. cholerae* stimulation at the doses indicated here (Figure 11).

Figure 24. Bone marrow-derived MyD88$^{-/-}$ DCs were impaired in their ability to express CD40 and MHCII molecules in response to stimulation with *V. cholerae*. Bone marrow progenitors from WT and MyD88$^{-/-}$ mice were cultured with 10 ng/ml of both GM-CSF and IL-4 over 5 days. Cultures were then stimulated with the indicated ratios of *V. cholerae* for 24 hours and cells were evaluated for their ability to express CD40 (A) and MHCII (B) by flow cytometry. Results are shown as the MFI of three independent experiments ± SEM. *$p<0.05$. Unstim denotes cells that received mock treatment comprised of media alone.

A


CD40 expression (MFI)
40
30
20
10
0
WT
MyD88-/-
*
Unstim
10:1 V.cholerae
100:1 V.cholerae

B

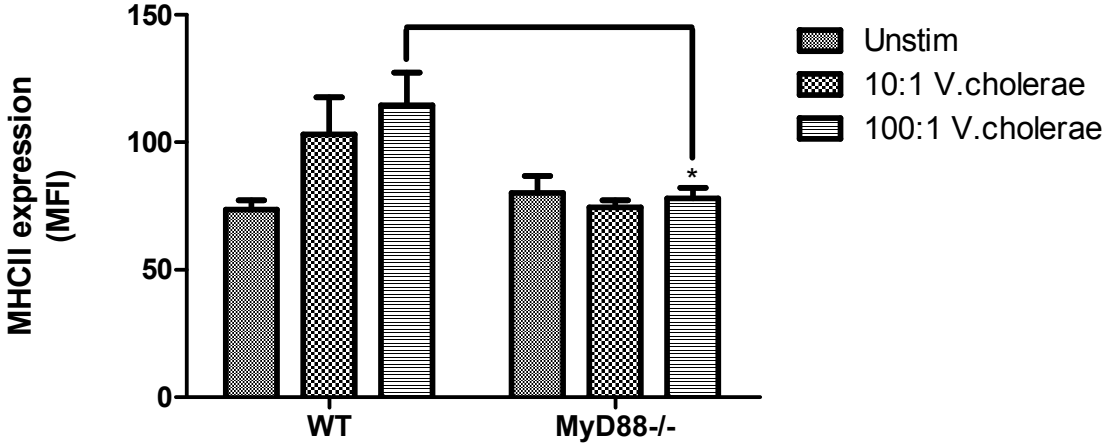

MHCII expression (MFI)
150
100
50
0
WT
MyD88-/-
*
Unstim
10:1 V.cholerae
100:1 V.cholerae

Figure 25. Bone marrow-derived DCs from MyD88$^{-/-}$ mice were impaired in their ability to express CD80, CD86, CD40 and MHCII cell surface molecules in response to stimulation by whole-cell *V. cholerae*. Bone marrow progenitors from C57BL/6 WT mice (filled grey histograms) and MyD88$^{-/-}$ mice (red overlay) were cultured with 10 ng/mL of both GM-CSF and IL-4 over 5 days. Cultures were then stimulated with a 100:1 ratio of whole-cell *V. cholerae* for 24 hours and cells were evaluated for their ability to express cell surface markers CD80 (A), CD86 (B), MHCII (C) and CD40 (D) by flow cytometry. Results shown are one representative histogram of three independent experiments.

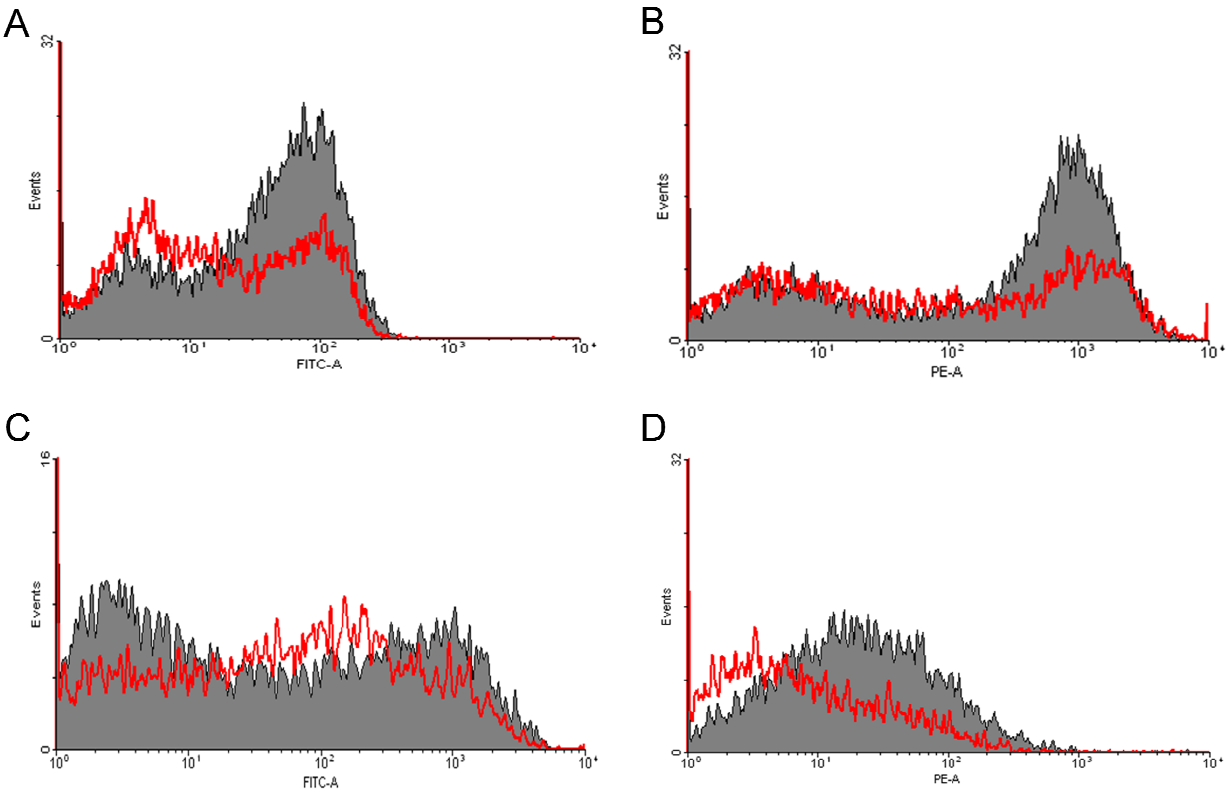
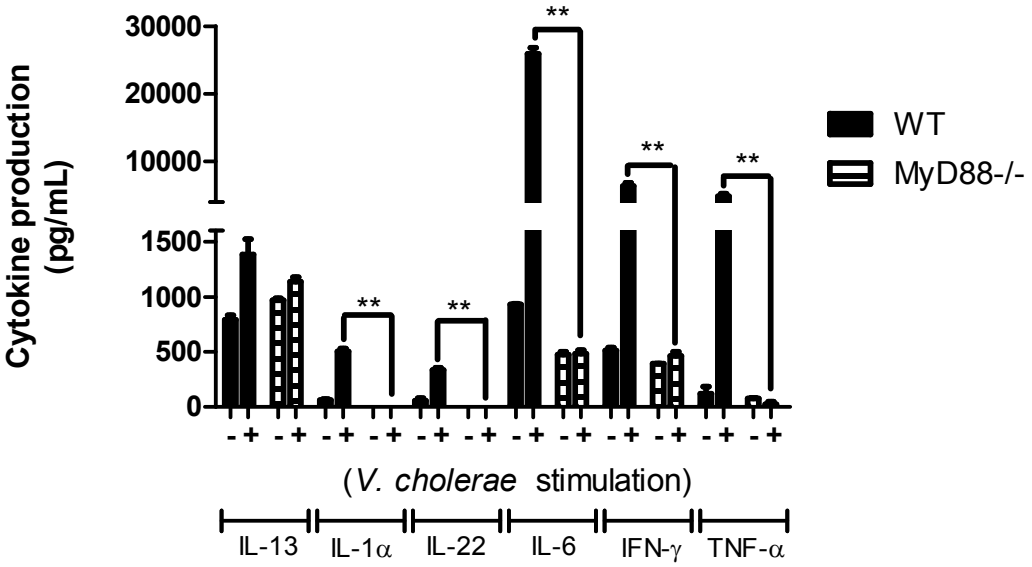

A
B
C
D
Events
FITC-A
PE-A
32
16
0
10^0
10^1
10^2
10^3
10^4

Figure 26. Bone marrow-derived MyD88$^{-/-}$ DCs were inhibited in their ability to secrete cytokines in response to *V. cholerae* stimulation. Bone marrow progenitors from MyD88$^{-/-}$ mice were cultured with 10 ng/ml of both GM-CSF and IL-4 over 5 days. Cultures were then stimulated with a 100:1 ratio of *V. cholerae* for 24 hours. Cell culture supernatants were collected on day 6 and frozen until tested by cytokine array. Results are shown as the mean cytokine production of duplicate samples from three pooled independent experiments ± SEM. ** $p<0.01$.


Cytokine production (pg/mL)
30000
20000
10000
1500
1000
500
0
**
**
**
**
WT
MyD88-/-
- + - + - + - + - + - + - + - + - + - + - + - +
(V. cholerae stimulation)
IL-13
IL-1α
IL-22
IL-6
IFN-γ
TNF-α

3.3 Immune responses against the CTB component of the Dukoral vaccine

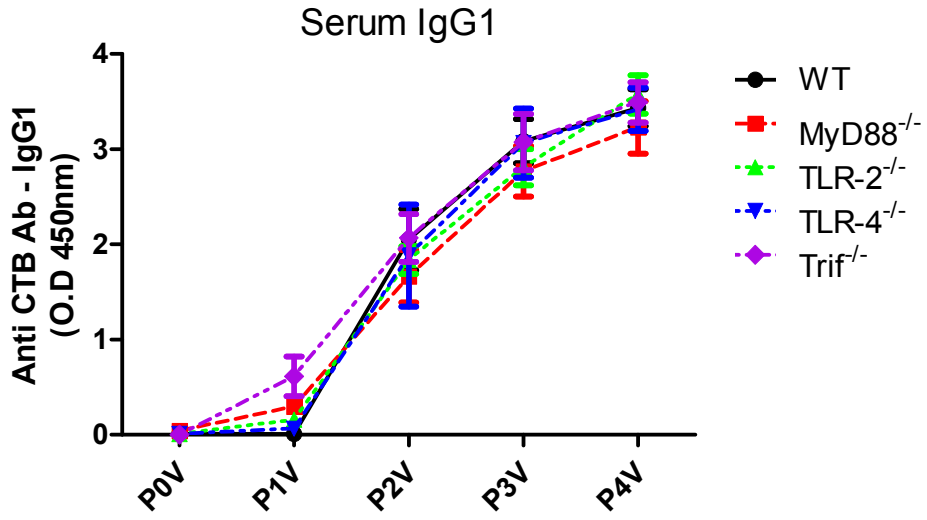
3.3.1 MyD88, Trif, TLR-2 and TLR-4 signaling did not mediate the production of CTB-specific serum IgG or fecal IgA antibodies following oral immunization with Dukoral

Following the results obtained against the *V. cholerae* component of the Dukoral vaccine, I sought to determine whether TLR signaling mediated the generation of humoral and cellular immune responses against the soluble CTB component of the Dukoral vaccine. After four oral immunizations with Dukoral, TLR mutant mice did not show any significant impairment in the induction of CTB-specific serum IgG1 and IgG2c antibody at any stage following immunization (Figure 27). Trif $^{-/-}$ mutant mice developed CTB-specific IgG2c antibody post-2nd vaccination, while TLR-2$^{-/-}$ and TLR-4$^{-/-}$ mice showed induction of CTB-specific IgG2c antibody post-3rd vaccination. However, this earlier induction of CTB-specific IgG2c antibody was not statistically significant. WT and MyD88 mutant mice generated CTB-specific IgG2c antibody production only after the 4th vaccination with Dukoral. Similar results were obtained when mice were immunized orally with CTB alone (Figure 28).

Following oral immunization with Dukoral, MyD88$^{-/-}$ ($p=0.0254$) and TLR-2$^{-/-}$ mice ($p=0.0358$) also showed significantly inhibited CTB-specific fecal IgA responses, but only when tested at an undiluted concentration (Figure 29). The additional dilutions of fecal supernatant tested did not demonstrate statistically significant impairment in CTB-specific fecal IgA antibody, compared to the WT mice.

Figure 27. Generation of CTB-specific serum IgG1 (A) and IgG2c (B) antibodies following oral immunization with Dukoral did not require TLR signaling. TLR mutant mice (TLR-$2^{-/-}$, TLR-$4^{-/-}$, MyD$88^{-/-}$ and $Trif^{-/-}$) were immunized orally with Dukoral ($3x10^9$ *V. cholerae* with 10 μg CTB) on days 0, 10, 20 and 30. Serum was collected 9 days after each vaccination, and CTB-specific serum IgG1 (A) and IgG2c (B) was measured by ELISA. Results are shown as the mean O.D 450 nm ± SEM. P0V – P4V are as defined in the legend to Figure 2.

A


Serum IgG1
Anti CTB Ab - IgG1 (O.D 450nm)
4
3
2
1
0
P0V
P1V
P2V
P3V
P4V
WT
MyD88-/-
TLR-2-/-
TLR-4-/-
Trif-/-

B

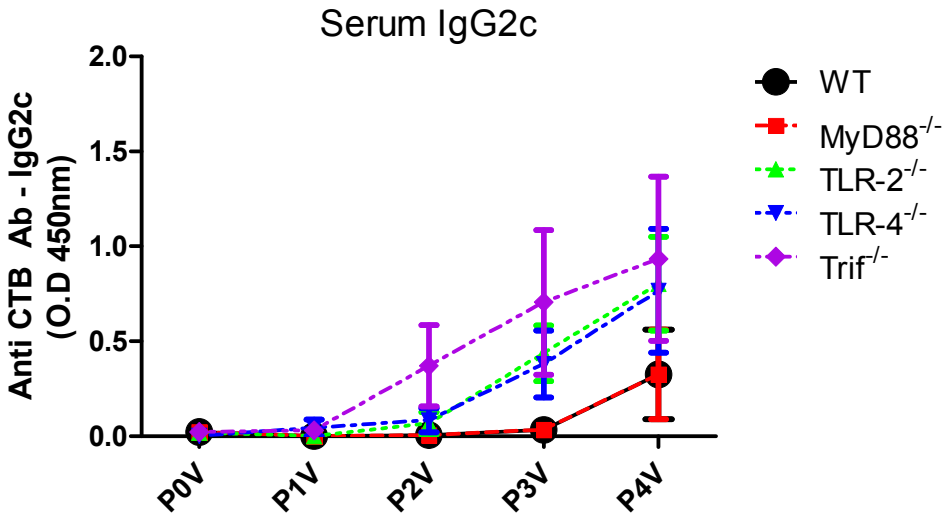
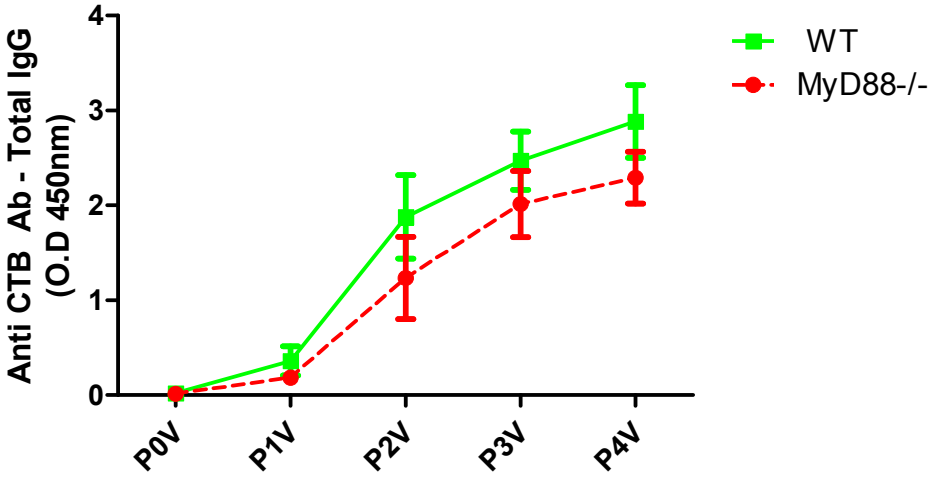
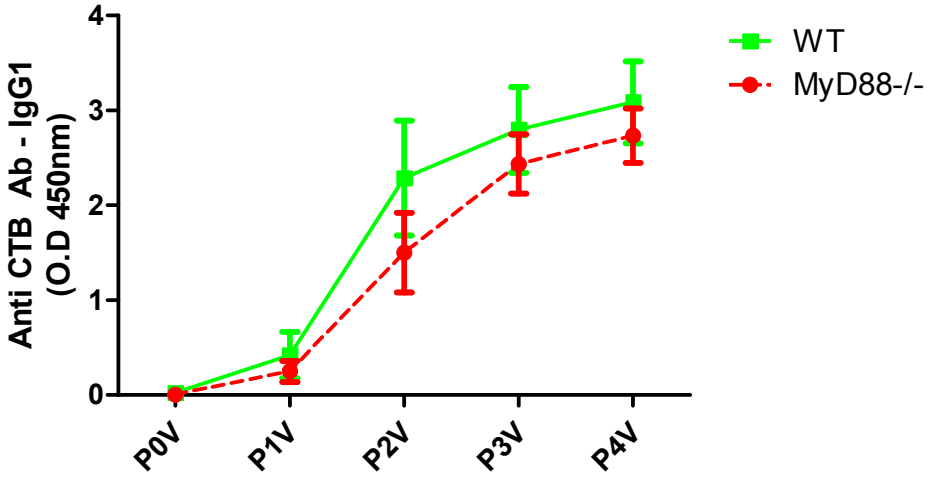

Serum IgG2c
Anti CTB Ab - IgG2c (O.D 450nm)
2.0
1.5
1.0
0.5
0.0
P0V
P1V
P2V
P3V
P4V
WT
MyD88-/-
TLR-2-/-
TLR-4-/-
Trif-/-

Figure 28. MyD88 signaling was dispensable for CTB-specific serum antibody production following oral immunization with CTB. MyD88$^{-/-}$ and WT mice were immunized orally on days 0, 10, 20, and 30 with 10 μg CTB. Serum was collected 9 days after each vaccination, and CTB-specific total serum IgG (A), IgG1 (B) and IgG2c (C) responses were measured by ELISA. Results are shown as the mean O.D 450 nm ± SEM. P0V – P4V are as defined in the legend to Figure 2.

A


Anti CTB Ab - Total IgG
(O.D 450nm)
0
1
2
3
4
P0V
P1V
P2V
P3V
P4V
WT
MyD88-/-

B


Anti CTB Ab - IgG1
(O.D 450nm)
0
1
2
3
4
P0V
P1V
P2V
P3V
P4V
WT
MyD88-/-

C

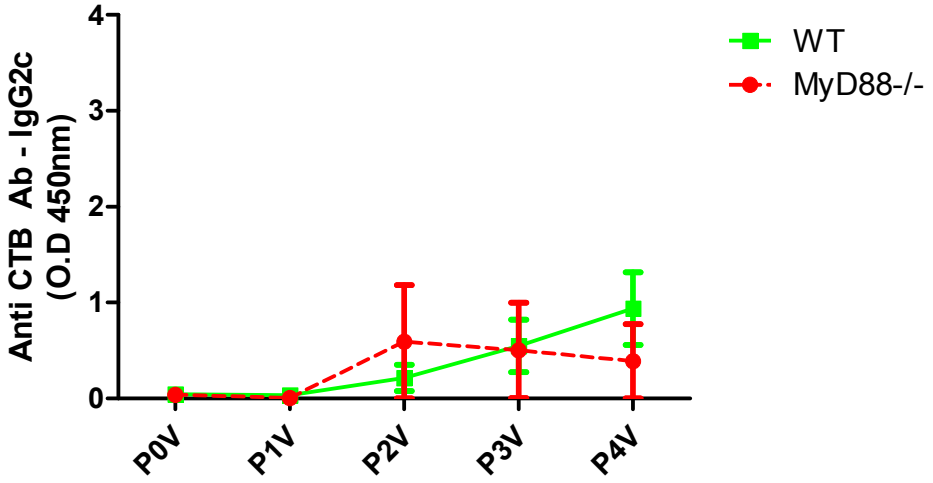
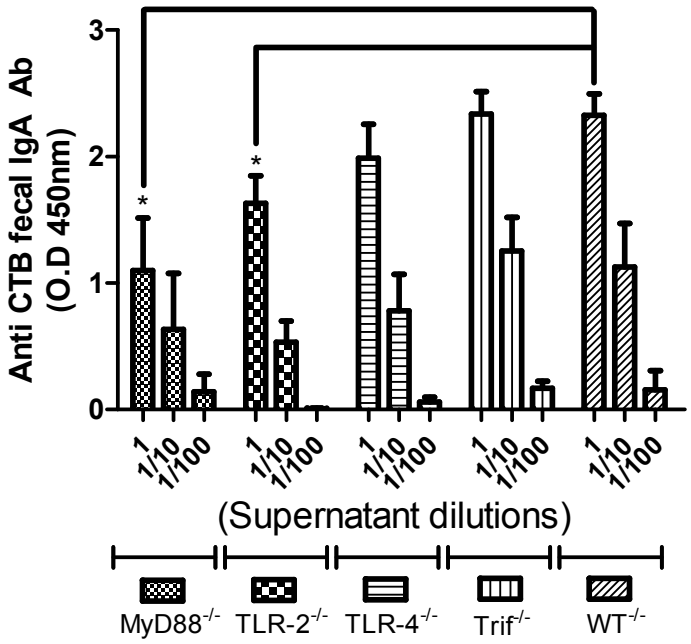

Anti CTB Ab - IgG2c
(O.D 450nm)
0
1
2
3
4
P0V
P1V
P2V
P3V
P4V
WT
MyD88-/-

Figure 29.TLR signaling did not mediate the generation of CTB-specific fecal IgA antibody production following oral immunization with Dukoral. TLR mutant mice (TLR-$2^{-/-}$, TLR-$4^{-/-}$, MyD$88^{-/-}$ and $Trif^{-/-}$) were immunized orally with Dukoral ($3x10^9$ *V. cholerae* with 10 μg CTB) on days 0, 10, 20, and 30. Fecal pellets were collected pre-vaccination and 9 days after the last vaccination. Fecal supernatants were extracted as described in Materials and Methods. CTB-specific fecal IgA was measured by ELISA at the indicated dilutions (1, undiluted, 1/10,10-fold diluted, 1/100, 100-fold diluted). Results shown are the post-4^{th} vaccination mean O.D 450 nm ± SEM, corrected for pre-vaccination background signal. * $p < 0.05$.


Anti CTB fecal IgA Ab
(O.D 450nm)
3
2
1
0
*
*
1
1/10
1/100
(Supernatant dilutions)
MyD88-/-
TLR-2-/-
TLR-4-/-
Trif-/-
WT-/-

As the remaining dilutions did not show any statistically significant impairment, I believe that the statistical difference observed for the undiluted concentration may represent an isolated observation. Similar results were observed when MyD88$^{-/-}$ and WT mice were orally immunized with CTB alone (Figure 30). Thus, overall, these results suggest that TLR signaling was not critical to the production of CTB-specific fecal IgA antibodies.

3.3.2 MyD88 signaling shaped CTB-specific antibody responses following intramuscular immunization with Dukoral or CTB alone

In light of the results which showed TLR signaling to be dispensable for CTB-specific serum IgG and fecal IgA antibody production following oral immunization, I wished to determine if TLR signaling was important for CTB-specific serum antibody production following IM immunization with Dukoral.

After four intramuscular immunizations with Dukoral, the results showed that MyD88$^{-/-}$ mice demonstrated impaired serum CTB-specific antibody production (Figure 31). MyD88$^{-/-}$ mice demonstrated significantly decreased total IgG induction post-1st ($p<0.0001$) and post-2nd ($p=0.0017$) vaccination, as well as decreased IgG1induction post-1st ($p<0.0001$) and post-2nd ($p=0.0065$) vaccination. Subsequent boosting via 3rd and 4th immunizations allowed MyD88$^{-/-}$ mice to develop a post-4th vaccination response comparable to that seen in WT mice. Conversely, CTB-specific IgG2c production in MyD88$^{-/-}$ mice was not boosted after subsequent immunizations, and was significantly inhibited even after the 4th vaccination ($p=0.0004$). Together, these results suggest that MyD88 signaling mediates the induction of CTB-specific IgG1 and IgG2c antibodies, albeit at different time points following immunization.

Figure 30. MyD88 signaling was dispensable for CTB-specific fecal antibody production following oral immunization with CTB alone. MyD88$^{-/-}$ and WT mice were immunized orally on days 0, 10, 20, and 30 with 10 μg CTB. Fecal pellets were collected pre-vaccination an 9 days after the last vaccination. Fecal supernatants were extracted as described in Materials and Methods, and CTB-specific fecal IgA responses were measured by ELISA. Results are shown as the mean O.D 450 nm of undiluted fecal supernatant ± SEM.

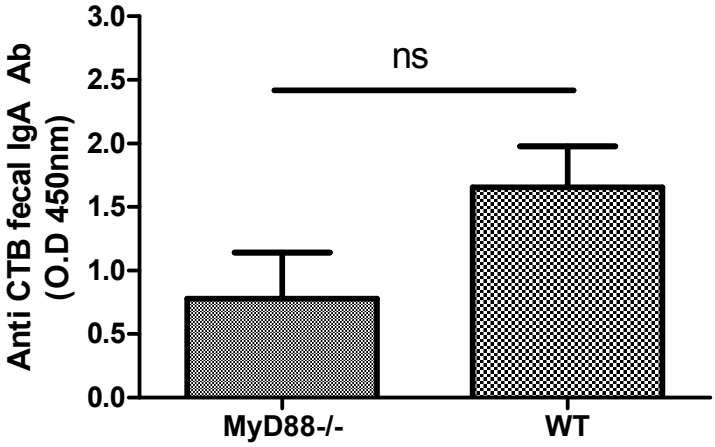
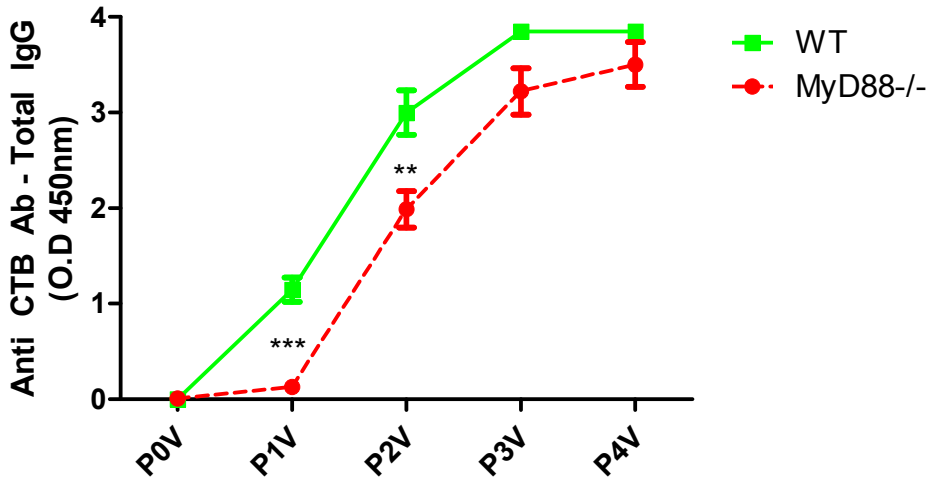
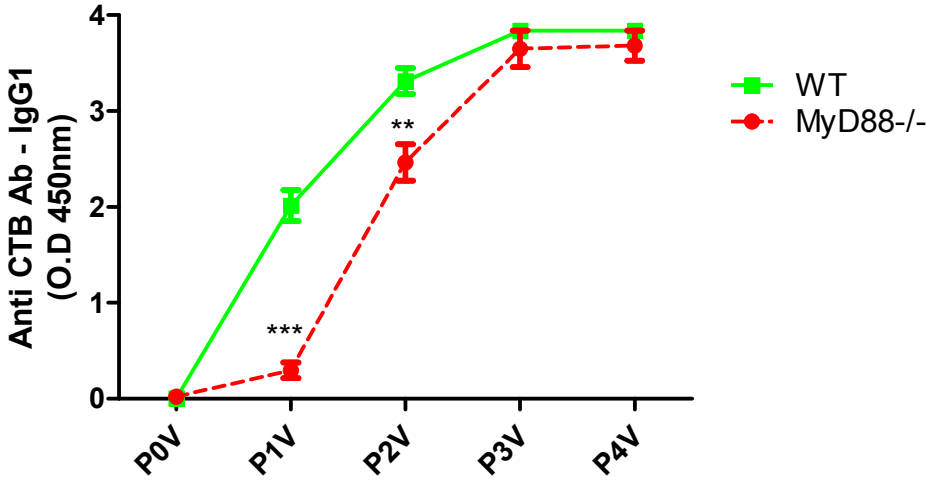

Anti CTB fecal IgA Ab
(O.D 450nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ns
MyD88-/-
WT

Figure 31. MyD88 signaling played a critical role in the induction of CTB-specific serum antibodies following intramuscular immunization with Dukoral. MyD88$^{-/-}$ and WT mice were immunized intramuscularly on days 0, 10, 20 and 30 with Dukoral (1x10^{7} *V. cholerae* with 10 μg CTB). Serum was collected 9 days after each vaccination, and CTB-specific total serum IgG (A), IgG1 (B) and IgG2c (C) responses were measured by ELISA. Results are shown as the mean O.D 450 nm ± SEM. **p<0.01, ***p<0.001. P0V – P4V are as defined in the legend to Figure 2.

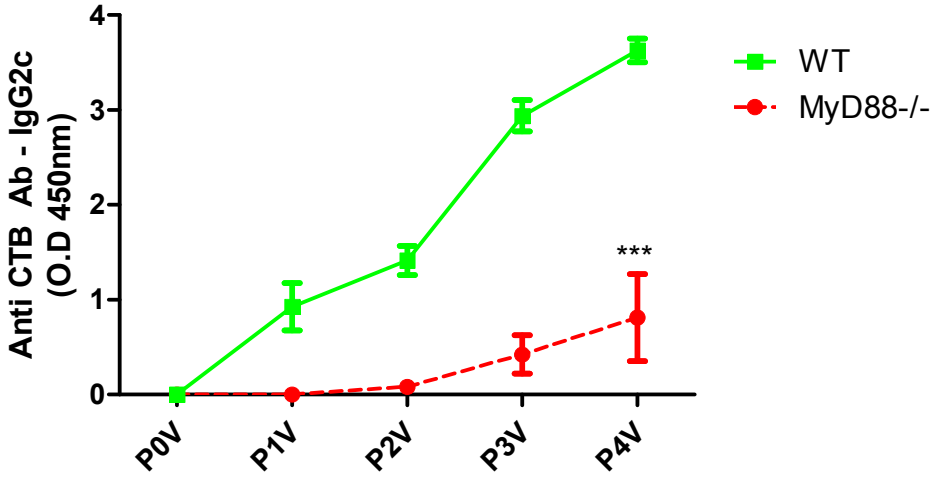
A


Anti CTB Ab - Total IgG
(O.D 450nm)
4
3
2
1
0
***
**
P0V
P1V
P2V
P3V
P4V
WT
MyD88-/-

B


Anti CTB Ab - IgG1
(O.D 450nm)
4
3
2
1
0
***
**
P0V
P1V
P2V
P3V
P4V
WT
MyD88-/-

C


Anti CTB Ab - IgG2c
(O.D 450nm)
4
3
2
1
0
***
P0V
P1V
P2V
P3V
P4V
WT
MyD88-/-

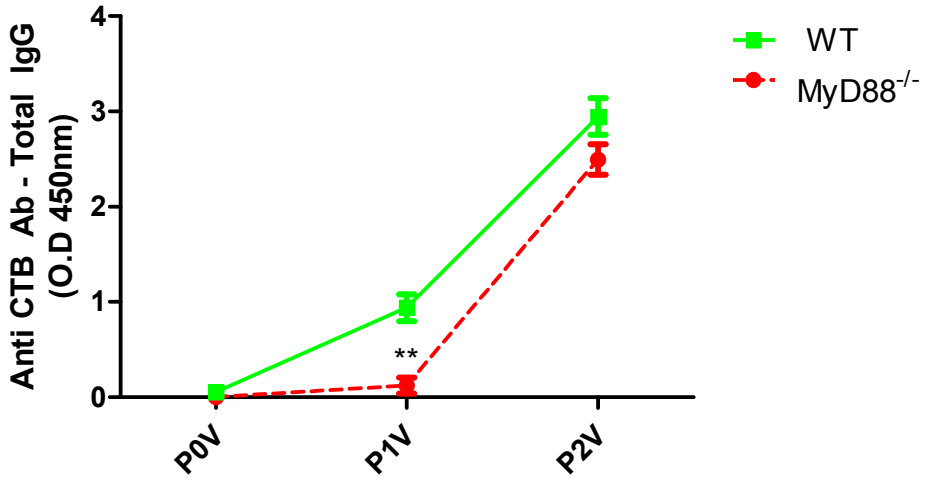
In a follow-up study, CTB-specific humoral responses were evaluated following the intramuscular administration of CTB alone. After two intramuscular immunizations with CTB, MyD88$^{-/-}$ mice showed significantly decreased production of CTB-specific total IgG ($p=0.0032$) and IgG1 ($p=0.0426$) responses, but not IgG2c (Figure 32). These findings were similar to those observed following intramuscular administration of the Dukoral vaccine (Figure 31). Thus, a differential involvement of TLR signaling in the generation of humoral immune responses against the CTB antigen was observed between intramuscular and oral immunization with both Dukoral and CTB alone. Overall, these results suggest that MyD88 signaling played a critical role in the generation of CTB-specific serum antibody responses when CTB is administered intramuscularly, compared to when CTB is given orally. This effect on CTB-specific serum antibody production was seen whether CTB was administered alone, or when administered as part of the complete Dukoral vaccine.

3.3.3 CD4+ T-cells and CD19+ B-cells from TLR mutant mice orally immunized with Dukoral were impaired in their ability to proliferate in response to CTB stimulation

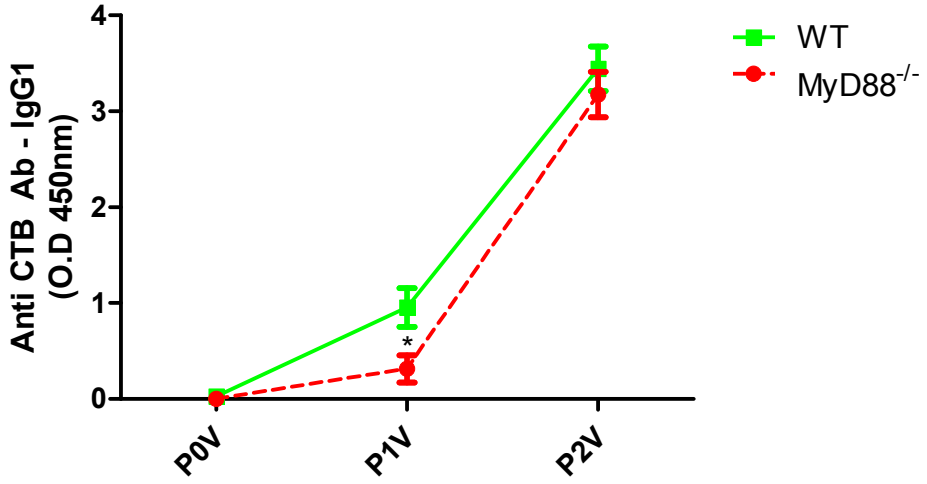
Having first evaluated humoral responses, I next sought to examine the requirement of TLR signaling on the induction of cell-mediated immune responses towards the CTB component of the Dukoral vaccine. The ability of splenocytes from WT and TLR mutant mice orally immunized with Dukoral to proliferate in response to CTB stimulation was evaluated by CFSE-dilution. The results demonstrated that CD4+ T-cells from TLR-4$^{-/-}$ mice ($p=0.0186$) were significantly inhibited in their ability to proliferate following stimulation with CTB. In contrast, CD4+ T-cells from Trif$^{-/-}$ mice were found to be significantly improved in their ability to

Figure 32. MyD88 signaling played a critical role in the induction of CTB-specific serum antibody production following intramuscular vaccination with CTB. MyD88$^{-/-}$ and WT mice were immunized intramuscularly two times with 10 μg CTB. Serum was collected 9 days after each vaccination, and CTB-specific total serum IgG (A), IgG1 (B) and IgG2c (C) responses were measured by ELISA. Results are shown as the mean O.D 450 nm ± SEM. *$p<0.05$, **$p<0.01$. P0V – P2V are as defined in the legend to Figure 2.

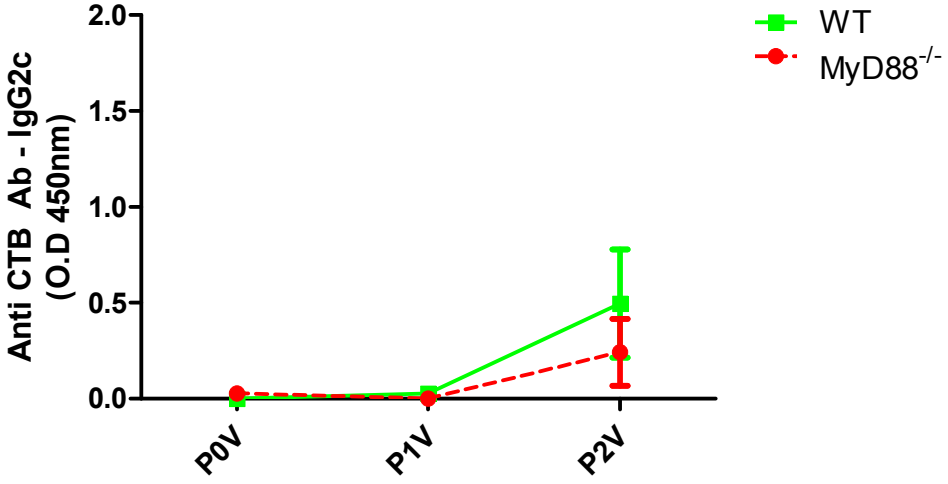
A


Anti CTB Ab - Total IgG
(O.D 450nm)
4
3
2
1
0
**
P0V
P1V
P2V
WT
MyD88-/-

B


Anti CTB Ab - IgG1
(O.D 450nm)
4
3
2
1
0
*
P0V
P1V
P2V
WT
MyD88-/-

C


Anti CTB Ab - IgG2c
(O.D 450nm)
2.0
1.5
1.0
0.5
0.0
P0V
P1V
P2V
WT
MyD88-/-

proliferate following stimulation with CTB (p=0.0049) (Figure 33). CD19+ B-cells from TLR-2$^{-/-}$(p=0.0221), TLR-4$^{-/-}$ (p=0.0315) and MyD88$^{-/-}$ (p=0.0242) mice were shown to be significantly inhibited in their ability to proliferate in response to CTB stimulation. Similar to the results obtained with CD4+ T-cells, CD19+ B-cells from Trif$^{-/-}$ mice were shown to be significantly enhanced (p=0.0023) in their ability to proliferate in response to CTB stimulation (Figure 34).

3.3.4 Splenocytes from mice orally immunized with Dukoral did not secrete IFN-γ or IL-4 following stimulation with CTB

Splenocytes from orally immunized TLR mutant and WT mice were stimulated with CTB for 72 hours to measure IFN-γ and IL-4 secretion by ELISPOT. The results showed that CTB stimulation was unable to induce any significant IFN-γ and IL-4 secretion (Figure 35).

3.3.5 The CTB component of the Dukoral vaccine induced maturation of WT bone marrow-derived DCs

I next determined the ability of the soluble CTB component of the Dukoral vaccine to induce maturation of DCs. DCs were induced from WT bone marrow cultures by stimulation with 10 ng/mL of both GM-CSF and IL-4. After 5 days, cultures were stimulated with increasing doses of soluble CTB for 24 hours, and DC maturation was evaluated by cell surface expression of CD80, CD86, CD40, and MHCII molecules. In addition, DC maturation was evaluated by measuring cytokine secretion in the culture supernatants by a 13-plex cytokine array. The results showed that soluble CTB was able to induce DC maturation as shown by increased cell-surface

Figure 33. TLR-4 positively regulated while Trif negatively regulated CD4+ T-cell proliferation in response to stimulation by CTB. TLR mutant mice were immunized orally with Dukoral ($3x10^9$ *V. cholerae* with 10 μg CTB) on days 0, 10, 20 and 30. Spleens were collected 2 weeks after the last vaccination, and splenocytes were cryo-preserved until tested. Splenocytes were labelled with 5 μM CFSE and stimulated with CTB for 5 days. Cell proliferation was evaluated by flow cytometry by the degree of CFSE dilution on fluorescently-labelled CD4+ T-cells. Results are shown as the % of CFSE-diluted from 10,000 gated events ± SEM.-, unstimulated; +, 10 μg CTB. *$p<0.05$, **$p<0.01$, ns , not significant.

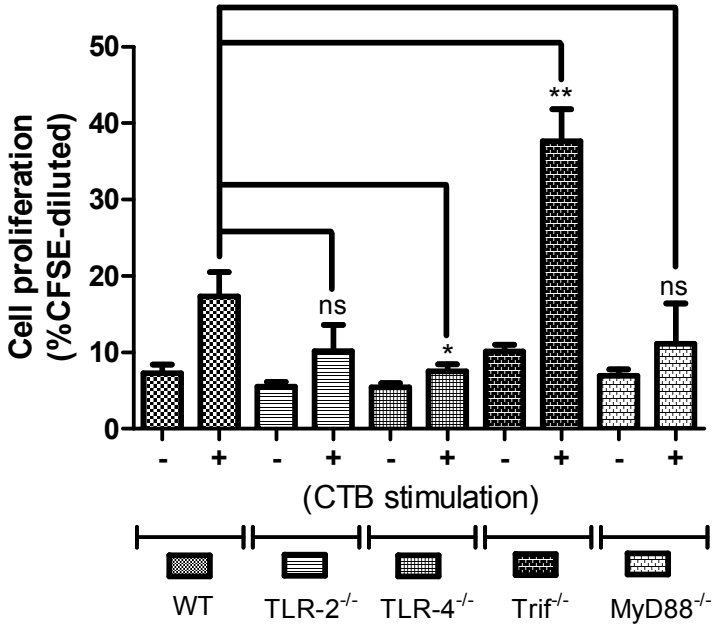

Cell proliferation
(%CFSE-diluted)
50
40
30
20
10
0
**
ns
*
ns
- + - + - + - + - +
(CTB stimulation)
WT
TLR-2-/-
TLR-4-/-
Trif-/-
MyD88-/-

Figure 34. TLR-2, TLR-4 and MyD88 positively regulated while Trif negatively regulated CD19+ B-cell proliferation in response to stimulation by CTB. TLR mutant mice (TLR-2$^{-/-}$, TLR-4$^{-/-}$, MyD88$^{-/-}$ and Trif$^{-/-}$) were immunized orally with Dukoral ($3x10^9$ *V. cholerae* with 10 μg CTB) on days 0, 10, 20 and 30. Spleens were collected 2 weeks after the last vaccination, and splenocytes were cryo-preserved until tested. Splenocytes were labelled with 5 μM CFSE and stimulated with soluble CTB for 5 days. Proliferation was evaluated by flow cytometry by the degree of CFSE dilution on fluorescently-labelled CD19+ B-cells. Results are shown as the % of CFSE-diluted from 10,000 gated events ± SEM.-, unstimulated; + , 10 μg CTB. *$p<0.05$, **$p<0.01$.

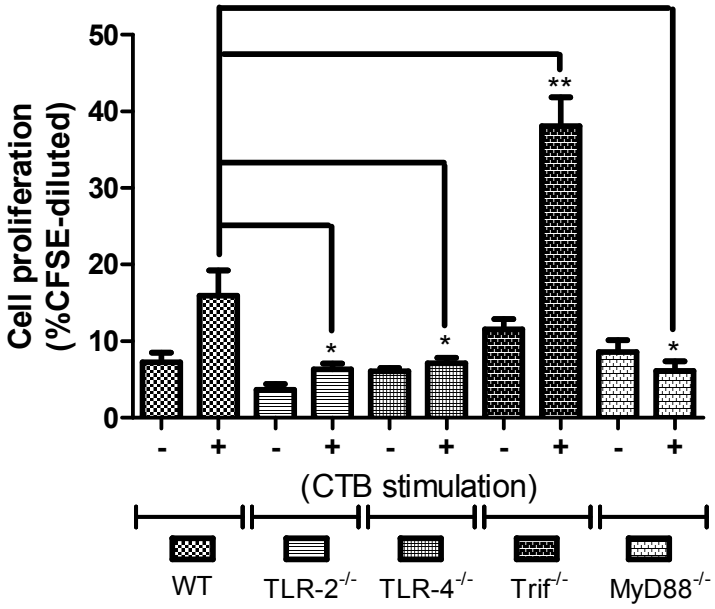
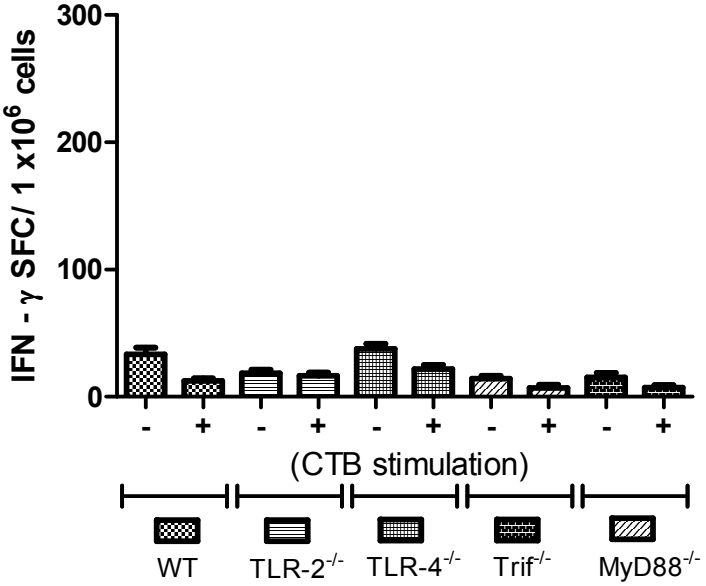
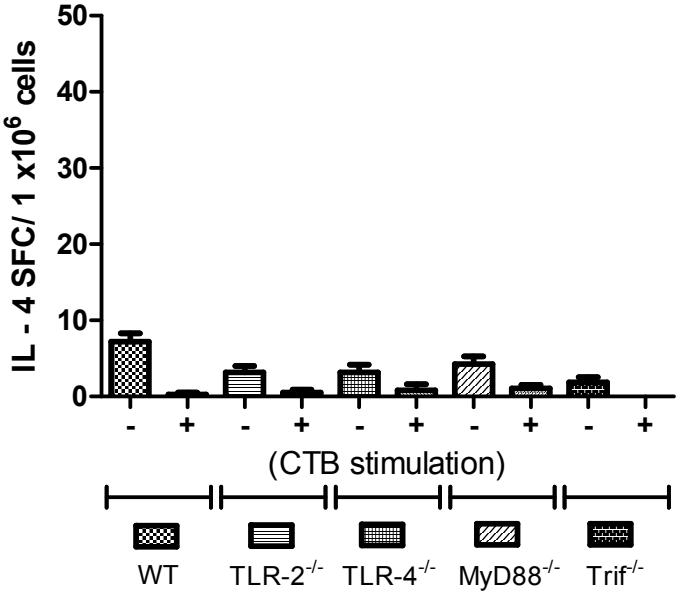

Cell proliferation
(%CFSE-diluted)
50
40
30
20
10
0
**
*
*
*
- + - + - + - + - +
(CTB stimulation)
WT
TLR-2-/-
TLR-4-/-
Trif-/-
MyD88-/-

Figure 35. Splenocytes from mice orally immunized with Dukoral did not secrete IFN-γ or IL-4 following stimulation with CTB. TLR mutant mice (TLR-$2^{-/-}$, TLR-$4^{-/-}$, $MyD88^{-/-}$ and $Trif^{-/-}$) were immunized orally with Dukoral ($3x10^9$ *V. cholerae* with 10 μg CTB) on days 0, 10, 20 and 30. Spleens were collected 2 weeks after the last vaccination, and splenocytes were cryo-preserved until tested Splenocytes were evaluated for their ability to secrete IFN-γ (A) and IL-4 (B) by ELISPOT. 250,000 splenocytes were seeded in triplicate wells and stimulated with soluble CTB for 72 hrs. Results are shown as IFN-γ or IL-4 spot-forming cells (SFC) / $1x10^6$ cells ± SEM. -, unstimulated; +, 10 μg CTB.

A


IFN - γ SFC/ 1 x10^6 cells
300
200
100
0
- + - + - + - + - +
(CTB stimulation)
WT
TLR-2-/-
TLR-4-/-
Trif-/-
MyD88-/-

B


IL - 4 SFC/ 1 x10^6 cells
50
40
30
20
10
0
- + - + - + - + - +
(CTB stimulation)
WT
TLR-2-/-
TLR-4-/-
MyD88-/-
Trif-/-

expression of co-stimulatory molecules CD80, CD86, CD40, whereas it did not appear to be an inducer of MHCII expression (Figure 36). A representative histogram is shown in Figure 37. Cell culture supernatants were tested against a panel of 13 cytokines (IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, IL-22, IL-27 and TNF-α) to measure cytokines secreted during DC maturation. Stimulation of DC cultures by soluble CTB was able to induce secretion of IL-13, IL-1α, IL-22, IL-27 and IFN-γ, in addition to very high levels of IL-6 and TNF-α (Figure 38). IL-4 secretion was also detected, however since IL-4 was added to the cell culture media it has been omitted from this analysis. Together, these results indicate that the CTB component of the Dukoral vaccine was able to induce maturation of DCs.

3.3.6 Maturation and cytokine secretion of dendritic cells in response to CTB stimulation occurred in a MyD88-dependant manner

Since the *V. cholerae* and CTB components of the Dukoral vaccine were able to induce maturation of WT bone marrow-derived DCs (Figure 36 and 38), I determined whether MyD88 knockout would inhibit DC maturation. Bone marrow-derived DCs from WT and MyD88$^{-/-}$ mice were stimulated with CTB, and the results demonstrated that DCs from MyD88$^{-/-}$ mice were inhibited in their ability to mature by being significantly impaired in their ability to up-regulate expression of CD80 following stimulation with 1 μg CTB ($p=0.0079$) and 10 μg CTB ($p=0.0106$). However, CD86 expression was not impaired by MyD88 knockout (Figure 39). CD40 expression was also shown to be significantly inhibited in MyD88$^{-/-}$ DCs following stimulation with 1 μg CTB ($p=0.0026$) and 10 μg CTB ($p=0.0039$), as was MHCII expression ($p=0.0078$) (Figure 40). A representative histogram is shown in Figure 41.

Figure 36. Bone marrow-derived DCs matured in response to stimulation by CTB. Bone marrow progenitors were cultured with 10 ng/ml of both GM-CSF and IL-4 over 5 days. Cultures were then stimulated with the indicated amounts of CTB for 24 hours. DCs were then evaluated for their ability to express CD80 (A), CD86 (B), CD40 (C) and MHCII (D) by flow cytometry. Results are shown as the MFI of three independent experiments ± SEM.

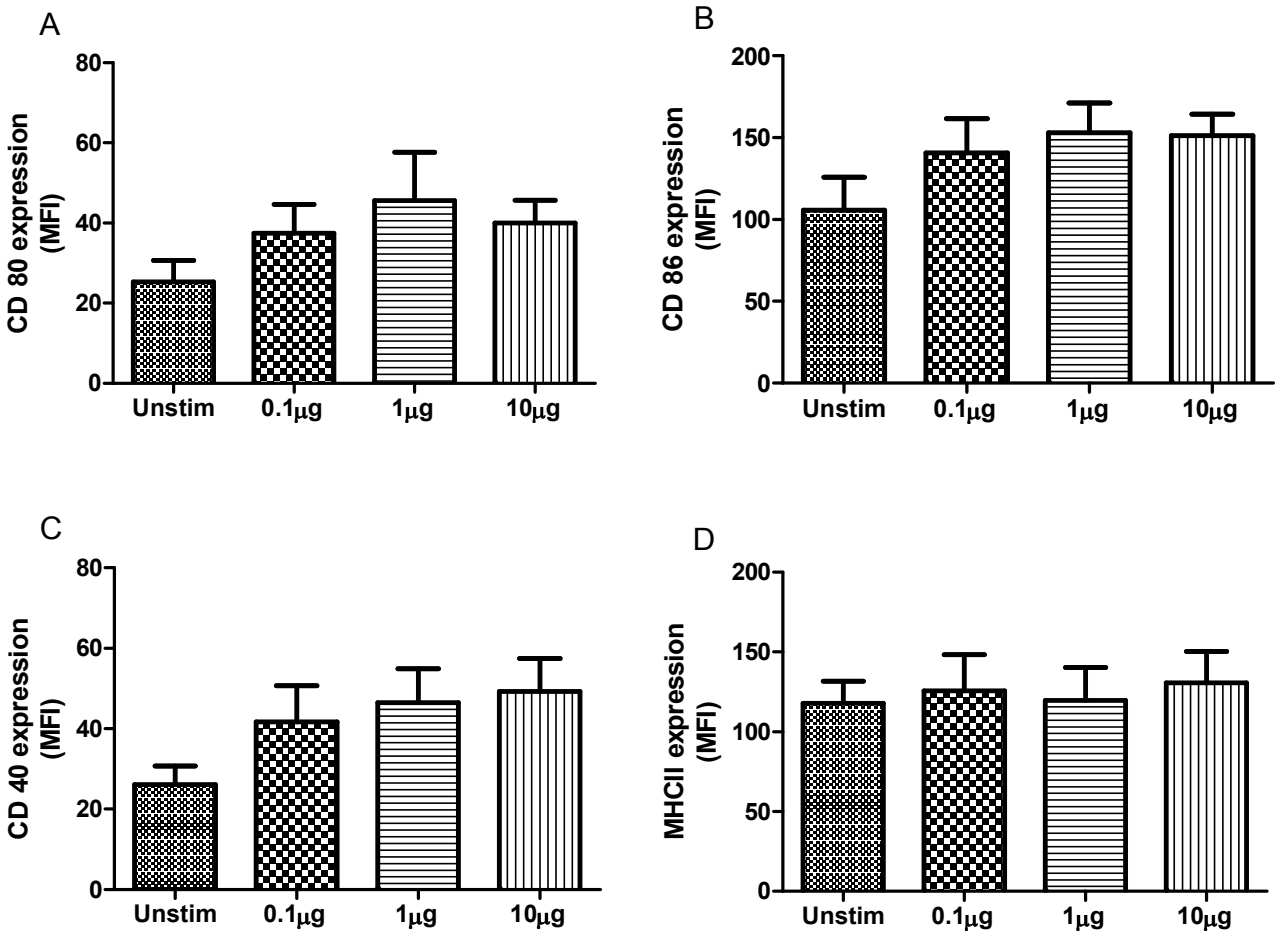

A
CD 80 expression (MFI)
80
60
40
20
0
Unstim
0.1μg
1μg
10μg
B
CD 86 expression (MFI)
200
150
100
50
0
Unstim
0.1μg
1μg
10μg
C
CD 40 expression (MFI)
80
60
40
20
0
Unstim
0.1μg
1μg
10μg
D
MHCII expression (MFI)
200
150
100
50
0
Unstim
0.1μg
1μg
10μg

Figure 37. Bone marrow-derived DCs matured in response to stimulation by CTB. Bone marrow progenitors from C57BL/6 WT mice were cultured with 10 ng/mL of both GM-CSF and IL-4 over 5 days. Cultures were then mock stimulated (filled grey histograms) or stimulated with 10 μg CTB for 24 hours (red histogram overlay) and cells were evaluated for their ability to express cell surface markers CD80 (A), CD86 (B), MHCII (C) and CD40 (D) by flow cytometry. Results shown are one representative histogram of three independent experiments.

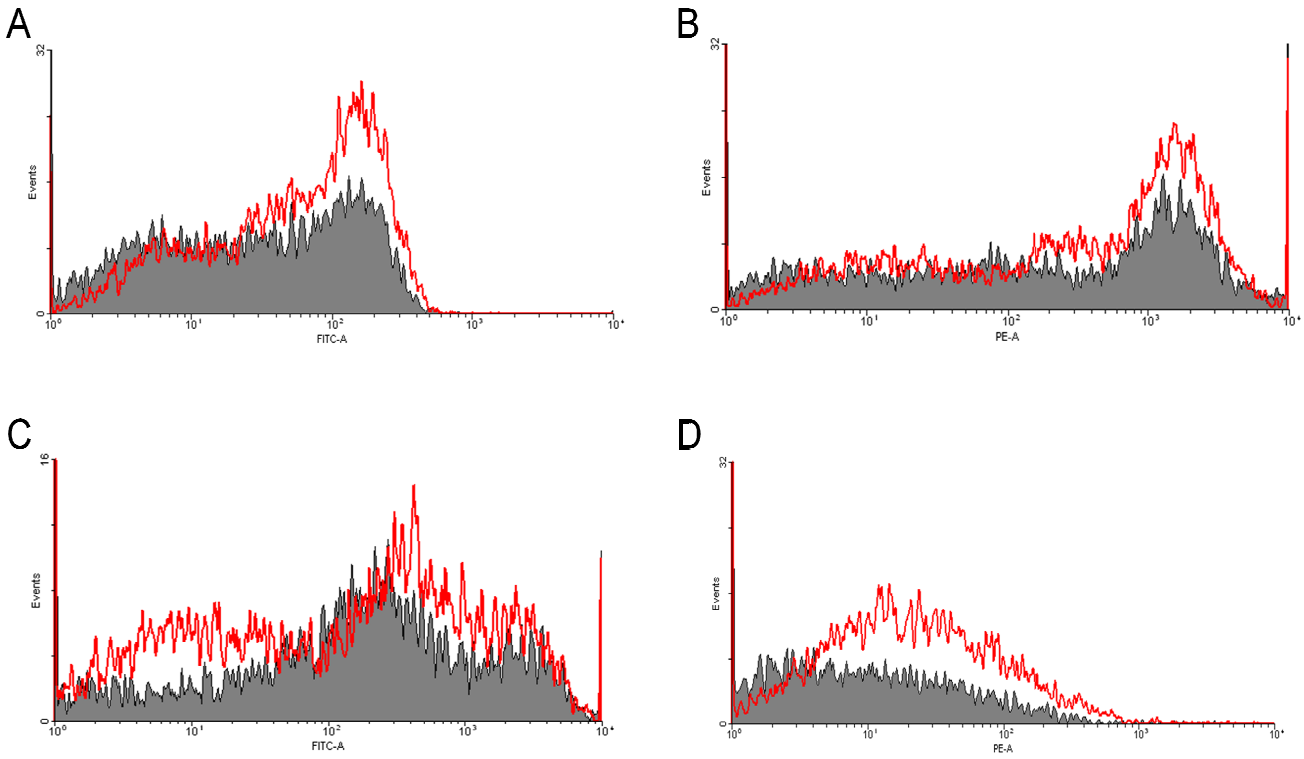

A
B
C
D
Events
FITC-A
PE-A

Figure 38. Bone marrow-derived DC cultures secreted multiple cytokines in response to CTB stimulation. Bone marrow progenitors were cultured with 10 ng/mL of both GM-CSF and IL-4 over 5 days. Cultures were then stimulated with a 10 μg of CTB for 24 hours. Cell culture supernatants were collected and frozen until tested by cytokine array. Results are shown as the mean of duplicate samples from three pooled independent experiments ± SEM.

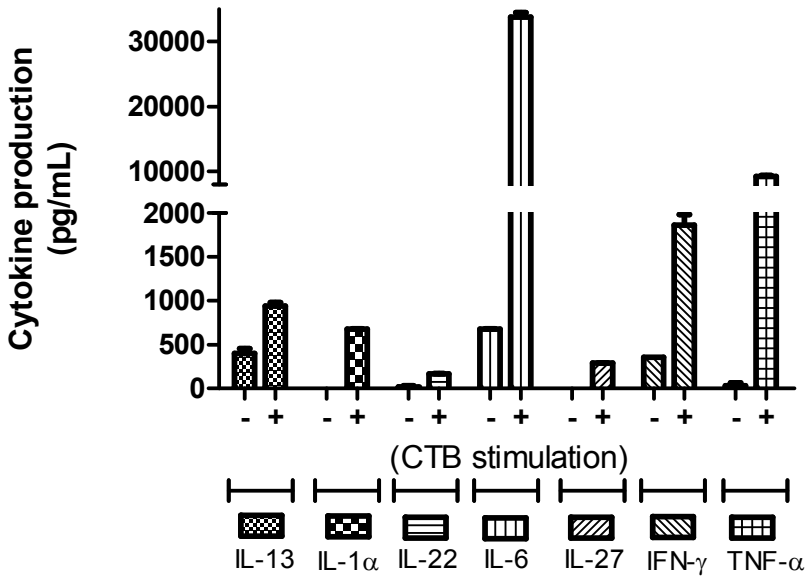
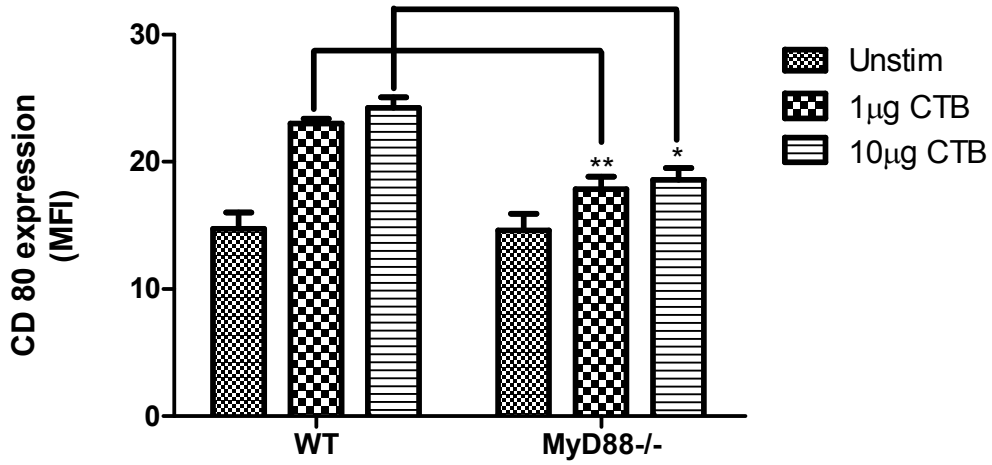

Cytokine production (pg/mL)
30000
20000
10000
2000
1500
1000
500
0
- + - + - + - + - + - +
(CTB stimulation)
IL-13
IL-1α
IL-22
IL-6
IL-27
IFN-γ
TNF-α

Figure 39. Bone marrow-derived MyD88$^{-/-}$ DCs were impaired in their ability to express CD80 but not CD86 co-stimulatory molecules in response to stimulation by CTB. Bone marrow progenitors were cultured with 10 ng/ml of both GM-CSF and IL-4 over 5 days. Cultures were then stimulated with the indicated concentrations of CTB for 24 hours and cells were evaluated for their ability to express co-stimulatory molecules CD80 (A) and CD86 (B) by flow cytometry. Results are shown as the MFI of three independent experiments ± SEM. *$p<0.05$, **$p<0.01$, ns: not significant. Unstim denotes cells that received mock treatment comprised of media alone.

A


CD 80 expression (MFI)
30
20
10
0
**
*
WT
MyD88-/-
Unstim
1μg CTB
10μg CTB

B

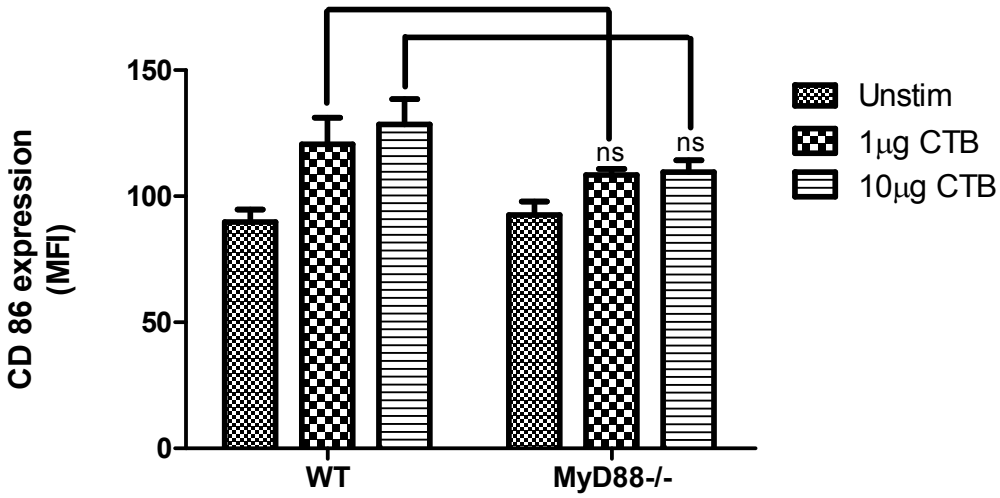
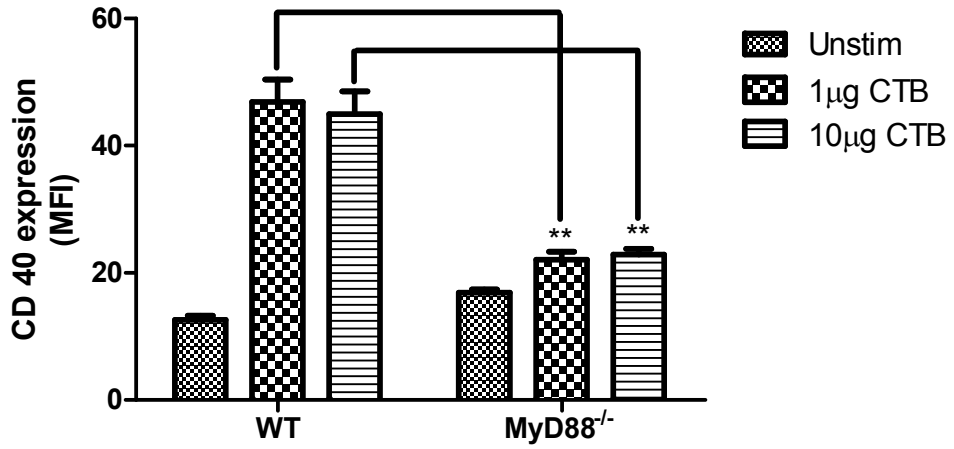

CD 86 expression (MFI)
150
100
50
0
ns
ns
WT
MyD88-/-
Unstim
1μg CTB
10μg CTB

Figure 40. Bone marrow-derived MyD88$^{-/-}$ DCs were impaired in their ability to express C40 and MHCII in response to stimulation by CTB. Bone marrow progenitors were cultured with 10 ng/ml of both GM-CSF and IL-4 over 5 days. Cultures were then stimulated with the indicated concentrations of CTB for 24 hours and cells were evaluated for their ability to express co-stimulatory molecules CD40 (A) and MHCII (B) by flow cytometry. Results are shown as the MFI of three independent experiments ± SEM. *$p<0.05$, **$p<0.01$. Unstim denotes cells that received mock treatment comprised of media alone.

A


60
40
20
0
CD 40 expression (MFI)
**
**
WT
MyD88-/-
Unstim
1μg CTB
10μg CTB

B

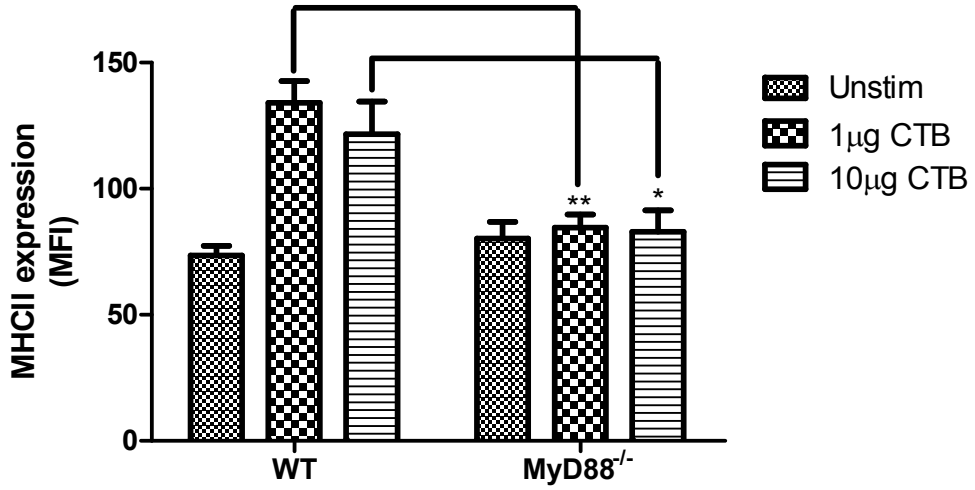
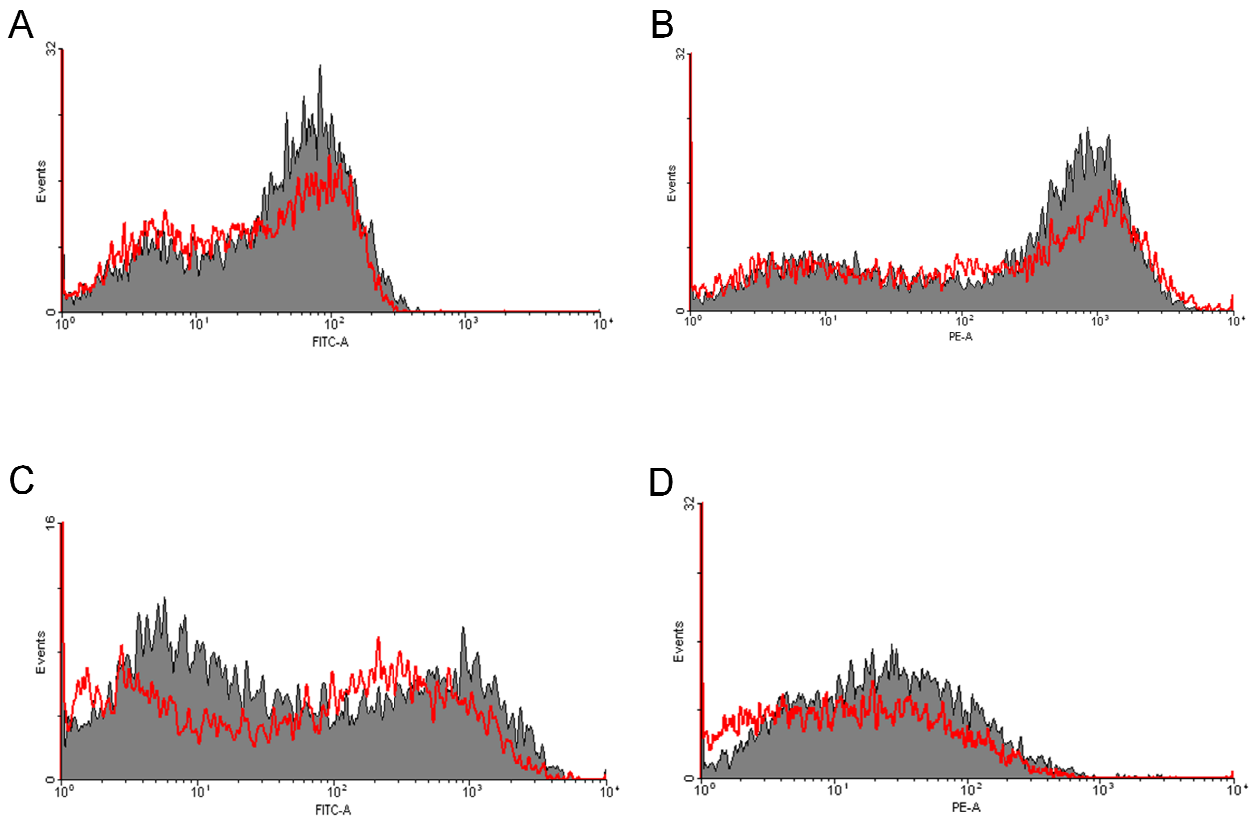
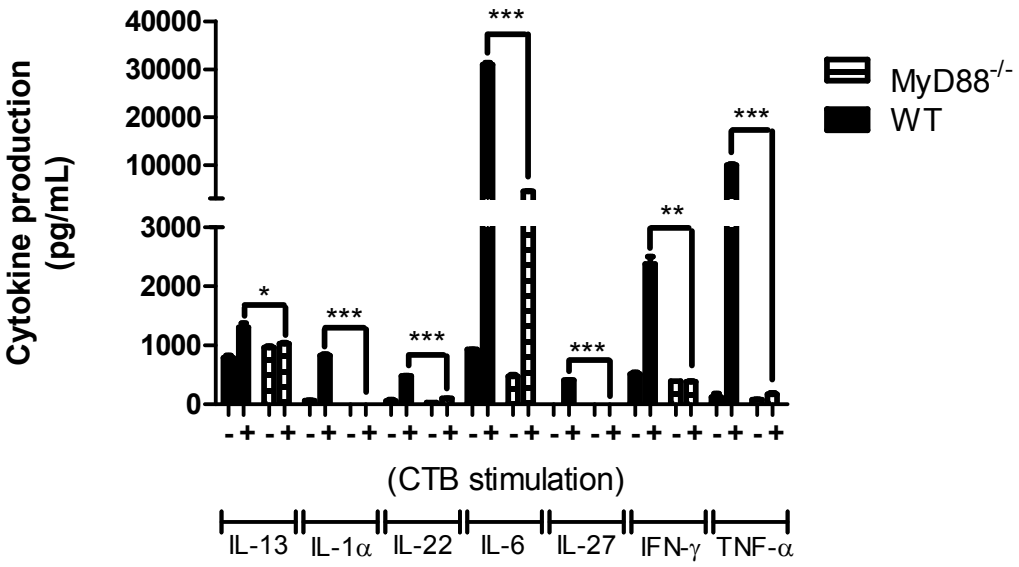

150
100
50
0
MHCII expression (MFI)
**
*
WT
MyD88-/-
Unstim
1μg CTB
10μg CTB

Figure 41. Bone marrow-derived DCs from MyD88$^{-/-}$ mice were impaired in their ability to mature in response to stimulation by CTB. Bone marrow progenitors from C57BL/6 WT mice (filled grey histograms) and MyD88$^{-/-}$ mice (red overlay) were cultured with 10 ng/mL of both GM-CSF and IL-4 over 5 days. Cultures were then stimulated with 10 μg CTB for 24 hours and cells were evaluated for their ability to express cell surface markers CD80 (A), CD86 (B), MHCII (C) and CD40 (D) by flow cytometry. Results shown are one representative histogram of three independent experiments.


A
B
C
D
Events
FITC-A
PE-A
32
16
0

The results showed that DCs from MyD88$^{-/-}$ mice were significantly inhibited in their ability to secrete IL-13 ($p=0.05$), IL-22 ($p=0.0007$), IL-6 ($p=0.003$), IFN-γ ($p=0.0041$) and TNF-α ($p=0.0004$), and were unable to secrete IL-1α ($p=0.0006$) and IL-27 ($p=0.0001$) following stimulation with CTB (Figure 42). Interestingly, despite being decreased, elevated levels of IL-6 still remained in the DC culture supernatant when stimulated with CTB, compared to when DC cultures were stimulated with *V. cholerae* (Figure 26). The remaining cytokines were not found above the limit of detection of the cytokine array kit. These results suggest that TLR signaling was important for the maturation of DCs in response to stimulation by CTB.

Figure 42. Bone marrow-derived MyD88$^{-/-}$ DCs were inhibited in their ability to secrete cytokines in response to CTB stimulation. Bone marrow progenitors were cultured with 10 ng/mL of both GM-CSF and IL-4 over 5 days. Cultures were then stimulated with 10 μg CTB for 24 hours. Cell culture supernatants were collected on day 6 and frozen until tested by cytokine array. Results are shown as the mean of duplicate samples from three pooled independent experiments ± SEM. *$p<0.05$, ** $p<0.01$, ***$p<0.001$.


Cytokine production (pg/mL)
40000
30000
20000
10000
3000
2000
1000
0
MyD88-/-
WT
*
***
***
***
***
**
***
- + - + - + - + - + - + - + - + - + - + - + - + - + - +
(CTB stimulation)
IL-13
IL-1α
IL-22
IL-6
IL-27
IFN-γ
TNF-α

CHAPTER IV: DISCUSSION

My results have shown that the induction of humoral responses (*V. cholerae*- and CTB-specific serum IgG and fecal IgA antibodies), following oral immunization with the Dukoral vaccine, as well as with CTB alone, occurred in a TLR-independent manner. Interestingly, a differential requirement of TLR signaling following intramuscular vaccination with Dukoral or CTB alone, in contrast to oral vaccination, was observed for the generation of CTB-specific serum IgG antibodies. The induction of cell-mediated immune responses (cell proliferation and cytokine secretion) was found to be TLR-dependent.

I also investigated the role of TLR signaling in DC maturation in response to stimulation with Dukoral vaccine components. My results showed that MyD88$^{-/-}$ DCs were inhibited in their ability to mature, as measured by the impaired cell-surface expression of CD80, CD86, CD40 and MHCII, as well as their ability to secrete cytokines following stimulation with Dukoral vaccine components. My results also showed that despite the inability of My88$^{-/-}$ DCs to mature in response to stimulation by Dukoral vaccine components, MyD88$^{-/-}$, and Trif$^{-/-}$, TLR-2$^{-/-}$, TLR-4$^{-/-}$ mice all generated *V. cholerae*-specific and CTB-specific serum IgG and fecal IgA antibody responses which were comparable to those seen in WT animals, following oral immunization with Dukoral. These results further suggest that in addition to TLR signaling, DC maturation may also not be critical for the induction of humoral immune responses to the orally administered Dukoral vaccine.

CTB as an orally administered adjuvant

There is evidence to suggest that CTB exhibits both adjuvant [233] [234] as well as tolerizing effects [214]. Therefore, it was of interest to determine whether orally administered CTB, given in the context of the Dukoral vaccine, acted as an adjuvant for humoral *V. cholerae*-specific immune responses. My results showed that orally administered CTB significantly enhanced *V. cholerae*-specific serum IgG as well as fecal IgA production following oral immunization with the Dukoral vaccine (Figure 10). In agreement with my findings, a recent study by Lei et al. showed that mice orally immunized with a bacterial vector expressing the avian influenza hemagglutinin (HA) protein, along with CTB, showed significantly higher HA-specific serum IgG and fecal IgA responses, compared to animals orally immunized without CTB [205]. Similar findings have also been reported following oral immunization of mice and guinea pigs using *Porphyromonas gingivalis* fimbrial antigens and foot-and-mouth disease virus [235] [233].

Several mechanisms underlying the adjuvant effect of CTB have been suggested. Lycke et al. found that administration of CT, but not CTB, resulted in significantly increased gut permeability with a concomitant increase in the immune response to an orally co-administered model antigen, keyhole limpet hemocyanin (KLH). The observed increase in gut permeability, attributed to the catalytic activity of the A subunit, was postulated as being the cause of the augmented anti-KLH immune response [236]. Others have suggested that coupling antigens to CTB improves their uptake across mucosal barriers [237]. Thus, whether CTB enhances immune responses via increased gut permeability remains to be more precisely determined. The in-vivo adjuvant effect the *E. coli* enterotoxin B subunit has been suggested to depend upon the binding

of its B subunit to its receptor, GM1, expressed on all eukaryotic cells [238]. Given the similarities between the *E. coli* and *V. cholerae* toxins, I hypothesize whether CTB may act in a similar fashion. CTB has also been shown to positively enhance the antigen-presentation abilities of macrophages and B-cells, in addition to dendritic cells, and to up-regulate the expression of co-stimulatory molecules CD40 and CD86 on macrophages [213]. Conversely, the tolerizing ability of CTB has been attributed to the induction of B-cell-dependent Foxp3(+) regulatory T-cells, and to the induction of B-cell-independent Foxp3(-) regulatory T-cells expressing immune-suppressive transforming growth factor beta (TGF-β) or IL-10 [239]. Inhibition of IL-6 production is also thought to be involved [239]. Although CTB may act at multiple points to exert its adjuvant effect, further studies are needed to precisely investigate the mechanisms underlying the adjuvant effect of CTB.

TLR signaling in the generation of humoral immune responses

My results showed that the generation of humoral immune responses to the orally administered Dukoral vaccine occurred in a TLR-independent manner for both *V. cholerae*-specific (Figure 11) and CTB-specific serum IgG antibodies (Figure 27). Serum *V. cholerae*-specific IgG1 and IgG2c, and *V. cholerae*-specific fecal IgA responses, in all groups tested (MyD88$^{-/-}$, Trif$^{-/-}$, TLR-2$^{-/-}$ and TLR-4$^{-/-}$) were found to be comparable to those seen in WT mice. Similar to the *V. cholerae*-specific serum IgG results, the agglutinating ability of *V. cholerae*-specific antibodies was also found to occur independently of MyD88 signaling, regardless of the route of immunization (Figure 14). Although orally immunized animals produced elevated levels of *V. cholerae*-specific serum antibodies, agglutinating antibody titers were significantly lower, compared to the mice immunized intramuscularly (Figure 15). The mechanisms responsible for

the differential induction of binding antibodies versus agglutinating antibodies are not clear at this time. As well, given that MyD88$^{-/-}$ mice and Trif$^{-/-}$ mice still contain functional Trif and MyD88 signaling molecules, respectively, and since the co-operative effect of MyD88 and Trif signaling is not yet well established, it is not possible to completely rule out the involvement of TLR signaling in the generation of humoral immune responses and agglutinating antibodies. Such confirmation will only be possible with studies with MyD88$^{-/-}$Trif$^{-/-}$ double-deficient animals.

As protection against *V. cholerae* infection has been attributed to intestinal IgA antibodies [179], I subjected fecal supernatants from orally immunized WT and TLR-deficient mice to the same agglutination assay used herein. However, the results were inconclusive as agglutinating antibodies specific for *V. cholerae* from fecal supernatants from any group, including WT mice, were not detectable. The reasons for this are not clear at this time. Such investigation should be re-examined in future work.

Interestingly, following intramuscular administration of the Dukoral vaccine, post-1st and post-2nd vaccination CTB-specific serum total IgG and IgG1 responses were significantly impaired in MyD88$^{-/-}$ mice (Figure 31). These observations were confirmed when CTB alone was administered intramuscularly (Figure 32). Despite the post-1st and post-2nd vaccination impairment in the generation of CTB-specific serum total IgG and IgG1 antibodies, subsequent immunizations were able to boost the response such that post-4th vaccination IgG1 responses in MyD88$^{-/-}$ animals were nearly identical to those seen in WT animals (Figure 31). Moreover, TLR dependence for the generation of humoral immune responses was lost when animals were

immunized orally with the same CTB antigen (Figure 28). In addition, MyD88$^{-/-}$ animals showed significantly impaired CTB-specific serum IgG2c antibody induction even after the 4th intramuscular vaccination with Dukoral (Figure 31 C). Barr et al. have recently suggested that defective IFN-γ production by T-cells may explain impaired induction of serum IgG2c antibodies in mice with MyD88-deficient B-cells [240]. Splenocytes from all TLR-deficient animals were significantly impaired in their ability to secrete IFN-γ in response to *V. cholerae* stimulation (discussed below). Whether this explains the observed defect in the generation of CTB-specific serum IgG2c antibodies following intramuscular vaccination with Dukoral remains to be determined. Taken together, these findings suggest that i) MyD88 signaling is important for the induction of CTB-specific serum IgG1 and IgG2c responses (and by extension, for the initiation of serum IgG responses to soluble proteins) when injected intramuscularly, and that ii) the requirement of MyD88 signaling for the induction of CTB-specific serum antibodies is lost if the antigen is administered orally.

To the best of my knowledge these results represent novel findings regarding the role of TLR signaling in the induction of humoral immune responses to the Dukoral vaccine. There is evidence for the independence of TLR signaling on the induction of immune responses to a related intestinal Gram-negative pathogen, *Salmonella*. Kweon et al. demonstrated that both serum and fecal antibody responses against an exogenous protein antigen, *Streptococcus* surface protein A (PspA), following oral immunization by a *Salmonella* vector expressing PspA, occurred independently of MyD88 signaling [174]. Subsequent findings by their group also showed that systemic and mucosal LPS-specific IgG and IgA responses, following oral immunization with an attenuated *Salmonella* strain, also occurred independently of MyD88 and

TLR-4 signaling [241]. Seibert et al. recently showed in a *Salmonella typhi* infection model (via intravenous injection with an attenuated *Salmonella* strain) that MyD88$^{-/-}$, TLR-4$^{-/-}$, and TLR-2$^{-/-}$TLR-4$^{-/-}$ and WT animals were able to generate comparable levels of antibody responses against whole-cell *Salmonella typhi* [175]. This is in agreement with my findings, which showed that MyD88 signaling was dispensable for the generation of whole-cell *V. cholerae*-specific serum IgG antibodies when Dukoral was administered orally or intramuscularly (Figure 11 and 13).

The involvement of TLR signaling in humoral antibody responses may also depend on the route of administration of the vaccine and on the nature of the antigen(s). Cervantes-Barragan et al. have previously shown TLR signaling to be important for immune responses to *Salmonella typhi* porins administered via intra-peritoneal injection [242] . Their findings indicated that mice deficient in MyD88, Trif, and TLR-4 exhibited significantly inhibited serum antibody responses against purified *Salmonella* porin proteins. Furthermore TLR-2 and TLR-4 signaling specifically on B-cells was critical for the anti-porin IgM response, while TLR-2 was also shown to be critical on B-cells for the anti-porin IgG response. Overall, their findings suggested that TLR signaling was required for serum anti-porin antibody responses, following intra-peritoneal injection, and that deficiencies in TLR signaling particularly affected the induction of IgG2a/c and IgG3 antibody isotypes. Similarly, Massari et al. demonstrated that the ability of neisserial porins to induce CD86 and MHCII expression on B-cells was TLR-2- and MyD88-dependent [243]. Of interest, Barr et al. have recently demonstrated that the induction of the IgG2c antibody isotype, following parenteral immunization, was absolutely dependent upon MyD88 signaling specifically in B-cells, and this defect in IgG2c production was thought to be due to a defect in

IFN-γ production by T-cells [240]. My results from intramuscular immunization of MyD88$^{-/-}$ mice with Dukoral also showed similar findings, as the induction of CTB-specific IgG2c antibodies was found to be significantly inhibited even after the 4th immunization (Figure 31 C).

Protection against *Salmonella* infection appears to be mediated to a larger extent by cell-mediated immune responses [174-176,241]. Since cell-mediated immune responses are regulated by TLR signaling, the absence of MyD88 signaling may very well explain the indispensability of TLR signaling for protection against *Salmonella* infection. This is in contrast to my current findings, which showed that serum from orally immunized MyD88$^{-/-}$ mice was not impaired in its ability to agglutinate live *V. cholerae*. These findings suggest that MyD88 signaling may be dispensable for protection from *V. cholerae* infection. As protection against *V. cholerae* is antibody mediated [179], and all TLR-deficient and WT groups of animals tested in our study showed comparable levels of *V. cholerae*- and CTB-specific serum IgG and fecal IgA antibodies, I hypothesize whether MyD88 may also be dispensable for protection from *V. cholerae* challenge. It was not possible for me to conduct a challenge study with *V. cholerae* as I did not have access to a suitable model nor access to live pathogenic *V. cholerae*, in order to assess the requirement of TLR signaling for survival against *V. cholerae* challenge. Recently, Nygren et al. have developed an adult mouse challenge model capable for assessing the protective efficacy of experimental cholera vaccines [185]. Future use of this model may prove useful in concretely elucidating the requirement of MyD88 signaling in protection from challenge by *V. cholerae*.

Of particular interest was the finding that MyD88$^{-/-}$ DCs were still able to secrete IL-6 into the supernatant following stimulation with CTB (Figure 42). A recent study also demonstrated MyD88-independent production of IL-6 [244]. Friis et al. observed continued IL-6 production by Caco-2 cells following *C. jejuni* infection, despite MyD88-knockdown by short

interfering RNA complexes [244]. IL-6 has been described to have profound effects on B-cells by promoting plasma cell differentiation and antibody production [245], as well as enhancing intestinal IgA production [100]. IL-6 has also been shown to increase expression of endothelial leukocyte adhesion molecules such as VCAM-1 and ICAM-1, thereby promoting leukocyte retention and accumulation [246, 247]. Taken together, I speculate whether such residual IL-6 production in MyD88$^{-/-}$ animals, following the oral administration of Dukoral, may be contributing to the induction of antibody responses comparable to those seen in WT animals, by directly stimulating MyD88$^{-/-}$ B-cells. Additional cytokines apart from IL-6, including those not examined here, may also be involved. Further studies are necessary to explore these findings. Furthermore, previous findings have demonstrated the ability of CTB-linked antigen to enhance the antigen-presenting ability of B-cells [213, 248]. Taking these findings together, I hypothesize whether the combined effects of IL-6 production and CTB stimulation may be acting in a compensatory fashion to stimulate B-cells in the absence of TLR signaling, thereby providing a possible explanation for how MyD88$^{-/-}$ animals were able to generate *V. cholerae*- and CTB-specific antibody responses comparable to those seen in WT mice, following oral immunization. Whether such B-cell stimulatory activities are also responsible for the serum and fecal antibody responses observed in the other TLR-deficient animals evaluated in this study also remains to be determined.

In summary, the requirement of TLR signaling for the generation of humoral immune responses may depend on many factors such as the antigen/ adjuvant combination, as well as the route of immunization. My results suggest that TLR signaling may be critical for the induction of humoral responses, particularly if the antigen is administered parenterally. As such, the TLR-

dependence for the induction of humoral responses to other pathogens or antigens will likely have to be determined individually, and for various routes of administration.

TLR signaling in the generation of cell-mediated immune responses

My results have shown TLR signaling to be critical to the generation of cell-mediated immune responses. CD4+ T-cell proliferation in response to *V. cholerae* stimulation was TLR-2 and MyD88-dependent, while CD19+ B-cell proliferation was TLR-2, TLR-4 and MyD88-dependent (Figure 17) CD4+ T-cell proliferation in response to CTB stimulation was TLR-4-dependent, while CD19+ B-cell proliferation was TLR-2, TLR-4 and MyD88-dependent (Figure 33 and 34). Findings from the literature have previously shown that TLR signaling plays an important role in cell-mediated immune responses [249,250].

Splenocytes from Trif$^{-/-}$ mice were unimpaired in their ability to proliferate in response to stimulation by whole-cell *V. cholerae* (Figure 17). These findings suggest that both CD4+ T-cell and CD19+ B-cell proliferation in response to stimulation by *V. cholerae* occurred preferentially via the MyD88-dependent pathway.

A peculiar finding was that CD4+ T-cells and CD19+ B-cells from Trif$^{-/-}$ mice were significantly enhanced in their ability to proliferate following stimulation with CTB (Figure 33 and 34). Hasan et al. have shown that type I interferon production induced by TLR-3 and TLR-4-mediated Trif signaling was inhibitory for cell cycle entry and proliferation [251, 252] and that such cell cycle arrest was blocked when the production of type I IFNs was blocked. A recent study by Seregin et al. evaluated the molecular mechanisms responsible for the observed adjuvant effect of a recombinant protein derived from *Eimeria tenella* (rEA). Their results showed that Trif-deficient mice exhibited dramatic increases in immune cell activation,

compared to WT animals, following stimulation with rEA protein, and such effects were seen across multiple cell types [253]. Their results also showed that the presence of Trif suppressed the release of pro-inflammatory cytokines by DCs in response to stimulation by multiple TLR ligands (TLR-4, TLR-7/8 and TLR-9 ligands) [253]. Previous findings by this group also demonstrated the ability of Trif to negatively regulate the production of antigen-specific IgG antibodies to an injected antigen [254]. Given the evidence for the inhibitory effects of Trif in antigen-induced cell proliferation, I speculate whether Trif may be also acting as a negative regulator in the context of my results with CTB-induced proliferation. In addition to the improved cell proliferation observed in response to CTB stimulation, Trif$^{-/-}$ animals did not show any decreased *V. cholerae*-specific or CTB-specific fecal IgA antibody production (Figure 12 and 29). As well, Trif$^{-/-}$ animals showed an earlier induction of CTB-specific serum IgG2c antibodies compared to WT animals or other TLR-deficient animals (Figure 27), although this observed increase was not statistically significant. Further studies are needed to address the role of Trif in antigen-induced cell proliferation and antibody production, as well as the larger role of Trif in cell–mediated immune responses.

As might be expected, based on their well-described role of TLR signaling in the production of proinflammatory cytokines, all groups of TLR-deficient mice tested (MyD88$^{-/-}$, TLR-2$^{-/-}$, TLR-4$^{-/-}$, and Trif$^{-/-}$) were significantly impaired in their ability to secrete IFN-γ in response to *V. cholerae* stimulation (Figure 18). These results suggest that IFN-γ production utilizes both the MyD88- and Trif-dependent pathways. My results also showed that TLR-2$^{-/-}$ animals were significantly impaired in their ability to produce IL-4 in response to stimulation by *V. cholerae* (Figure 19). These results suggest that i) TLR-2 may be the primary receptor for *V. cholerae*-induced IL-4 production, and ii) Trif and MyD88 may perhaps be acting in

compensatory fashion to allow for the continued production of IL-4 in the absence of the other. Previous findings by Hajishengallis et al. have demonstrated the ability of CTB to signal via TLR-2. Their results showed that CTB induced cytokine production in THP-1 cells in a TLR-2-dependent manner [222]. Such findings provide evidence in support of the TLR-simulating ability of CTB. Further work is required to confirm these findings.

In my hands, under the conditions described herein, CTB stimulation was not an inducer of either IFN-gamma or IL-4 secretion in splenocytes from either WT or TLR-deficient mice (Figure 35). CTB was shown to induce IFN-γ in DCs, as discussed below.

TLR signaling in the maturation of bone marrow-derived DCs

Since the development of humoral responses following oral immunization with Dukoral was found to be independent of TRL signaling, in contrast to the development of cell-mediated immune responses, and since DCs play a critical role in the development of both humoral and cell-mediated immune responses, I investigated the role of TLR signaling in the maturation of WT and MyD88$^{-/-}$ bone-marrow derived DCs in response to stimulation by Dukoral vaccine components. DC maturation was measured by the expression of cell surface co-stimulatory molecules and cytokine secretion. My results showed that DC maturation in responses to stimulation by *V. cholerae* was found to be MyD88-dependent (Figures 23, 24, 26, and 39, 40 and 42). The cell surface expression of CD80, CD86, CD40 and MHCII molecules was inhibited in MyD88$^{-/-}$ DCs, as was the secretion of inflammatory cytokines during the maturation process, following stimulation with *V. cholerae*. However, in response to stimulation with CTB, expression of CD80, CD40, and MCHII, but not CD86, was found to be MyD88-dependent. It remains to be seen whether the induction of CD86, in response to stimulation with CTB, occurs via the Trif-dependent pathway. All cytokines induced during the DC maturation process were

significantly inhibited in MyD88$^{-/-}$ DCs following stimulation with *V. cholerae* and CTB. In spite of the fact that maturation of DCs was MyD88-dependent, humoral responses following oral immunization with Dukoral, however, occurred independently of TLR signaling, as well as of DC maturation. Whether MyD88$^{-/-}$ DCs are inhibited from maturing in-vivo, following oral immunization with Dukoral, remains to be ascertained. Overall, these findings indicate a critical role of MyD88 signaling in the maturation of DCs in response to stimulation by *V. cholerae* and CTB.

CHAPTER V: CONCLUDING REMARKS AND FUTURE DIRECTIONS

Only a limited number of orally administered vaccines have been licensed, and in many instances the mechanistic details underlying the protective immune responses, including the requirement of TLR signaling, remain largely unknown. Herein, I investigated the requirement of TLR signaling in the induction of humoral and cell-mediated immune responses to the orally administered Dukoral vaccine. My results have demonstrated that antibody production, and in collaboration with Helen Tabor from the Winnipeg National Microbiology Laboratory, antibody agglutinating ability, following oral immunization with the Dukoral vaccine as well as with CTB alone, occurred in a TLR-independent manner. However, the initiation of CTB-specific humoral immune responses, following intramuscular immunization with Dukoral as well as with CTB alone, was found to occur in a MyD88-dependent manner. Thus, the requirement of TLR signaling in the induction of humoral responses may vary, depending on the nature of the antigen as well as the route of vaccination employed. Cell-mediated immune responses to the Dukoral vaccine were found to be TLR-dependent. A peculiar finding was that serum and fecal humoral responses in MyD88$^{-/-}$ animals were comparable to those seen in WT animals, despite the fact that MyD88$^{-/-}$ DCs were impaired in their ability to mature. Overall, my findings suggest that humoral immune responses (antibody production and agglutinating ability) to the Dukoral vaccine, following oral immunization, occurred independently of TLR signaling as well as of DC maturation.

My findings have raised several questions. First, my results showed a differential requirement of TLR signaling for the induction of humoral responses to the CTB antigen following intramuscular immunization, but not following oral immunization. The reasons for this are not clear and remain to be determined. Similarly, the reasons behind the TLR-independence

of *V. cholerae*-specific and CTB-specific humoral responses following oral immunization are also not clear and need to be investigated. Whether the Dukoral vaccine is able to directly stimulate B-cells to allow for the continued induction of humoral responses, during TLR-deficiency, should be addressed in the future.

Evidence from the literature has shown TLR signaling to be critical for antibody affinity maturation and the induction of robust germinal center (GC) reactions [255,256]. Future studies should aim to measure the antibody affinity of *V. cholerae*- and CTB-specific antibodies from TLR mutant animals, as defects in antibody avidity may not be detected during conventional ELISA analysis.

The cell-mediated immune responses described herein pertain mainly to cytokine production and cell proliferation following the stimulation of splenic lymphocytes. However, I have not determined the role of TLR signaling in the generation of cell-mediated immune responses within the context of mucosal immunity. The evaluation of such responses from within the intestinal tract of orally immunized animals would be beneficial for providing a more complete picture of the effect of TLR-deficiency on mucosal immune responses. Indeed, I attempted to answer this question. Intestinal tracts from orally immunized mice were collected, digested and cryo-preserved as per a previously described protocol [257]. However, I was not able to recover a sufficient number of cells to perform any cellular assays. Such investigations should be undertaken in future studies in order to provide a more complete picture of the effect of TLR-deficiency on intestinal/mucosal cell-mediated immune responses.

My findings also showed that MyD88$^{-/-}$ DCs were impaired in their ability to mature in response to stimulation by Dukoral vaccine components. In order to confirm these observations in-vivo, future studies should examine the maturation state of DCs from orally immunized TLR-

deficient animals.

Results with the CTB component of the Dukoral vaccine have suggested that CTB may have TLR-stimulatory capabilities. Several protein antigens (atypical TLR ligands) such as *E. coli* fimbrial proteins and *Neisseria* porins have been shown to exhibit TLR-stimulatory activities [243,258]. As such, it is possible that CTB may also be able to signal via one or multiple TLRs. In future studies, these findings should be expanded, under strict experimental conditions, to more precisely elucidate the TLR-stimulating abilities of the CTB antigen and its identification as a type of TLR ligand.

In light of my findings which showed that MyD88$^{-/-}$ and Trif$^{-/-}$ animals exhibited unimpaired humoral responses, future studies should investigate the use of MyD88$^{-/-}$Trif$^{-/-}$ double-deficient mice, in order to provide confirmation whether mucosal and systemic humoral immune responses to the Dukoral vaccine occur absolutely independently of any TLR signaling. Also of interest would be to evaluate if the resulting humoral immune responses are able to occur in the absence of T-cell help. Such studies would provide further insight into whether the Dukoral vaccine stimulates B-cells directly. Lastly, the challenge model developed by Nygren et al. should be employed in order to answer the question of whether TLR signaling is critical for protection from *V. cholerae* challenge. Such findings would provide more precise in-vivo evidence on the role of TLR signaling in the induction of protective immune responses to the orally administered Dukoral vaccine.

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CONTRIBUTION OF COLLABORATORS

Dr Helen Tabor and her technician, Morganne Jerome, of the National Microbiology Laboratory in Winnipeg, Manitoba, performed the *Vibrio cholerae* agglutination assay described herein.

CURRICULUM VITAE

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EDUCATION

University of Ottawa, Faculty of Medicine, Ottawa, Ontario
(September 2010 – present)

- MSc in Microbiology and Immunology
- University of Ottawa Excellence Scholarship recipient
- Completion of University of Ottawa French Language proficiency certificate

University of Ottawa, Faculty of Science, Ottawa, Ontario
(2002 – 2007)

- Honours BSc in Biopharmaceutical Science (Genomics specialization), 2007

AWARDS

University of Ottawa Excellence Scholarship, $6000 per year	2010 & 2011
Ontario Graduate Scholarship, $15,000 per year	2011
uOttawa BMI Poster Day award, 1st place MSc, $100	2011
NSERC Canada Graduate Scholarship, $17,500 per year	2010
International Paris AIDS Vaccine conference scholarship, (undisclosed)	2009
University of Ottawa Entrance Scholarship, $200	2002

RESEARCH EXPERIENCE

Variation Biotechnologies Inc., Ottawa, Ontario 2007 - 2010
Research Technician II

- Performed multiple immunological assays in support of pre-clinical vaccine development activities for various viral pathogens
- WSIB-certified member of the health and safety committee
- Assisted with the writing and editing of scientific manuscripts and grant applications
- Trained laboratory personnel for working within a BSL2+ environment

Boehringer Ingelheim Inc, Laval, Quebec May – August 2006
Summer intern

- Summer internship conducting in-vitro research on experimental HIV inhibitor compounds

LABORATORY SKILLS

Designing and executing mouse immunization studies
Mouse immunization (oral, IM, IP and sub-cutaneous routes)
General assay development
Mammalian tissue culturing (cell lines and primary cells)
Flow cytometry (intra-cellular & cell-surface staining)
ELISA & ELISPOT
CFSE proliferation assay
Molecular cloning/PCR/Site-directed mutagenesis PCR
DNA/RNA purification
Antibody and protein purification
Fluorescent microscopy
Influenza virus propagation (egg-based and cell-based methods)
Influenza virus neutralization assay development

PUBLICATIONS

1. Innovative bioinformatics approaches for developing peptide-based vaccines against hypervariable viruses.
Sirskyj D, Diaz-Mitoma F, Kumar A, Golshani A, Azizi A.
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2. Detection of Influenza A and B Neutralizing Antibodies in Vaccinated Ferrets and Macaques Using Specific Biotin-Streptavidin Conjugated Antibodies.
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3. Rapid detection of anti-hepatitis A virus neutralizing antibodies in a microplate enzyme immunoassay.
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4. Inverse association of repressor growth factor independent-1 with CD8 T cell interleukin (IL)-7 receptor [alpha] expression and limited signal transducers and activators of transcription signaling in response to IL-7 among [gamma]-chain cytokines in HIV patients.
Benoit A, Abdkader K, **Sirskyj D**, Alhetheel A, Sant N, Diaz-Mitoma F, Kumar A, Kryworuchko M. AIDS. 2009 Jul 17;23(11):1341-7.
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5. Disruption of the gamma c cytokine network in T cells during HIV infection.
Sirskyj D, Thèze J, Kumar A, Kryworuchko M.
Cytokine. 2008 Jul;43(1):1-14. Epub 2008 Apr 15. Review.
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BOOK CHAPTER

Sasmita Mishra, **Danylo Sirskyj**, Ashok Kumar. *Monocytic cell survival during infection.* Recent Development in Immunology, 2008: 331- 367.
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COMPUTER SKILLS

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Experience with VectorNTI sequencing software and Corel Draw 8 software
Experience with Cyflogic, CellQuest and FCS Express flow cytometry analysis software
Experience with GraphPad Prism v5.0 statistical software

ADDITIONAL TRAINING

University of Ottawa
Biosafety Level 3 certified user 2009

WSIB Level I and II safety training
Certified Safety Committee Worker representative 2008 – 2010

Performance Management Consultants 2007 - 2008
Time Management Course
Communication Skills Course
Proofreading and Editing Course