Mechanistic studies of the adjuvant effects of CTA1-DD and the native cholera toxin:

Impact of cell targeting and tissue localization

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Abstract

Vaccines are the most effective means of preventing infectious diseases and improving global health. However, few vaccines have successfully been developed for protection at mucosal surfaces where most pathogens gain access. The reason for this poor outcome has been the lack of immunoenhancers, or adjuvants, that allow for efficient mucosal immunizations. Empirical data has identified cholera toxin (CT) as one of the most effective adjuvant molecules known today. Because of its inherent toxicity, clinical use of CT is precluded. The closely related CTA1-DD adjuvant share the same dependence on the ADP-ribosylating enzymatic activity of the A1 subunit, however the differential binding properties of CTA1-DD renders the molecule safe and non-toxic. The aim of this thesis work has been to increase the knowledge about how adjuvants function by studying CT and the CTA1-DD adjuvant. To delineate key elements required for the adjuvant effects, we explored their *in vivo* distribution in tissues and the dependence on specific components of the immune system.

We found that both CT and CTA1-DD localizes to the marginal zone macrophages (MZMs) of the spleen after iv. injection. To investigate the importance of this finding we treated mice with clodronate liposomes, depleting the MZMs, and found that immunizations with CT or CTA1-DD generated unperturbed immune responses in the treated mice, suggesting that this cell subset is dispensable for their adjuvant effect.

Following initial accumulation in MZMs, CTA1-DD localized to the follicular dendritic cell (FDC) network. This correlated with the ability of CTA1-DD to activate complement primarily via the alternative pathway, allowing the adjuvant to bind to the complement receptors 1 and 2 (CR1/CR2) on FDCs. We found that adjuvanticity was dramatically reduced in *Cr2* knockout mice, where this localization is absent. This prompted us to isolate FDCs from mice immunized with CTA1-DD and assess their activation status using RT-PCR. We found that a number of genes important for the ability of FDCs to support germinal center (GC) formation were up-regulated. Whereas FDCs are highly involved in orchestrating the GC reaction it was feasible that a direct effect of CTA1-DD on FDC functions promoted GC formations.

Conventional dendritic cells (DCs) are believed to be essential for generating follicular helper T (Tfh) cells, but it is unknown to what extent CTA1-DD affects this process. Unexpectedly, when using the CD11c-DTR mouse model to deplete DCs, we found Tfh cell priming appeared to be normal in terms of expansion and phenotype, however a significant reduction in the expression of the Tfh cell transcription factor Bcl-6 was recorded following immunization. Despite potentially reduced Tfh function, we observed that the ability of CTA1-DD to promote antibody production and GC formations was still significant. We speculate that this was the result of a compensatory mechanism employed by the CTA1-DD adjuvant, possibly via the activation of FDCs.

Finally, we examined the immunomodulatory properties of CT. CT is generally considered a Th2 adjuvant and has been reported to inhibit Th1 responses by down-regulating IL-12 production. Here we demonstrated that CT rather induces a mixed Th1/Th2/Th17 response, independently of IL-12. Interestingly, i.v immunization with CT completely blocked the ability to respond to a subsequent immunization, and both Th1 and Th2 responses were inhibited, arguing that an early event in the priming process was impaired. This correlated well with the observation that $CD11b^+$ DCs were activated, thus compromising their ability to process additional antigens. In addition we found that the $CD8\alpha^+$ DC population was depleted following CT-administration and could therefore not be involved in the adjuvant effect of CT. Finally, reconstituting CT-treated mice with DCs re-established their ability to respond to a subsequent immunization.

In conclusion, we have demonstrated that the differential binding properties of the related adjuvants, CT and CTA1-DD, critically affects the mechanisms by which they modulate immune responses. This underpins the importance of targeting adjuvants to specific components of the immune system in order to efficiently deliver stimulation and avoiding toxic side effects, an important insight when designing future vaccines.

Keywords: adjuvants, vaccines, CTA1-DD, cholera toxin, follicular dendritic cells, dendritic cells, Th1, Th2, Th17, Tfh, germinal centers, complement.
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Original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-III):

I. **Complement activation and complement receptors on follicular dendritic cells are critical for the function of a targeted adjuvant**
Mattsson J, Yrlid U, Stensson A, Schön K, Karlsson MC, Ravetch JV, Lycke NY.
J Immunol. 2011 Oct 1;187(7):3641-52

II. **The adjuvant function of cholera toxin is independent of IL-12 and mediated by $CD11b^{+}CD11c^{+}$ dendritic cells inducing not only Th2- but also Th1 and Th17 responses**
Mattsson J, Schön K, Yrlid U, Lycke NY.
Manuscript

III. **CTA1-DD adjuvant targets follicular dendritic cells and up-regulates the expression of germinal center-promoting genes**
Mattsson J, Gustafsson T, Dahlgren M, Stensson A, Johansson-Lindbom B, Yrlid U, Lycke NY.
Manuscript

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Abbreviations

ADP	Adenosine diphosphate
APC	Antigen presenting cell
ARF	Adenosine diphosphate ribosylating factor
Baff	B lymphocyte activating factor
BCR	B cell receptor
C4BP	C4 binding protein
CR1/2	Complement receptor 1/2
CSR	Class switch recombination
CT	Cholera toxin
CTB	Cholera toxin subunit B
CTL	Cytotoxic lymphocyte
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DTR	Diphteria toxin receptor
DTx	Diphteria toxin
DZ	Dark zone
ER	Endoplasmatic reticulum
ERAD	ER associated protein degradation
FcR	Fc receptor
FDC	Follicular dendritic cell
FOB	Follicular B cell
GC	Germinal center
GFP	Green fluorescent protein
HPRT	Hypoxanthine guanine phosphoribosyltransferase
IFN	Interferon
IL	Interleukin
in	Intranasal
iTreg	Inducible T regulatory cell
iv	Intravenous
LPS	Lipopolysaccharide
LT	Heat labile enterotoxin
LTB	Heat labile enterotoxin subunit B
LZ	Light zone
MAC	Membrane attack complex
MARCO	Macrophage receptor with collagenous structure
MBL	Mannose binding lectin
MHCII	Major histocompability complex class II
MMM	Metallophilic macrophages
MPLA	Monophosphoryl lipid A
MZ	Marginal zone
MZB	Marginal zone B cells
MZM	Marginal zone macrophages
NLRP3	NOD-like receptor protein 3
NP-CGG	(4-hydroxy-3-nitrophenyl)acetyl (NP)-chicken γ-globulin (CGG)
nTreg	Natural T regulatory cells
OVA	Ovalbumin
PC	Plasma cell
PRR	Pattern recognition receptor

RA	Retinoic acid
SHM	Somatic hypermutation
S1P	Sphingosine 1-phosphate
SRA	Scavenger receptor A
STAT	Signal transducer and activator of transcription
TI	T cell independent
TCR	T cell receptor
TD	T cell dependent
Tfh	T follicular helper
Th	T helper
TLR	Toll like receptor
TNFα	Tumor necrosis factor α
WT	Wild type

Introduction

During the 20th century, the average life expectancy has dramatically increased worldwide, from an average life span of only about 30 years in the early 1900 to almost 65 years at the turn of the century [1]. The principal reason for this remarkable progress has been the control of infectious diseases by the invention of antibiotics and vaccines [2]. Vaccines are unique in that they provide prophylactic protection against diseases, many of which lack effective therapeutic treatment. Up until recently, vaccine development was largely a process of trial and error using formulations based on attenuated or killed pathogens, often mixed with the adjuvant alum. This strategy has worked remarkably well in the past, many of these vaccines have been highly successful. However some diseases such as HIV or malaria represent a challenge that will require a new approach to vaccine development. In order to be successful, these new vaccines will require rationally selected conserved antigens that, in contrast to whole cell vaccines, are safer and will be able to confer protection against infections that fail to generate natural immunity. In addition, such vaccines will require new types of immunoenhancers, or adjuvants, that can modulate the immune response not only by enhancing the effect of a vaccine, but also by influencing the quality of the response. Furthermore, in addition to systemic immunity, protection against many infections require local mucosal defenses. In order to avoid inappropriate immune responses against harmless environmental antigens, mucosal surfaces are biased towards the development of tolerance. Therefore, powerful adjuvants are needed in order to boost the efficiency of vaccines delivered by mucosal routes.

The mechanisms behind the immunostimulatory effect of adjuvants used in human vaccines are still not fully understood. For example, the by far most widely used adjuvant, alum, has been included in vaccines for over 80 years, nevertheless its impact on the immune system remain unclear. In order to develop new adjuvants that will be included in future vaccines, it is critical to increase the knowledge about how existing adjuvants modulate the immune response.

The aim of my thesis has been to explore the immunostimulatory mechanisms behind two related adjuvants; cholera toxin (CT) and CTA1-DD. We have investigated the targeting properties of the two adjuvants as well as their dependence on selected cell types- and other components of the immune system. For example the ability of CTA1-DD to promote the formation of large and numerous germinal centers (GC), crucial in the development of high quality memory B cells, was correlated to the activation of complement and the binding of the adjuvant to follicular dendritic cells (FDC). We also investigated the importance of conventional dendritic cells (DC) and their role in the differentiation of T follicular helper cells in this process. Despite the fact that the key factor behind the adjuvant function of both CTA1-DD and CT is the enzymatic activity of the shared A-subunit, their effect following immunization is fundamentally different. While CT is highly toxic and paradoxically can potentiate immune responses as well as induce a state of hyporesponsiveness following administration, CTA1-DD is non-toxic and stimulates immune responses without exhibiting

the immunosuppressive effect as seen with CT. Thus, a fundamental question that we address is whether CT and CTA1-DD promote immune responses by acting on the same target cells or if they employ different cell types and mechanisms for their immunoenhancing effects. Furthermore we challenge previous reports describing CT as an adjuvant that primarily promotes Th2- and not Th1 responses. In contrast we demonstrate a balanced induction of Th1, Th2 and Th17 immune responses following immunization with CT. In the following sections I will describe key aspects of adjuvants and the immune system relevant to this thesis work.

Vaccines

The basis for vaccination was set at the end of the 18th century by Doctor Edward Jenner. At this time it was widely believed that dairymaids, who often contracted cowpox, were also protected against smallpox. In a classical experiment, Jenner inoculated an 8 year old boy with matter from a cowpox lesion derived from the hands of a young dairymaid. The boy developed mild fever but soon recovered. When Jenner inoculated the boy again, this time with matter from a fresh smallpox lesion, no disease developed. Jenner concluded that the boy was protected [3]. However, Jenner was not the first to induce immunity by inoculation. A practice where non-immune individuals were inoculated with smallpox intradermally, referred to as variolation, had been used long before Jenner's experiments. But, variolation was risky, a significant number of inoculated persons developed the disease and the risk of contracting other infections was considerable [4]. The importance of Jenner's discovery, which remains to be fundamental in the field of vaccinology, was that it is possible to infer protection against a severe disease using a similar agent that cause mild- or no symptoms. The initial work of Jenner and his successors resulted in the eradication of smallpox in 1977. Today, vaccination is recognized as one of the greatest public heath achievements of the 20th century. The eradication of smallpox was followed by an almost complete elimination of polio and a major reduction in the prevalence of a number of diseases including diphtheria, tetanus, pertussis, yellow fever, *Haemophilus influenzae* type B, measles, mumps, rubella, typhoid fever and rabies [5].

Vaccines can be broadly divided into live attenuated and nonliving vaccines. The live attenuated vaccines, such as the smallpox vaccine, are comprised of weakened versions of the pathogen, closely mimicking the natural infection, but with mild or no symptoms. Such vaccines are highly efficient and confer long lasting protection because they provide immunological memory typified by an ability to mount a strong response within a few days. However there are apparent risks with attenuated vaccines as they can cause severe infections, particularly in immunocompromised individuals. There is also a risk that the attenuated organism can revert to a highly virulent pathogen [6]. Hence, live attenuated vaccines are unsuitable when dealing with pathogens that mutate rapidly, such as HIV, or for those that exist in many different serotypes, e.g. dengue fever. Of course, attenuated vaccines against infections that naturally provide no- or only partial protection against reinfection are unlikely to be successful [7].

Nonliving vaccines can be further classified as whole cell - or subunit vaccines. Such vaccines provide a better safety profile as compared to attenuated vaccines. As the name implies, whole cell vaccines contain the killed pathogen that has been inactivated using chemicals, high temperatures or radiation. Subunit vaccines on the other hand are comprised of one or several antigens purified from the pathogen or produced recombinantly. The subunit approach offers several advantages in addition to their improved safety features. They can be designed to protect against multiple serotypes of a pathogen by including a variety of antigens in the vaccine formulation or by directing the response towards conserved epitopes present on many strains. However they are also less immunogenic and most often require adjuvants to generate a strong immune response.

The majority of licensed vaccines available on the market today are administered via parenteral routes, either intramuscularly or subcutaneously. Although efficient at generating systemic immunity, such vaccines are often poor at inducing mucosal responses [8, 9]. Mucosal surfaces represent the primary entry point for a large number of pathogens, therefore mucosal immunity is much warranted to prevent mucosal as well as systemic infections. Furthermore, mucosal vaccines are needle free, eliminating the risk of spreading infections by contaminating pathogens and they most often imply improved compliance, especially in children and in individuals that suffer from a fear of needles.

Despite the advantages of mucosal immunization there are only 7 licensed mucosal vaccines available, 6 delivered by the oral route and one intranasal (in), all of which are live attenuated or whole dead formulations [10]. This reflects the inherent challenges of delivering antigen- and initiating immune responses at mucosal surfaces. A mucosal vaccine candidate must be able to penetrate mucosal barriers such as the mucus layers or epithelial cells. In addition it must be protected from degradation so that it can reach the immune inductive sites in the mucosal immune system. The degradation of antigens is primarily an issue associated with oral vaccines, due to the local enzyme enriched environment in the gut. To overcome this problem oral vaccines use large quantities of antigen and often include buffers to counter the acidity of the stomach. Notable disadvantage with oral vaccines is their varying efficiency as well as their often poor induction of memory responses [11]. In. vaccination offers an advantage over oral vaccines in that lower amounts of antigen are required and that both mucosal and systemic protection is effectively generated [12, 13].

A major challenge in the development of mucosal vaccines is to overcome the largely tolerogenic environment of mucosal surfaces. In order to prevent immune responses towards environmental antigens or commensal bacteria, mucosal surfaces generally induce tolerance as opposed to immunity. The tolerogenic property of mucosal surfaces is largely maintained by the production of retinoic acid (RA) and TGF-β by epithelial cells. Together, these two factors imprint local DCs to adopt a tolerogenic phenotype [14]. These DCs produce RA and TGF-β that synergize to induce the formation of T regulatory cells (Tregs) in draining lymph nodes [15-17]. Tregs suppress immune responses by migrating to effector sites where they secrete TGF-β and IL-10 [16, 18, 19]. Additionally they can induce the expression of IL-27 in DCs, generating Tr1 cells which also secrete IL-10 [20, 21]. Furthermore, large doses of

antigen can result in the deletion of specific T cells by apoptosis or generate a state of unresponsiveness termed anergy [22, 23]. In the steady state condition, these mechanisms ensure that mucosal tolerance and homeostasis is maintained. Therefore, in order to induce immunity a mucosal vaccine candidate must be able to overcome this disposition. However, there are currently no mucosal adjuvants licensed for human use, which precludes the development of mucosal vaccines.

Adjuvants

Adjuvants are substances that serve to potentiate the immunogenicity of vaccines. They comprise a wide variety of molecules and complex formulations that have the ability to enhance the magnitude- as well as the longevity of the immune response. By including adjuvants in vaccines the antigen dose can be dramatically lowered and the number of immunizations can be reduced. Furthermore, the choice of adjuvant can modulate the immune response in several ways. For example, a vaccine directed against an extracellular pathogen will be more dependent on a strong humoral (Th2) response as compared to intracellular organisms where a cytotoxic (Th1) response is more desirable. Skewing the immune system towards either type of response by choosing an appropriate adjuvant can be essential for the efficacy of the vaccine [24].

Based on their mode of action, adjuvants are often classified as being either delivery systems or immunostimulants. Delivery systems present antigen in a more accessible form and include aluminum salts, oil emulsions, virus like particles (VLPs), liposomes and micro- or nanoparticles. Immunostimulats on the other hand, potentiate the immune response by activating innate immunity, most often through pattern recognition receptors (PRRs). Examples of immunostimulants are microbial components, bacterial toxins and their derivatives, endogenous danger signals such as cytokines, or molecules released as a result of tissue damage or inflammation. However this division is not mutually exclusive as many adjuvants fall into both categories [25].

The first report of mixing antigen with foreign substances to augment the activity of a vaccine was published in 1916 [26]. A majority of these early adjuvants were oil emulsions, and their discovery was soon followed by aluminum-based formulations [27]. Even today aluminum salts are still the most widely used adjuvants in human vaccines. In fact, apart from aluminum salts there are only 4 adjuvants licensed for the use in humans; MF59, AS03, AS04 and liposomes [28]. This also reflects a lack of knowledge about adjuvants and which mechanisms they use to modulate the immune response. A better understanding of how adjuvants function will provide the tools needed to rationally design better and safer adjuvants for the development of new vaccines.

Aluminum salts

Aluminum salts, commonly referred to as alum, has been used for over 80 years. The adjuvant is employed in a number of human vaccines including vaccines against diphtheria, tetanus, pertussis, hepatitis A and B, *Haemophilus influenza* type B, polio, *Streptococcus pneumonia*, human papilloma virus among others [28, 29]. Alum has an excellent safety record and generates strong humoral responses. However it is a potent inducer of Th2-skewed immunity

and is therefore unsuitable in vaccines against pathogens that require a Th1-driven cytotoxic T lymphocyte (CTL) response [30]. The mechanism behind the adjuvanticity of alum has been elusive, and the issue remains to be controversial. It was traditionally believed that alum increased antigen accessibility to antigen presenting cells (APCs) by forming a depot of persisting antigen [31]. However this notion has been challenged by many recent reports, including studies that have excised the injection depot without any negative effects on the immune response [32]. Moreover, alum was shown to induce the production of IL-1β and IL-18 in a caspase-1 dependent manner, which was linked to the release of uric acid , leading to the recruitment of monocyte derived inflammatory DCs [33, 34]. These findings have been correlated to the activation of the NOD-like receptor protein 3 (NLRP3) inflammasome by alum, either directly or via the release of uric acid [35, 36]. However, there are conflicting reports showing that NALP3 is only essential for the activation of early innate responses but dispensable for the subsequent production of IgG antibody titers [37-39]. In an alternative model, the ability of alum to activate DCs *in vivo* was linked to alum-antigen complexes that bind to lipid moieties in the cell membrane leading to the up-regulation of CD86 and ICAM-1 [40]. In addition, DNA released from dying cells, may activate damage-associated molecular patterns (DAMPs) which also have been implicated in the adjuvant function of alum [41]. Thus, the immunomodulatory effects of alum are still not fully understood and are likely to be complex, involving multiple pathways.

Squalene based formulations; MF59 and AS03

MF59 (Novartis) is an oil in water emulsion based on microvesicles of squalene, a precursor to cholesterol, that can be derived from plants or from the liver of some fish species. MF59 is included in two vaccines licensed for the use in humans, a seasonal influenza vaccine and a vaccine against the H1N1 influenza strain [42]. The mechanism behind the adjuvant effect of MF59 is poorly known. It is not believed to involve depot formation since the persistence of antigen is not affected by the addition of MF59 [43]. However, MF59 was shown to induce the up-regulation a large number of genes involved in inflammation, cell migration and antigen presentation [44]. In agreement with these results, there are a number of reports demonstrating a massive influx of cells into the muscle tissue at the site of immunization, including macrophages, monocytes, DCs and neutrophils [44-47]. Antigen is then taken up by the infiltrating cells and transported to the draining lymph nodes [46, 47]. Furthermore the adjuvant effect of MF59 has been shown to be independent of NALP3- inflammasome but dependent on the adaptor protein MyD88 [48].

Similar to MF59, the AS03 (adjuvant system 03, GlaxoSmithKleine biologicals) is an oil in water emulsion based on squalene, that also contains DL-α-tocopherol, a form of vitamin E with immunomodulating activity. AS03 is included in a vaccine against H1N1 influenza [49]. The mode of action of AS03 has not been well documented; but it generates a mixed Th1/Th2 response. Similar to MF59, it causes local inflammation with the expression of numerous chemokines and cytokines at the injection site. This was shown to facilitate the influx of antigen loaded monocytes and DCs to the draining lymph node. The addition of DL-α-tocopherol appears to result in an increased antigen up-take by APCs and an augmented antibody response [50].

Monophosphoryl lipid A

Monophosphoryl lipid A (MPLA) is a modified form of lipopolysaccharide (LPS) from *Salmonella minnesota* [51]. It binds to toll-like receptor 4 (TLR-4) in a similar manner as native LPS, resulting in the activation of the transcription factor NF-κB and the production of pro-inflammatory cytokines. However, in contrast to LPS, where immunomodulation is accompanied with toxicity, MPLA retains the immunoenhancing effects of LPS with significantly reduced toxicity [52]. This has been attributed to a difference in the signaling pathway employed by the two molecules, where LPS signals via both the adaptor molecules MyD88 and Trif, but MPLA preferentially uses Trif [53]. MyD88 signaling induces a more rapid and potent pro-inflammatory response, resulting in a potentially more severe inflammation, compared to that induced by Trif. Interestingly, the generation of Il-1β is dependent on MyD88- but independent of Trif signaling; and, hence, not induced by MPLA [54]. MPLA was first used in an experimental oil in water formulation, RIBI. In this system TLR-4 signaling was required for full adjuvant effect; however a residual adjuvant function was present, attributed to the vehicle solution itself [55].

AS04 (adjuvant system 04, GlaxoSmithKleine biological) is a combination of alum and MPLA that is licensed for the use in humans and is included in two vaccines against human papilloma virus and hepatitis B respectively. The addition of MPLA to alum generates an enhanced inflammatory response, which allows for a more balanced Th1/Th2 response as compared to when alum is used alone [56].

Cholera toxin

Vibrio cholera is the causative agent of cholera, an acute diarrheal disease responsible for thousands of deaths every year in regions with poor sanitary conditions [57]. The hallmark of cholera infections, is the massive out flux of water and electrolytes from the upper part of the small intestine, which is mediated by CT [58]. CT is a member of the AB_5 family of toxins, which also includes the heat labile enterotoxins (LT-1 and LT-II) from *Escherichia coli*, shiga toxin from *Shigella dysenteriae*, pertussis toxin from *Bordella pertussis* among others [59]. The AB_5 toxins are composed of an A subunit and a pentameric B subunit. The A subunit can be further divided into the A1 and the A2 domains, linked via a disulfide bond. The A1 subunit harbors the enzymatic activity and the A2 subunit is non-covalently linked to the B subunit. The B subunit of CT (CTB) binds to Gm1 gangliosides, present on virtually all nucleated cells [60-63]. Upon binding to the cell, the toxin is endocytosed and delivered to the endoplasmatic reticulum (ER) by retrograde vesicular transport via the golgi apparatus [64, 65]. In the ER the disulfide bond between the A1 and A2 subunits is reduced, the A1 subunit is then unfolded and separated from the A2 and B subunits, a process that is facilitated by the protein disulfide isomerase [66]. In order to be transported from the ER to the cytosol, CT hijacks the ER-associated degradation (ERAD) pathway [67]. ERAD is a protein quality control system that mediates the degradation of misfolded proteins by the proteasome in the cytosol. It is still unclear how CT is brought to the cytosol and avoids ubiqutination and subsequent degradation, this is possibly mediated via the Sec61 channel [68, 69]. In the cytosol, the A1 subunit refolds, where it can bind to ADP-ribosylating factors (ARFs), causing a confirmation change that greatly enhances the efficiency of the enzyme [70, 71].

The A1 subunit then catalyzes the transfer of an ADP-ribose moiety from nicotinamide adenine dinucleotide (NAD) to the α subunit of the G protein Gs, causing it to lose its GTPase activity, thus becoming chronically active [72, 73]. In turn, Gsα activates adenylate cyclase which converts ATP to the second messenger cAMP [74]. cAMP has a wide range of effects in the cell. However the mechanism behind the severe fluid loss has been ascribed to the activation of the cystic fibrosis transmembrane conductance regulator (CFTR) in epithelial cells. Activating the CFTR leads to an increased Cl^- secretion, which is accompanied by the osmotic movements of water into the lumen of the gut [75].

In addition to the toxic effects induced by CT, the holotoxin is also a powerful adjuvant when admixed with- or conjugated to antigen. This ability was first demonstrated by administrating the toxin intravenously (iv) [76]. In subsequent studies, potent adjuvant activity has been reported for numerous systemic and mucosal delivery routes [77-85]. However the use of CT in human vaccines is precluded by its toxicity. As described, oral administration results in severe diarrhea. Furthermore, upon in. delivery CT can traffic to the central nervous system via olfactory nerves, causing inflammation in the brain [86-88]. This has been associated with the development of Bell's palsy, or facial paralysis in humans. An LT-adjuvanted influenza vaccine administered in. was removed from the market due to a few cases of Bell's palsy in vaccinated individuals [89]. Furthermore, human trials of a detoxified mutant of the LT toxin, LTK63, were recently halted for the same reason [90]. Hence, it appears that GM1-binding holotoxin-derived adjuvants should not be given in. because of the risk for neurotoxic side effects.

Although not definitely proven in experimental animals, the immunoenhancing ability of CT is believed to be mediated by the direct effects of the holotoxin on DCs [91-98]. Upon CT administration, DCs are activated, as demonstrated by their enhanced expression of co-stimulatory molecules including CD80, CD86 and CD40 as well as the major histocompatibility complex II (MHC II). This allows for efficient antigen presentation and priming of naïve T cells [85, 95, 99]. Furthermore, CT has been shown to promote the enhanced expression of CCR7 and CXCR4 on DCs which facilitates their migration into T cell areas where they can interact with cognate T cells [92, 100]. The maturation status and the expression of co-stimulatory molecules on DCs is further potentiated by the induction of IL-1β [101]. CT is often reported to generate a Th2 dominated immune response to co-administered antigens based on the production IL-4, IL-5, IL-6 and IL-10, generating mainly IgG_1, IgA and IgE antibody responses [81, 96, 99, 102, 103]. This Th2 skewing has been attributed to the down regulation of IL-12 by the inhibition of the transcription factor IRF8 [104]. However, there are numerous conflicting reports describing Th1 responses induced by CT, including IFN-γ production and the effective induction of CTLs [78-80, 105-108]. Adding to the complexity, there are also a number of recent studies demonstrating a Th17 response following CT immunization [79, 109-111].

As aforementioned, the molecular mechanisms behind the toxic effects of CT, are relatively well established. In contrast, the underlying basis for the adjuvant effect of CT is still incompletely understood. Site directed mutagenesis have been a useful tool in deciphering the

relative contribution of the enzymatic activity as well as the role of Gm1 ganglioside binding in the immunomodulating ability of CT [61]. CT mutants that lack Gm1 ganglioside binding display substantially diminished toxicity [112]. Although these constructs were not tested for adjuvant function, the importance of Gm1 ganglioside binding in the adjuvant activity of CT has been supported by studies using Gm1 ganglioside deficient mice. In these mice, immunization with CT failed to induce T cell proliferation as well as antibody responses [94]. In addition, a functional CTA1 subunit also plays an important role in mediating the full immunoenhancing effect of CT. This is evident in studies revealing a poor adjuvant effect of CTB alone and supported by elegant studies by Giuliani et al showing that mutated LT with some enzymatic activity (LTR72) were more adjuvant active than mutants with no enzymatic activity (LTK63) [80, 113] . Importantly, the CTA1-DD fusion protein (see below), which exhibits comparable adjuvant activity to CT holotoxin, provides strong evidence that the ADP-ribosylating ability of the A1-subunit induces potent immune responses [114]. Noteworthy, it is currently unclear how adjuvanticity in mutated enzymatically inactive AB_5 holotoxins is retained, especially in comparison to the weak adjuvant ability of the CTB or LTB molecules [82, 115, 116]. Furthermore, the requirement for cAMP production in the adjuvant effect of CT is not entirely clear. It has been suggested that other attributes of the A subunit, distinct from its enzymatic activity, such as intracellular transport or the interaction with ARFs could account for the adjuvant function in mutated enzymatically inactive AB_5 toxins [61, 117].

CTA1-DD

To circumvent the toxicity of CT in future human vaccines , the fusion protein CTA1-DD was developed [114]. CTA1-DD is composed of the enzymatically active A1 subunit from CT and a DD moiety from *Staphylococcus aureus* protein A. Contrary to the holotoxin, which binds to Gm1 gangliosides as previously described, the DD domain targets Fc- and Fab fragments on immunoglobulins, preferentially of the IgG subclasses [114, 118]. Due to the different binding properties of CTA1-DD it is completely non-toxic and safe even after in. administration, because it cannot bind Gm1 ganglioside and, hence does not accumulated in the olfactory nerve or bulb[114, 119].

Upon immunization, CTA1-DD generates a mixed Th1/Th2 response, resulting in augmented T cell proliferation, GC formation, antibody production as well as CTL activity [114, 120-122]. CTA1-DD has been tested and proven to be safe using a variety of mucosal and parenteral immunization protocols in both mice and macaques [123]. Furthermore, the fusion protein has been used in combination with a large number of antigens and has been shown to confer protective responses in different disease models including chlamydia, rotavirus, influenza and *Helicobacter pylori* [106, 124-127]. In addition to mixing the adjuvant with relevant antigens, CTA1-DD offers the possibility to incorporate peptide epitopes into the fusion protein itself. The significance of this concept was demonstrated using the universal influenza vaccine candidate, matrix protein 2 (M2e). Mice immunized in. with the CTA1-M2e-DD vaccine were fully protected against a lethal dose of live challenge influenza virus [126].

The adjuvant activity of CTA1-DD is dependent on the ADP-ribosylating A1 subunit as demonstrated by the inability of the enzymatically inactive mutant, CTA1R7K-DD, containing a single point mutation in Argenine (R) -7 to Lysine (K), to promote immune responses [118]. Interestingly, this inactivated construct promotes tolerance as opposed to immunity. By incorporating known epitopes involved in autoimmune diseases into the molecule, disease progression can be prevented or significantly ameliorated as was recently shown using a mouse model for rheumatoid arthritis [128]. This concept is currently being further evaluated for the treatment of type 1 diabetes and multiple sclerosis.

Given that CTA1-DD binds to immunoglobulins, there is the potential that the fusion protein could promote the formation of immune complexes when administered *in vivo*. Therefore, the involvement of immune complex formation in the adjuvant effect of CTA1-DD was assessed using mice deficient in the Fc-receptors FcγRIIb and FcεR. These mice displayed unaltered immune responses when immunized with the adjuvant, suggesting that immune complex formation was not involved in the adjuvant function of CTA1-DD [122]. Importantly, CTA1-DD appeared not to be bound to immunoglobulins in serum following injections [122]. However, it was recently documented that *ex vivo* generated CTA1-DD/IgG immune complexes could enhance the adjuvant function mediated by mast cells *in vivo* [129].

CTA1-DD was originally constructed to target B cells [114]. The fusion protein has been shown to bind both mouse and human B cells, resulting in the up-regulation of the co-stimulatory molecule CD86 [114, 130]. However, CTA1-DD can also promote immune responses independently of B cells, as demonstrated by comparable levels of T cell proliferation in mice deficient in B cells as compared to WT mice, indicating that other APCs, presumably DCs, are also activated by CTA1-DD [126]. Thus, CTA1-DD affects multiple cell types and its function is not restricted to the binding of B cells as was originally hypothesized. The present thesis work focuses on the *in vivo* localization of CTA1-DD and the dependence on DCs and FDCs respectively for the adjuvant function.

The complement system

The complement system consists of a complex array of proteins found in plasma and on cell surfaces. The term “complement” originates from its ability to complement the antibacterial effects of antibodies. The effector functions of the complement system include opsonization of foreign substances, direct lysis of pathogens via the membrane attack complex (MAC) and the pro-inflammatory activities of its cleavage products, termed anaphylatoxins. Furthermore, complement receptors on B cells and FDCs are involved in enhancing B cell activation and GC reactions.

Complement activation can be initiated via three distinct pathways; the classical, lectin and alternative pathways (Fig. 1). The classical pathway is dependent on complement fixing antibodies bound to a foreign substance; the Fc region of bound antibodies can bind to the so called C1 complex which generates the C3 convertase by an autocatalytic process. The C3 convertase is central to all three complement pathways, its function is to cleave C3 into C3a (anaphylatoxin) which has a pro-inflammatory function and C3b which functions as an

opsonin. Moreover, the C3 convertase promotes further complement activation, and ultimately the formation of the MAC complex [131]. The lectin pathway is similar to the classical pathway, but it is initiated by mannose-binding lectin (MBL) or ficolins rather than antibodies. MBL and ficolins can bind to carbohydrates on pathogenic surfaces, forming a complex with MBL-associated serine proteases which are analogous to the C1 complex of the classical pathway [132, 133].

In contrast to the classical- and lectin pathways, the alternative pathway is initiated by spontaneous hydrolysis of C3, occurring mainly on the surface of microorganisms. Together with factor B and factor D, cleaved C3 forms the C3 convertase, generating further C3 deposition in an analogous way to the classical- and lectin pathways [134].

Further deposition of cleaved C3 in association with C3 convertases, forms the C5 convertase, which constitutes the basis for the formation of the MAC complex. The MAC complex induces cell lysis of pathogens by forming pores in the cell membrane [135].

To prevent inappropriate complement activation on endogenous cells there are a number of factors regulating the complement cascade. These components include factor I and the decay-accelerating factor (DAF) which acts to inactivate bound C3- and C4 cleavage products or C3 convertases, respectively. In order to prevent inactivation of complement bound to foreign substances, these proteins require cofactors that are only expressed on host cells. These cofactors include CD46, complement receptor 1, C4 binding protein and factor H [136].

Components of activated complement are recognized by a variety of receptors. The anaphylatoxins bind G-protein-coupled receptors primarily expressed on granulocytes, macrophages, monocytes, neutrophils, mast cells and DCs corresponding to the pro-inflammatory properties of these molecules [137]. Opsonizing proteins are recognized by the complement receptors 1-4 and CRig. Complement receptor 1 (CR1/CD35) and 2 (CR2/CD21) are splice forms of the *Cr2* gene [138]. The former is expressed on erythrocytes, monocytes, neutrophils and B cells whereas the latter is found mainly on B cells and FDCs. CR1 and CR2 are important in the transport- and deposition of immune complexes on FDCs, a process which will be further discussed in the section describing GCs. CR2 is also included in a co-receptor complex with the BCR (B cell receptor), together with CD19 and CD81 (TAPA-1). This co-receptor substantially reduces the threshold for activation of naïve B cells, significantly improving the ability of B cells to respond to low affinity antigens [139]. CR3 and CR4 are heterodimers composed of CD11b/CD18 and CD11c/CD18, respectively. They are mediating phagocytosis of complement coated pathogens, CR3 is mainly expressed on monocytes, neutrophils and NK cells whereas CR4 is primarily found on macrophages [140, 141]. CRIg is involved in clearing complement opsonized substances and is expressed on Kupffer cells in the liver [142]. Together the complement system has a wide range of effects and is involved in both innate- and adaptive immune functions.

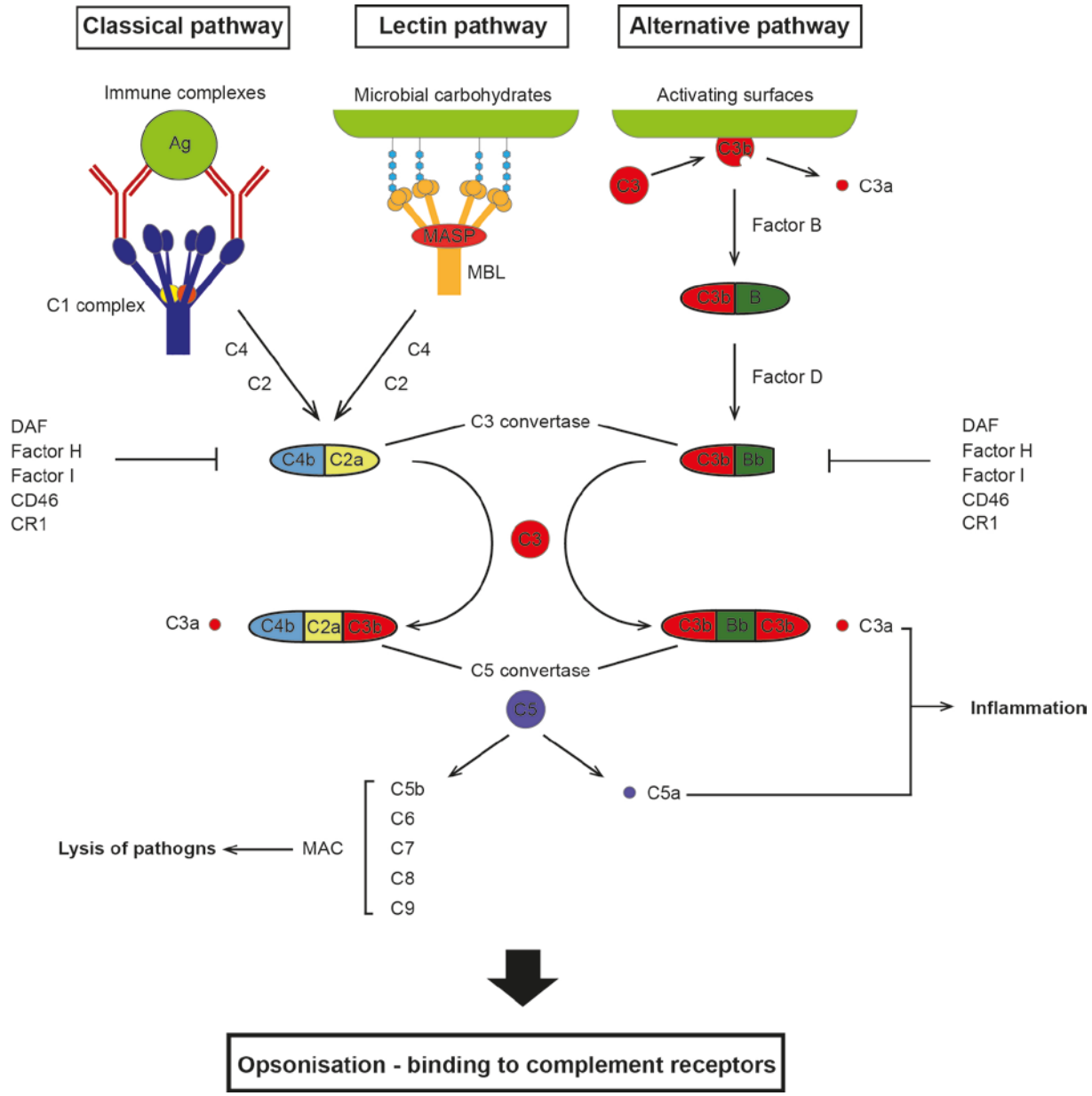

Figure 1. Schematic overview of the complement system.

The cell types and the microanatomy of the spleen

The primary function of the spleen is to filter blood in order to launch a rapid response against blood borne pathogens or to remove debris such as old and dying erythrocytes. It is also the main inductive site for immune responses initiated using the intraperitoneal- or intravenous immunization routes. Blood enters the spleen via the splenic artery which branch into several central arterioles. The central arterioles branches further to smaller vessels that empties directly into the red pulp or into the marginal sinus. The blood is then filtered thru the marginal zone (MZ) and further into the red pulp where it passes into venous sinuses that collect into the efferent vein, vena linealis. There are three functionally and phenotypically

distinct compartments of the spleen, the red pulp, the white pulp and the MZ [143]. The most abundant cell type in the red pulp are the red pulp macrophages; they have an important function in phagocytizing erythrocytes and iron recycling [144]. Other cell types found in the red pulp include DCs, natural killer cells, plasma cells as well as a small number of B- and T cells.

The white pulp host B cell follicles as well as organized T cell areas (also referred to as the periarteriolar lymphoid sheets, PALS). The B cell follicles comprise a large population of follicular B cells as well as a small number of FDCs and follicular stromal cells. The latter two cell types secrete the chemokine CXCL13, which attracts B cells via stimulation of their CXCR5 receptors [145]. During an immune response GCs can form in the center of B cell follicles, giving rise to high affinity memory B cells as well as antibody secreting plasma cells. Moreover, tingible body macrophages found in the GC remove apoptotic cells generated during the GC reaction [146]. The T cell areas contain $CD4^+$ and $CD8^+$ T cells and DCs. CCL19 and CCL21 are secreted by stromal cells in the T cell area, thereby recruiting CCR7 expressing T cells [147]. MOMA-2^+ macrophages are present in both B cell follicles and T cell areas, although incompletely studied, they have been shown to be important in providing a local source of complement C3 [148].

The MZ is located to the interface between the white pulp and the red pulp, it contains the marginal sinus which allows for a close contact between the slowly percolating blood and cells in the MZ. The cells of the MZ are unique to this compartment and have specialized functions which allow them to rapidly respond to- and control blood borne pathogens. These cell types include two macrophage subtypes, the marginal metallophilic macrophages (MMM) and the marginal zone macrophages (MZM), marginal zone B cells (MZB) and DCs [149].

Marginal zone B cells

MZB cells constitute a population of non-recirculating cells, phenotypically and functionally distinct from follicular B cells (FOB). Naïve MZB cells differ from FOB cells in that they are IgM^{hi}, $CD21^{hi}$, $CD23^{lo}$ and $CD1d^{hi}$, whereas FOB cells can be identified as being IgD^{hi}, $CD21^{int}$, $CD23^{hi}$ and $CD1d^{lo}$ [150]. The relatively high expression of CR2 on MZB cells allows them to bind and transport immune complexes into the follicle; this function is important in GC formation and will be further discussed in the section describing antigen localization to FDCs. The MHC 1-like molecule CD1d, expressed on MZB cells, enables the presentation of lipid antigens to invariant natural killer (iNKT) cells and has been implicated in the production of anti-lipid antibodies by MZB cells [151, 152]. In addition, MZB cells have been shown to be involved in the formation of antibodies towards T cell independent (TI) antigens, mice that lack MZB cells display reduced IgM, IgG_3 and IgG_{2a} antibody titers to TI antigens, while titers against T cell dependent (TD) antigens remained unaffected [153]. These responses are dominated by extrafolliclular plasma cells and occur within a few days following immunization [154, 155]. This unique ability of the MZB cells to launch rapid responses has been attributed to their low threshold of activation as compared to FOB cells [156]. However, the activity of MZB cells is not restricted to TI antigens, they can also respond to TD antigens and participate in GC reactions where they undergo somatic hypermutation and class switching [157, 158].

Macrophages of the marginal zone

There are two distinct populations of macrophages in the MZ, the marginal zone macrophages (MZM) and the marginal metallophilic macrophages (MMM). They are distinguished by their differential expression of surface markers as well as by their location in the MZ. MZM are found in the outer MZ whereas MMM are found on the inner side, in close contact with the marginal sinus, bordering the white pulp [149]. MMM are identified by the antibody MOMA-1 which recognizes sialoadhesin (CD169, siglec-1) [159, 160]. Sialoadhesin is a receptor for sialylated bacteria and has been shown to mediate the phagocytosis of sialylated strains of *Neisseria meningitides* [161]. MZM can be identified by the antibody ERTR-9 which recognizes the specific intracellular adhesion molecule-grabbing nonintegrin receptor 1 (SIGN-R1). SIGN-R1 binds to polysaccharides of capsulated bacteria and has been shown to facilitate the uptake of *Streptococcus pneumoniae* [162-164]. Another marker for MZM is the macrophage receptor with collagenous structure (MARCO), a scavenger receptor which binds a range of bacterial products including LPS, thereby mediating the phagocytosis of blood borne bacteria by MZM [165-167]. Furthermore MZM express the closely related scavenger receptor A (SR-A), which also is an important phagocytic receptor, recognizing various bacterial products [168-172].

Both MZM and MMM are highly phagocytic and have been shown to trap a wide range of particulate- and non-particulate antigens [162, 164, 165, 173-182]. This ability is crucial in preventing the hematogenic spread of blood borne infections as demonstrated by the reduced pathogen clearance and diminished survival of systemically infected mice lacking these macrophage subsets [162, 173, 174, 180, 182]. With the exception of some reports where the specific role of the receptors SIGN-R1 or MARCO have been investigated [162, 165, 180], many of the studies have used clodronate liposomes to deplete MZM and MMM [173, 174], or alternatively employed osteopetrotic (op/op) mice, which correspondingly lacks both subsets [182]. Thus, it is difficult to distinguish between the respective roles of MZM or MMM in pathogen clearance in the systems that have been studied.

Although essential for removing disseminated pathogens from the blood, MZM and MMM do not appear to have a role in priming T cells since T cell priming was intact in mice deficient in both MZM and MMM populations [173, 174, 182]. This notion is also supported by their apparent lack of MHC II molecules [183]. However, MZM and MMM may have other roles in the induction of immune responses. For example, they have been shown to be the major producers of type 1 interferons following systemic injection with *Herpes simplex* virus [183]. Furthermore, MMM have been implicated in the transfer of antigens to $CD8^+$ DC, enabling cross presentation and CTL induction [184].

Dendritic cells

In the spleen, classical DCs are found in the red pulp, the T cell areas and in the MZ. They can be divided into three distinct subtypes based on their function as well as their expression of surface molecules; $CD8a^+CD11b^-DEC205^+$ DCs, $CD8\alpha^-CD11b^+DEC205^-CD4^-$ and $CD8\alpha^-CD11b^+DEC205^-CD4^+$ DCs, here I will refer to the former as $CD8\alpha^+$ DCs and the latter two as $CD8\alpha^-$ DCs [185-187]. $CD8\alpha^-$ DCs are primarily located to the splenic bridging channels

and in the red pulp, whereas $CD8\alpha^+$ DCs are found in the T cell areas [188, 189]. However, recently a $CD8\alpha^+$ DC population was described to reside in the MZ and identified by the langerin (CD207) marker [190].

$CD8\alpha^+$ and $CD8\alpha^-$ DCs differ in their ability to present antigen to T cells. $CD8\alpha^+$, but not $CD8\alpha^-$ DCs can take up apoptotic cells and are capable of cross presenting exogenous cell associated- as well as soluble antigen on MHC class I molecules [189, 191-193]. This correlates with the superior ability of $CD8\alpha^+$ DCs to prime MHC class I restricted CD8 T cells. Conversely, $CD8\alpha^-$ DCs more efficiently induces proliferation of CD4 T cells that recognizes peptides presented on MHC class II. This dichotomy has been demonstrated *in vitro*, using either $CD8\alpha^+$ or $CD8\alpha^-$ DCs as APCs in culture [193], or *in vivo* by targeting antigen to the respective DC population [189] or by using knockout mice lacking the $CD8\alpha^+$ DC population [194]. In addition to their divergent capacity with regard to APC function, $CD8\alpha^+$ and $CD8\alpha^-$ DCs also differ in their respective cytokine secretion profiles. $CD8\alpha^+$ DCs are able to secrete IL-12 upon activation, inducing Th1 T cells and IFN-γ production, whereas $CD8\alpha^-$ DCs elicit a more Th2 skewed response, mainly resulting in the secretion of IL-4 and IL-10 [195-197].

The induction of polarized T cell responses is not only determined by the intrinsic differences between the different DC subtypes. In fact there is a significant plasticity within the DC population to modulate the immune responses depending on the various activation stimuli. DCs express a wide array of PRRs, including TLRs, NOD-like receptors (NLRs), C-type lectin receptors etc. [198]. Different microbial products bind to different receptors and therefore influence the cytokine pattern released by DCs and consequently the polarization of T cell responses. For example LPS from *Escherichia coli* signals via TLR4, promoting the secretion of IL-12 which drives Th1 induction. In contrast LPS from a different bacterium, *Porphiromonas gengivalis* signals via TLR2 which generates Th2 type of responses [199]. Together, these intrinsic and extrinsic signals govern the polarizing activities by DCs, enabling the induction of tailored responses specifically aimed at combating different types of pathogens.

DCs of the spleen are resident, non-migratory cells that arrive as precursors via the blood stream [200]. Under steady state conditions the majority of splenic DCs are immature, that is they express low levels of MHC- and T cell co-stimulatory molecules and produce low amounts of cytokines [201-203]. However upon exposure to microbial products they can be induced to mature, up-regulating their levels of MHC- and co-stimulatory molecules, i.e CD40, CD80 and CD86 [186, 188].This activation correlates with the expression of CCR7 which facilitates the migration into T cell areas where they can prime naïve T cells [204]. In contrast to the spleen, lymph nodes also harbor a population of migratory DCs in addition to the resident population. The migratory DCs comprise a heterogeneous population of mature DCs that continuously travel from the periphery to draining lymph nodes [205-207]. In lymph nodes they can present peripheral antigen directly to T cells, or alternatively transfer antigens to resident DCs which in turn can prime naïve T cells [208, 209].

T cell differentiation

Following CD4 T cell priming activated T cells can differentiate into distinct subsets that are distinguished by their diverse effector functions and cytokine secretion patterns. The induction of these subpopulations is influenced by the local cytokine milieu at the priming event [210]. Central to this process is the activation of the signaling transducer and activator of transcription (STATs) proteins, these transcription factors are induced by signaling thru cytokine receptors and in turn regulate the expression of the different master transcription factors that define subset differentiation. Up until relatively recently only the Th1 and Th2 subsets had been described, but today we know that several additional lineages exist including the Th17-, Treg and T follicular helper (Tfh) cell subsets [211]. An overview of the different CD4 T cell subsets, the cytokines involved in their differentiation and their secretion profiles is presentment in (Fig. 2).

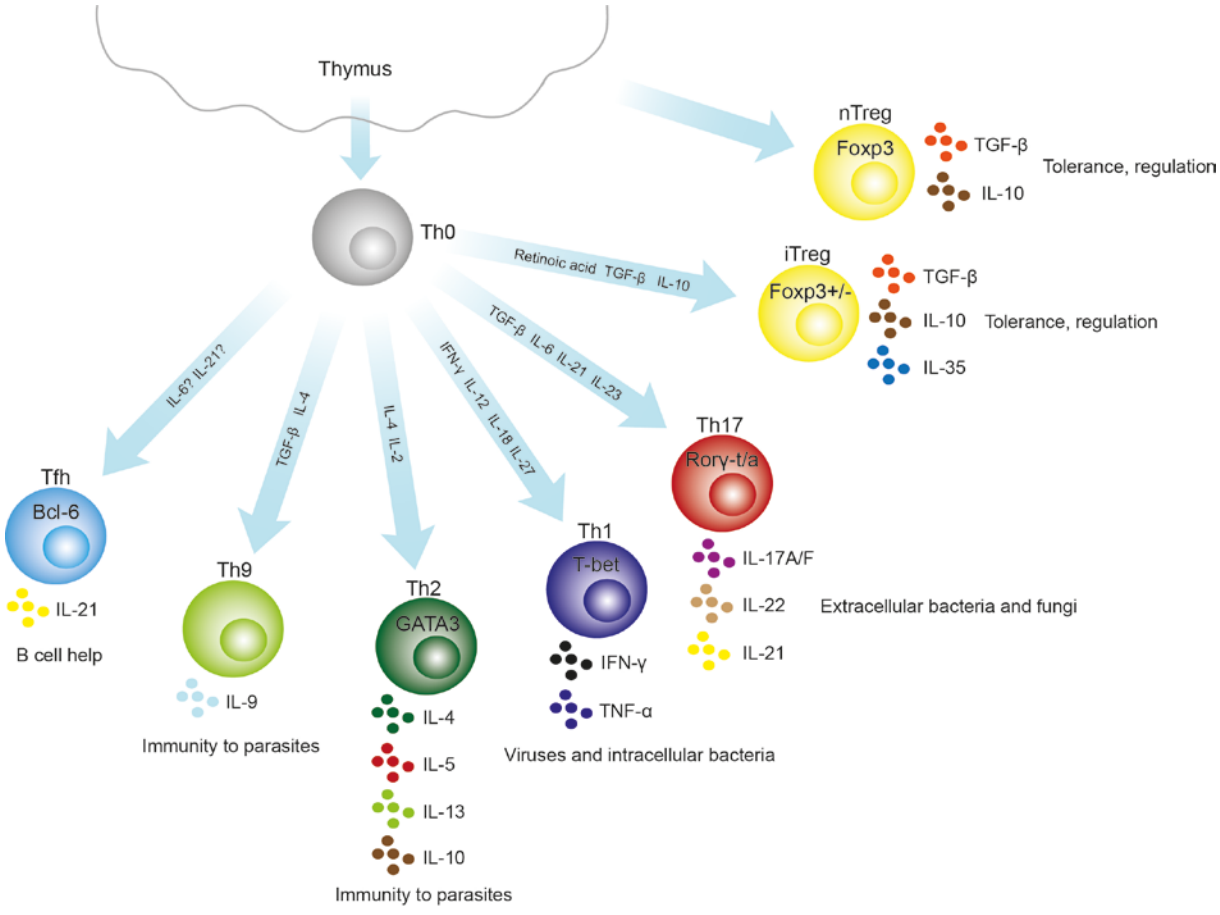

Figure 2. CD4 T cell differentiation.

Th1

Th1 cells are primarily involved in the protection against viruses and intracellular bacteria; their effector functions include the activation of macrophages as well as the expansion of cytolytic CD8 T cells. IFN-γ is the signature cytokine produced by Th1 cells and it is also involved in the initial induction of Th1 differentiation. IFN-γ activates STAT1, which promotes the expression of the Th1 master regulator, T-bet. T-bet drives transcription of the

IFN-γ gene; this creates a positive feedback loop which amplifies the Th1 response [212-214]. T-bet also functions to negatively regulate the Th2 transcription factor GATA-3, which further reinforces the Th1 lineage commitment [215]. Additionally, T-bet mediates the up-regulation of the IL-12 receptor on the T cell [213]. IL-12 is involved in a second signaling pathway that drives Th1 differentiation, this pathway is dependent on the induction of STAT4 which promotes the secretion of IFN-γ thus augmenting Th1 development [216]. Consequently, mice deficient in IL-12, IL-12R, T-bet or STAT4 exhibit severely diminished Th1 responses [216-219].

In addition to IL-12 and IFN-γ, other factors can also influence Th1 differentiation. For example, IL-18 and IL-27 synergizes with IL-12 to drive Th1 induction, this is illustrated by the impaired ability to form Th1 responses in mice deficient in IL-18 or IL-27 [220-223]. Furthermore Notch receptors and their ligands have been implicated in Th1 development. Notch 1 and Notch 2 are activated by the ligands Delta-like (Dll) 4 and 1 respectively [224]. Their involvement in Th1 differentiation has been demonstrated by inhibiting Notch signaling, resulting in diminished Th1 responses [225] or by inducing the expression of Dll4 or Dll1 on DCs which promotes Th1 development [226, 227]. Interestingly, the expression of Dll4 on $CD8\alpha^-$, but not $CD8\alpha^+$ DCs, has been shown to be induced by LPS, allowing for IL-12 independent Th1 differentiation [228].

Th2

Th2 cells are generated in response to extracellular parasites such as helminthes or nematodes. A hallmark of Th2 differentiation is the secretion of IL-4, IL-5 and IL-13, which ultimately results in the recruitment of mast cells and eosinophils and promotes immunoglobulin isotype switching from IgM to IgG_1 and/or IgE. Differentiation of Th2 cells can be induced by IL-4, which activates STAT6 which in turn induces the expression of GATA-3, the master transcription factor for Th2 development [229, 230]. GATA-3 promotes the secretion of IL-4 from the Th2 cells which drives further Th2 differentiation. The source of the IL-4 responsible for early STAT6 activation remains unclear. Basophils are known to secrete IL-4 and are therefore likely to contribute to Th2 development, however there is much controversy regarding the extent of their contribution [231]. Under some conditions, *in vivo* Th2 development can occur in the absence of STAT6, suggesting that signals other than IL-4 are able to drive Th2 differentiation [232, 233]. A second model for Th2 induction revolves around the notion that weak TCR (T cell receptor) signaling generally favors Th2- as opposed to Th1 development. This has been demonstrated using peptides that interact with the TCR with low affinity or by using peptides of low concentration [234, 235]. Under these circumstances TCR signaling causes a slight increase in GATA-3 expression and simultaneously induces the production of IL-2. IL-2 activates STAT5 which synergizes with GATA-3 to produce IL-4 which further drives Th2 differentiation [235]. In contrast, strong TCR signaling reduces IL-2 expression and GATA-3 expression [235]. Additionally the notch ligand Jagged 1 and its receptors Notch 1 and 2 are essential in the development of Th2 responses [226]. This is mediated by GATA-3 expression which is induced upon Notch signaling [236].

Th17

Th17 responses are important in protection against extracellular pathogens such as bacteria or fungi. Th17 development is initiated by the combination of TGF-β and IL-6 [237]. TGF-β induces the expression of RORγt, the Th17 master regulator, which synergizes with RORα to promote Th17 development [238, 239]. TGF-β also induces the expression of Foxp3, the transcription factor involved in Treg development [240]. The balance between the induction of inflammatory Th17 cells or anti-inflammatory Tregs depends on the presence or absence of IL-6 [241]. IL-6 is a potent inducer of STAT3 which drives the expression of RORγt, thus promoting the preferential development of Th17 cells as opposed to Tregs [242]. STAT3 also induces the secretion of IL-21 which acts in an autocrine manner to potentiate Th17 development by further activation of STAT3 and RORγt [242]. Furthermore both STAT3 and RORγt up-regulates the IL-23 receptor on Th17 cells, IL-23 is important in sustaining Th17 differentiation by promoting STAT3 activation [242].

Th17 cells produce the signature cytokines IL-17A/IL-17F and IL-22. IL-17 is a pro-inflammatory cytokine that recruits neutrophils and macrophages to the inflamed tissues, whereas mice lacking IL-17 or the IL-17 receptor display an increased susceptibility to a number of infectious diseases [243]. IL-22 has a wide range of effects, it can both regulate- and induce inflammation, it also has an important protective role by improving barrier functions at mucosal sites [244-246].

T regulatory cells

To maintain homeostasis immune responses must be controlled, this is critical in order to dampen inflammation following an infection or to induce tolerance to self- or environmental antigens. This is largely accommodated by the activities of regulatory T cells (Tregs). Tregs can be divided into two main categories, those that are derived from the thymus, termed natural Tregs (nTreg) and those that are formed in the periphery termed inducible Tregs (iTreg). Both nTregs and iTregs express the transcription factor Foxp3 [247, 248]. nTregs are believed to be derived from T cells that express TCRs with high affinity for self-antigens [249, 250]. iTregs on the other hand are induced by tolerogenic factors in the periphery, one such factor, TGF-β induces the expression of Foxp3 which is required for Treg suppressor function and maintenance [240]. There is also a subset of $Foxp3^-$ iTregs, termed Tr1 cells, they are induced by IL-10, and secretion of IL-10 is also their major effector function [251, 252]. The mechanisms by which Tregs regulate immune responses are diverse and rely on both the secretion of cytokines as well as cell-contact dependent suppression. Secreted inhibitory cytokines include IL-10, TGF-β and IL-35 [19, 253, 254], in addition Tregs express the cytotoxic T lymphocyte antigen-4 (CTLA-4) which can down-modulate the stimulatory capacity of DCs [255, 256], furthermore, Tregs can mediate suppression by direct killing of target cells via Granzyme B induced cytolysis [257].

Additional Th subsets

In addition to the T cell subtypes described above, two additional lineages have recently been defined, the Tfh- and the Th9 subset. Tfh cells are essential in orchestrating GC responses and will be further discussed in the next section. Th9 cells can be induced by IL-4 and TGF-β,

they secrete the cytokine IL-9 and are involved in the protection against intestinal parasites [258-260]. Whether the Th9 cells should be considered a separate subset, or if they are, in fact, a subpopulation of Th2 cells, is a matter of debate. However, recently the transcription factor PU.1 was shown to be required for Th9 induction, suppressing GATA-3[258, 261].

Germinal centers

Within a few hours after being antigen-activated via the BCR, B cells migrate to the B/T cell zone border where they receive essential co-stimulatory signals from cognate T helper cells [262]. Migration to the border area is facilitated by an altered receptor expression; CCR7, the receptor for the chemokines CCL19 and CCL21, produced in the T cell zone, is up-regulated [263]. Furthermore the expression of the Epstein-Barr virus-induced G-protein coupled receptor 2 (EBI2) which directs B cells towards the peripheral regions of the B cell follicle is also increased [264]. B cells are prevented from migrating further towards the T cell zone by their maintained expression of CXCR5, enabling them to respond to the chemokine CXCL13, which is produced in B cell follicles. In parallel to the positioning of activated B cells, cognate T cells migrate to the B/T cell border in a similar manner, they down-regulate their expression of CCR7 and subsequently up-regulate CXCR5 [265, 266]. After having received T cell help, B cells can adopt one of two fates; they can either become short lived extrafollicular plasma cells or participate in a GC reaction [267]. Extrafollicular plasma cells provide an early wave of low affinity antibodies that are important in protection during the initial stages of an infection prior to the formation of antibodies with higher affinity. These cells down-regulate their expression of CXCR5 and CCR7 and up-regulate CXCR4, which facilitates their positioning to the bridging channels and red pulp of the spleen or in the medulla of lymph nodes [268]. In contrast, the pre-GC B cells migrate into the B cell follicles, as a consequence of down-modulating their expression of CCR7 and EBI2, hence the GC reaction can develop a few days after initial antigen encounter [264].

GCs are specialized regions of rapid cell division that form in response to infection or immunization within the B cell follicles of secondary lymphoid organs. Their primary purpose is to generate plasma cells and memory B cells of high affinity. Structurally, GCs can be further divided into two distinct regions, the light zone (LZ) and the dark zone (DZ), originally based on their histological appearance [269]. B cells of the LZ are termed centrocytes and those residing in the DZ are called centroblasts. The distinction between the LZ and DZ is maintained by the expression of CXCR5 and CXCR4. $CXCR4^{hi}$ centroblasts are attracted to the DZ by the chemokine CXCL12 which is expressed at higher levels in the DZ as compared to the LZ, whereas $CXCR4^{lo}$ centrocytes migrate towards CXCL13 which is more abundantly expressed in the LZ [270].

The purpose of the compartmentalization between the LZ and the DZ was originally proposed by MacLennan in a classical model [271]. This model describes how the selection- and generation of high affinity B cells in the GC is accomplished; rapidly dividing centroblasts in the DZ generate random point mutations in their Ig variable (V-) regions by somatic hypermutation (SHM). This process produces cells that express BCRs of variable affinity for a given antigen. Following a number of consecutive rounds of division, the centroblasts then

travel to the LZ and are transformed into centrocytes. The centrocytes express their mutated BCRs and compete for antigen trapped on FDCs that reside in the LZ. Competition for antigen ensures that only cells with the highest affinity will bind enough antigen to receive the essential survival signals required to avoid apoptosis. Thus, only the high affinity GC B cells are able to survive and subsequently become plasma- or memory cells. Evidence supporting this model was initially provided by experiments in which cells were labeled using bromodeoxyuridine (BrdU) and subsequently observed at various time points. Labeled cells were found to initially accumulate in the DZ followed by their appearance in the LZ, suggesting a movement of cells from the DZ to the LZ [272]. Recent studies using multiphoton microscopy, allowing cells to be tracked *in vivo*, have confirmed that GC B cells constantly traffic between the two compartments in a highly dynamic fashion [273-275]. However these experiments were limited by the low number of recorded cells, and were unable to confirm the model of selection. Recently an elegant study used photoactivation to selectively induce the fluorescence of green fluorescent protein (GFP) in transgenic B cells of either the LZ or the DZ. This study revealed that the DZ is indeed the primary site for division and that cells traffic primarily from the DZ to the LZ with a less prominent movement form the LZ to the DZ, supporting the original model of selection [276].

Somatic hypermutation

Somatic hypermutation is driven by the enzyme activation induced cytedine deaminase (AID). This enzyme is expressed in activated B cells and initiates SHM by cysteine (C) deamination at certain hotspots in the Ig V genes [277]. AID replaces the removed C with a uracil (U) base which can either be recognized as a thymine in subsequent replication, generating an AT pair instead of the original CG par, or targeted by base excision- or mismatch repair systems. Base excision repair excises the U, facilitated by the uracil DNA glycoselase (UNG). This will create an abasic site which allows for the insertion of any base to repair the damaged DNA [278]. Moreover, the mismatch repair system can generate mutations outside the mismatched UG base pair, by employing the heterodimer MSH2/MSH6 and Exonuclease1 (Exo1) [279]. Mutations in the V regions are random and will therefore generate B cell clones that express BCRs with a range of affinities, some mutations will inevitably result in decreased affinity for a given antigen, while others increase the affinity, emphasizing the importance of a stringent selection process.

Class switch recombination

Antibodies have different effector functions depending on which isotype they belong to. These differences comprise the ability to activate complement, differential binding to Fc receptors, varying half-lives and tissue localization. Class switch recombination (CSR) is the process by which the different Ig isotypes are generated. This is accomplished by replacing one heavy chain constant (C_H) region with another. Naïve B cells express either IgM or IgD, switching to other isotypes is induced following antigen encounter and subsequent activation by T cells, or alternatively by T cell independent stimuli such as TLR ligands [280]. Similar to SHM, switching is dependent on AID. The C_H locus is arranged in a linear fashion with all the C_H exons aligned in a row. Upstream of individual C_H genes are the so called switch (S) regions, these regions are targeted by AID to generate CSR [281, 282]. To initiate CSR, the S

regions must first be transcribed. Naïve B cells transcribe the Cμ-Cϑ gene segment by default. In addition, the simultaneous transcription of one of the other C_H genes can be induced upon activation and stimulation with different cytokines, this is referred to as germline transcription [280]. Following the induction of germline transcription, AID mediates the conversion of C to U at multiple sites in both of the transcribed S regions. As with the process of SHM this can result in the removal of the mismatched U bases by UNG. These abasic sites are targets for the apurinic/apyrimidinic endonuclease (APE) which can induce double stranded DNA breaks [283, 284]. The breaks in the two S regions are then recombined by nonhomologous end-joining, excising the intervening DNA segment which results in the joining of the Ig V region with a new C_H [285].

Tfh

CSR is not restricted to GCs, in fact isotype switching can occur very early after the activation of B cells, prior to the formation of GCs [286]. This early commitment to different isotype classes is influenced by the local cytokine milieu. For example Th1 T cells secrete IFN-γ which stimulates switching to IgG_{2a} whereas IL-4 secreted from Th2 cells promotes switching to the IgG_1 and IgE isotypes [287]. However T cells are not only required in the early stages of B cell activation but are also found within GCs. These GC T cells belong to a specialized subset, termed follicular helper T (Tfh) cells. Their differentiation is controlled by the master regulator Bcl-6 which antagonizes transcription factors of other T cell subsets. Thus Tfh cells express low levels of the Th1, Th2 or Th17 effector cytokines [288]. In contrast Tfh cell secrete IL-21, a cytokine that promotes survival in GC B cells. Consequently IL-21 knockout mice display an aberrant GC formation [289, 290].

The role of Tfh cells in the formation of GCs has been demonstrated using mice that specifically lack Bcl-6 expression in T cells. These mice do not form functional Tfh cells and correspondingly fail to develop GCs in response to T cell dependent antigens [291-293]. Thus Tfh cells deliver critical survival signals in order to maintain GC reactions. In addition to IL-21 secretion, the key survival factor provided by Tfh cells is co-stimulation via CD40 signalling. CD40:CD40L interactions are critical for the maintenance of GCs [294]. Furthermore, it is likely that the competition for CD40 signals among GC B cells is the limiting factor that drives selection of high affinity clones. In this model only B cells with the highest BCR affinity would competitively acquire sufficient antigen from FDCs to be able to present it on MHC II and receive CD40 signalling by cognate interaction with Tfh cells [295, 296]. This concept was recently supported in a study where antigen was delivered to GC B cells via the DEC-205 receptor, independent of the BCR [297]. Accordingly, B cells were able to acquire similar amounts of antigen and present it on MHC II irrespective of their respective BCR affinity. It was shown that low affinity B cell clones were able to proliferate to a similar extent as high affinity clones when antigen was provided as a conjugate to anti-DEC-205. This was correlated to an increased frequency of cognate T-B cell interactions in DEC-205 sufficient cells [297] and with the loss of affinity maturation [276]. Therefore, the acquisition of antigen is essential for the survival of GC B cells, when equal amounts of antigen is provided to all B cells regardless of BCR affinity this results in abolished selection and hence affinity maturation.

The factors influencing Tfh development are incompletely known. Some studies have suggested a dependence on IL-21 and IL-6 for Tfh induction [298, 299], however conflicting reports have been unable to confirm this finding [289, 290]. The expression of ICOSL is required for early Tfh induction; hence ICOS knockout mice have an impaired Tfh development [300]. In addition to the early priming of Tfh cells, cognate B cells provide a second signal essential for sustained Bcl6 expression at later time points [300, 301].

Long lived plasma cells and memory B cells

The end product of GCs is the generation of high affinity memory B cells and plasma cells (PC). Bcl-6, the master regulator for GC B cells is down-regulated by the expression of the transcription factor Blimp-1, which drives PC differentiation [302]. The signals determining PC commitment, as opposed to memory B cell formation, are incompletely known [303]. However the PC compartment is generally composed of the highest affinity clones when compared to GC- or memory B cells suggesting that PC versus memory B cell commitment is not merely stochastic [304]. PC leave the GC and can set residence in the bone marrow where they may reside for extended periods of time. These long lived PC provide serum antibody levels that persist long after the initial priming event [305]. In contrast, memory B cells reside mainly in B cell follicles and in the splenic MZ [306-308]. Upon booster immunization or reinfection, memory cells can be activated and initiate renewed GC reactions and PC formation. IgM memory cells have been shown to preferentially participate in GCs whereas switched IgG memory cells mainly generate PC after booster immunization [306].

Follicular dendritic cells

FDCs should not be confused with bone marrow derived classical DCs, in contrast to cDCs they are radiation resistant long lived stromal cells resident in primary B cell follicles or GCs [309]. They derive their name from their numerous and long dendritic processes that surround neighboring B cells, forming a network like appearance. Their development and maintenance is dependent on lymphotoxin and tumor necrosis factor-α (TNF-α) secreted by B cells. Consequently, mice deficient in lymphotoxin, TNF-α or their receptors lack FDCs [310-313]. In fact, FDCs disappear from spleens of mice where lymphotoxin signaling is blocked *in vivo* [314]. CR1 and CR2 are highly expressed on FDCs, furthermore the expression of FcεRII and FcγRIIb are induced upon maturation [315, 316]. This enables the trapping of antigen in the form of immune complexes which can be retained for long periods of time [317]. *In vivo* imaging studies have confirmed that B cells travel along the dendrites of FDCs where they can capture trapped antigen, supporting the notion that an important function of FDCs is to provide an antigen source available to GC B cells [275, 318]. The contacts between FDCs and B cells are believed to be mediated by the integrins ICAM-1 and VCAM-1. The importance of their expression on FDCs is demonstrated by their ability to prevent apoptosis in B cells [319]. Moreover, suboptimal levels of FDC-expressed ICAM-1 and VCAM-1 results in reduced proliferation of GC B cells [319].

Although antigen persistence on FDCs have been shown to be important for CSR, SHM and memory responses in some studies [320-323], there are also reports where the inability to form immune complexes and to retain antigen on FDCs failed to have an impact on these parameters [324]. However, the deposition of antigen on FDCs below the limit of detection cannot be excluded in this study.

Antigens can be delivered to FDCs by either cellular transport or via the conduit system. In the spleen, cellular transport is mediated by the MZB cells. Their localization to the MZ, in close contact with percolating blood, enables them to trap immune complexes via CR1 and CR2. The trapped immune complexes can then be transferred to FDCs [325]. This transport from the MZ to the follicles was shown to be mediated by the expression of the sphingosine 1-phosphate (S1P) receptor $S1P_1$ and CXCR5 on the MZB cells [326]. S1P is abundant in blood and therefore also found in the MZ. The MZB cells express high levels of $S1P_1$ receptor which facilitates their positioning to the MZ. Exposure to S1P down-modulates the $S1P_1$ receptor, allowing signals from the CXCR5 receptor to dominate. This causes the MZB cells to migrate into the follicle in response to CXCL13 where the trapped immune complexes can be transferred to FDCs. However, the concentration of S1P is low in the follicle, therefore the expression of the $S1P_1$ receptor can recover and consequently the MZB cells migrate back into the MZ again. This shuttling of the MZB cells mediates the transfer of complexed antigen into the follicle to be deposited on FDCs [326]. How antigen can be trapped on FDCs during a primary response in the absence of complement fixing specific antibodies was addressed in a recent study [327]. The deposition of particulate antigen in the form of VLPs was compared to that of soluble protein. Retention of both VLPs and protein antigens on FDCs required complement activation. However soluble protein antigens did not localize to FDCs in the absence of pre-formed specific antibodies. In contrast, VLPs were deposited on FDCs even in the absence of prior immunity, a process that was shown to be dependent on natural IgM [327]. Thus, the mechanism by which antigen localizes to FDCs varies depending on the type of antigen, a process that can be mediated by the innate humoral immune system. Similar to the antigen transport by MZB cells in the spleen, non-cognate B cells can trap antigen-immune complexes from the subcapsular sinus macrophages in lymph nodes. The B cells bind the immune complexes via complement receptors and transfer the antigen into the follicle where it is retained on FDCs [322, 328]. In addition, antigen can be transported via the conduit system. Conduits are small tubular structures that perforate both B cell follicles and the T cell areas in lymph nodes and in the spleen. Due to their small size they can only accommodate antigen smaller than approximately 70 kD [329]. Transport via the conduits is fast, allowing for the transfer of antigen into the follicles within minutes. The close contact between FDCs and conduits is likely to mediate the delivery of antigen for deposition to the FDCs [330, 331].

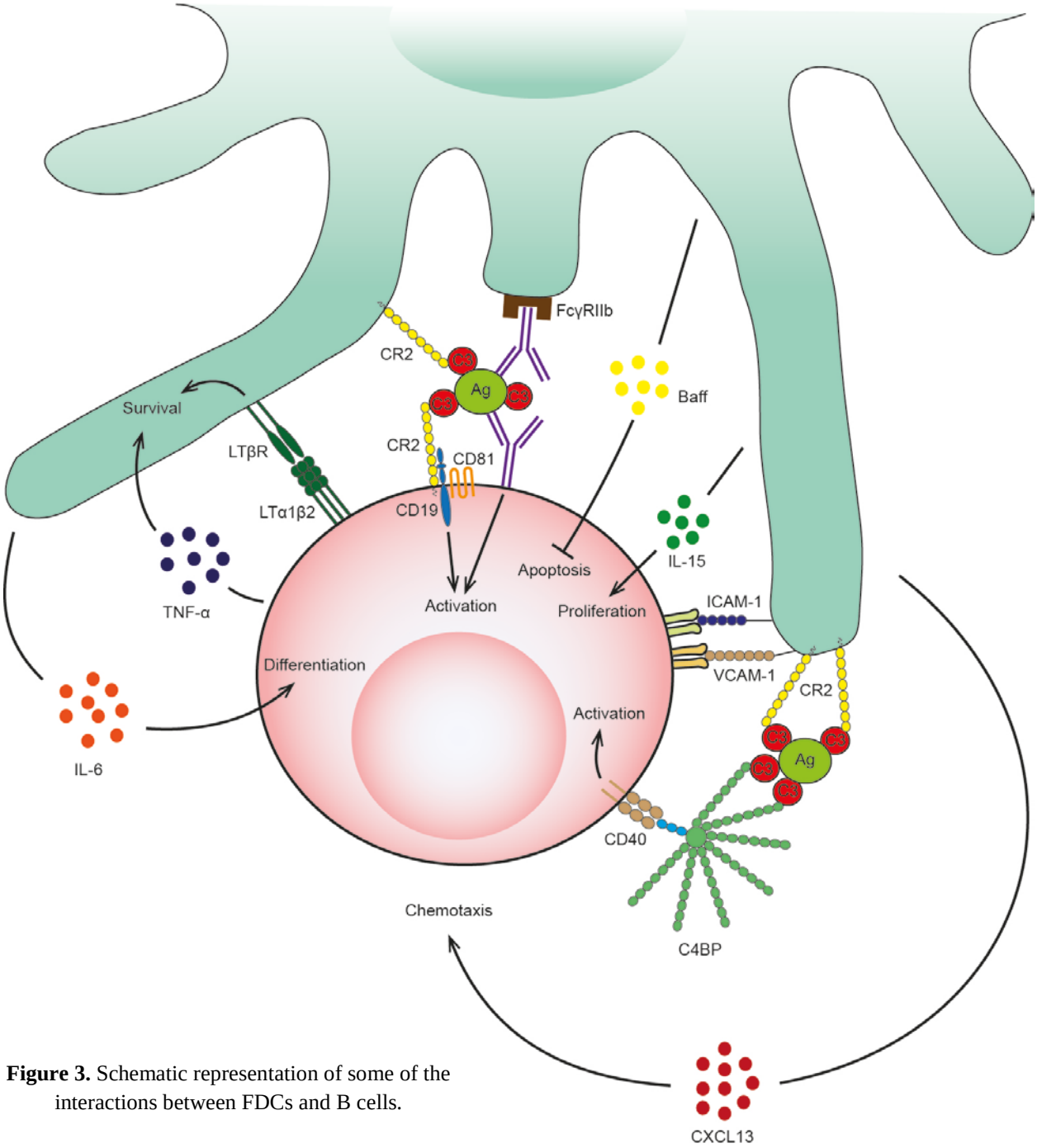

Figure 3. Schematic representation of some of the interactions between FDCs and B cells.

In addition to providing a source of antigen for GC B cells, FDCs provide a number of factors involved in GC homeostasis and B cell survival, as summarized in (Fig. 3). FDCs and follicular stromal cells secrete the chemokine CXCL13 which maintain the integrity of the B cell follicle as well as the GC organization [332]. FDCs have also been shown to be a source of the B cell activating factor (BAFF) which is important in mediating GC persistence by promoting B cell survival [333, 334]. Furthermore IL-6 and IL-15 have been shown to be produced by FDCs. Chimeric mice that lack FDC-expressed IL-6 display impaired GC formation, CSR and SHM [335, 336]. The role of IL-15 expressed by FDCs is less well

studied. However IL-15 produced by FDCs has been shown to promote GC B cell proliferation *in vitro* [337]. Upon maturation FDCs are known to up-regulate a large number of the genes discussed in previous sections, these include Baff, ICAM-1, VCAM-1, CXCL13, IL-6, IL-15, FcγRIIb, FcεRII, LTβR etc. [312, 315, 316, 333, 335, 338]. The mechanism behind FDC maturation is not well understood, but it is known to be induced by TLR ligation or by the binding of immune complexes via FcγRIIb [315, 338, 339]. There is also evidence suggesting that FDCs can be induced to mature by indirect activation via increased levels of lymphotoxin and TNF-α [340, 341].

FDCs are not only required to initiate and maintain T cell dependent GC reactions. They have also been shown to drive the formation of GCs in the absence of T cells [342-344]. This ability has been shown to be dependent on the localization of antigen-bound immune complexes to the FDCs as illustrated by the absence of GCs when immunizing with antigen unable to bind to FDCs [343]. Additionally, GCs can form in CD40L knockout mice, but not in mice lacking CD40, suggesting that an alternative CD40 ligand can rescue the GC response in these mice [345]. The complement receptor C4 binding protein (C4BP), expressed on FDC have been identified as a ligand for CD40 [346]. Blocking C4BP *in vitro* abolishes the ability of FDCs to drive T cell-independent GC responses, indicating that FDCs can promote GC in the absence of T cells by providing an alternative CD40-ligand, which is C4BP [343].

Aims

The aim of this thesis work was to acquire a better understanding of the mechanisms behind the adjuvant function of CT and CTA1-DD. Our approach was to study the adjuvant effects *in vivo* and to investigate the requirements for critical components of the immune system for the adjuvant function. By comparing similarities and differences between CT and CTA1-DD we hoped to gain insights into why these molecules are strong adjuvants.

The specific aims were:

- To characterize the *in vivo* localization of CT and CTA1-DD following immunization
- To compare the requirements for adjuvanticity between CT and CTA1-DD to better understand adjuvant actions; especially with regard to cell targeting and changes in cell distribution and migration
- To determine the importance of cell targeting for the immunomodulating effect and more specifically in the formation of germinal centers
- To assess the role of dendritic cells for the adjuvant function of CT and CTA1-DD

Key methodologies

This section describes the main experimental procedures used in this thesis work, a more detailed description can be found in each manuscript.

Mice and immunizations

With the exception of Cr2-/- mice, bred on the Balb/c background, the majority of mice used in this study were on the C57bl/6 background. Knockout and transgenic mice were bred and maintained at the experimental biomedicine (EBM) facility at the University of Gothenburg, Sweden. Wild type mice were obtained from Taconic Farms (M&B, Lille Skensved, Denmark). All mice were age- and sex matched and kept under specific pathogen free conditions in individually ventilated cages. Unless otherwise indicated, immunizations with the CTA1-DD adjuvant was administered at an optimal dose of 10µg, for the CT holotoxin, or the CT-OVA conjugate, mice were given 1µg. Admixed antigens were given as conjugates of the (4-hydroxy-3-nitrophenyl) acetyl (NP) hapten with chicken γ-globulin (CGG) or ovalbumin (OVA). The NP-hapten was used because of the well-defined and restricted humoral immune responses induced upon immunization. Secondary responses to NP-coupled antigens can generate antibodies with up to 10-times higher affinities as compared to that of primary responses [347-349]. Furthermore we took advantage of the availability of the NP-specific transgenic B1-8^{hi} IgH knock-in mice, bred to B6-GFP mice, in order to detect antigen specific B cells in lymphoid tissue following immunization [275, 350]. In order to reliably detect the adjuvants in lymphoid tissues, most immunizations were given as iv. injections.

Immunohistochemistry

Immunohistochemistry is an excellent tool to get qualitative information about the distribution of cells and other components in tissue. This technique has been pivotal to this study and has enabled us to describe cell migration- and activation, as well as the *in situ* distribution of the adjuvants CT and CTA1-DD. Spleens or lymph nodes were embedded in OCT-medium and snap frozen using isopenthane cooled by liquid nitrogen. For the detection of GFP^+ cells, samples were fixed in 4% paraformaldehyde prior to freezing in order to preserve GFP fluorescence. Sectioning was performed using a cryostat, sections were air dried and fixed in acetone. To prevent unspecific binding of antibodies to e.g. Fc-receptors, sections were blocked using normal horse serum and then incubated with antibodies as specified in Table 1. In order to reliably detect CT in tissue, we conjugated the holotoxin to ovalbumin and detected the conjugate using a rabbit anti-ovalbumin antibody followed by a secondary anti-rabbit antibody. Using this approach we were able to significantly enhance the signal as compared to when using unconjugated CT. CTA1-DD was detected using two alternative techniques; either by directly labeling the adjuvant with the fluorochrome AlexaFluor-488 or biotin, allowing for the direct visualization of the adjuvant, or by using an anti-CTA1-DD chicken IgY antibody. Given that chicken antibodies bind poorly to protein A, this eliminates the risk of unspecific binding via the DD-domain. Images were recorded using a conventional Leica DM LB microscope, or the Zeiss LSM 510 META- or 710 confocal microscope system available at the Centre for Cellular Imaging core facility at the University of Gothenburg.

Table 1. Antibodies used for immunohistochemistry.

Primary	Conjugate	Company	Secodary	Conjugate	Company
B220	biotin	BD Biosciences	streptavidin	TXRD	Dako
B220	FITC	BD Biosciences			
CD11c	__	BD Biosciences	α-hamster	Cy3	Jackson ImmunoResearch
CD11c	biotin	BD Biosciences	streptavidin	AlexaFluor-405	Invitrogen
CD86	biotin	BD Biosciences	streptavidin	AlexaFluor-488	Invitrogen
CD80	PE	BD Biosciences			
CD8α	AlexaFluor-647	BD Biosciences			
Collagen I	__	Millipore	α-rabbit	Cy5	Jackson ImmunoResearch
CR1/CR2	FITC	BD Biosciences			
CTA1-DD	__	Agrisera	α-chicken	DyLight-488	Jackson ImmunoResearch
ER-TR9	biotin	BMA biomedicals	streptavidin	AlexaFluor-488	Invitrogen
F4/80	FITC	Serotec			
GL7	FITC	BD Biosciences			
GL7	AlexaFluor-647	eBiosciences			
Gr-1	FITC	BD Biosciences			
Laminin	__	Sigma-Aldrich	α-rabbit	FITC, TXRD, Cy5	SouthernBiotech, Jackson ImmunoResearch
MARCO	FITC	Serotec			
MARCO	__	Serotec	α-rat	Cy3	Jackson ImmunoResearch
Mfge8	__	MBL International	α-hamster	Cy3	Jackson ImmunoResearch
Ovalbumin	__		α-rabbit	FITC, TXRD	SouthernBiotech
TO-PRO		Invitrogen			

Antibody assay

Unless otherwise stated, serum samples were collected 8d after the booster immunization, using antigen without the addition of adjuvants. Antibody responses were measured using ELISA. For the detection of NP- or OVA-specific antibodies, plates were coated with NP-BSA or OVA respectively. Following a blocking step, serum samples were added and a 3-fold dilution series was performed. Antibodies of different isotypes were detected using anti-mouse antibodes conjugated to alkaline phosphatase and developed using phosphatase substrate tablets diluted in ethanolamine buffer. Antibody titers were calculated by measuring the highest dilution giving an optical density value of 0,4 above background.

Preparation of fusion proteins and CT-conjugates

The fusion proteins CTA1-DD, or the enzymatically inactive form CTA1R7K-DD, and CTA1-OVA-DD, containing one copy of the $OVA_{323-339}$ peptide, were produced by transforming *E. coli* DH5 cells with the respective expression vectors. Bacteria were grown in 500ml cultures over night in SYPPG medium with carbenicillin at 37ºC. Following centrifugation, the fusion proteins were collected as inclusion bodies and extracted using 8M urea. Proteins were refolded by slowly diluting them in Tris-HCl, and purified by ion exchange- and size exclusion chromatography. Endotoxin levels were consistently low, at <100 units/mg.

CT-OVA and CTB-OVA conjugates were produced using the crosslinker *N*-succinimidyl 3-(2-pyridyldithio) propionate, (SPDP). CT (LIST Biological Laboratories), rCTB (provided by Dr. J. Holmgren at the Department of Microbiology and Immunology, University of Gothenburg, Gothenburg, Sweden) and OVA were coupled to SPDP and conjugated as described before [351]. Concentration of the conjugates, and the OVA content, was determined by ELISA against a standard dilution of CT, CTB and OVA. The molar ratio of OVA to CT or CTB was 4:1.

Cell sorting and quantitative real-time PCR

In order to analyze the mRNA expression of genes in OT-II transgenic CD4 T cells or FDCs, we sorted splenocytes into RLT buffer using a FACSAria II. For isolating OVA-specific T cells, single cell suspensions of spleens were prepared from $CD45.2^+$ recipient mice adoptively transferred with CFSE labeled $CD45.1^+$ OT-II T cells. OT-II cells were identified as being $CD4^+$, $CD45.1^+$, $CFSE^+$ and $B220^-$. FDCs were isolated as previously described [352]. Mice were irradiated and spleens were enzymatically digested using collagenase D, dispase I and DNAse I. FDCs were identified as being $CD21/CD35^+$ $Mfge8^+$ and $CD45.2^-$. RNA was prepared using the RNeasy micro kit (Qiagen) and converted to cDNA using the SuperScript III First-Strand cDNA kit (Invitrogen) or the QuantiTect reverse transcription kit (Qiagen).

Bcl-6 and CXCR5 expression in sorted OT-II T cells was normalized to β-actin and assessed by qRT-PCR. The following primers were used: CXCR5 forward; 5'AAC TAC CCA CTA ACC CCT GGA CAT'3, reverse; 5'AAG TTA CTG TCC TGT AGG GGA ATC T'3, Bcl-6

forward; 5'GTA CCT GCA GAT GGA GCA TGT'3, reverse; 5'CTC TTC ACG GGG AGG TTT AAG T'3, β-actin forward; 5'CCA CAG CTG AGA GGG AAA TC'3, reverse; 5'CTT CTC CAG GGA GGA AGA GG'3.

For the isolated FDCs, a custom made gene array plate from SABiosciences was used to assess the mRNA expression of selected genes, fold change between groups was normalized to HPRT housekeeping gene and calculated using the SABiosciences web portal.

Bone marrow chimeras

Bone marrow chimeras represent a powerful tool for generating mice that display different expression patterns of the same gene product in different cell types. For example, we have exploited the fact that FDCs are radiation resistant whereas B cells are sensitive to radiation. Therefore lethally irradiated Cr2-/- mice reconstituted with WT bone marrow lack CR1/CR2 on radiation resistant cells e.g. FDCs, whereas B cells display normal expression levels. This allowed us to assess the role of complement receptors expression specifically on FDCs.

The same rational was used when constructing the CD11c-DTR mice. Injecting DTx into native CD11c-DTR mice results in the death of the transgenic mice 6-7 days after injection. This is believed to due to the loss of an unknown radiation resistant cell type that expresses CD11c [353]. Therefore, chimeras of WT mice reconstituted with CD11c-DTR bone marrow were used in order to be able to follow the mice for longer periods of time following DTx injection.

A notable disadvantage with the CD11c-DTR model is the fact that both plasma cells and GC B cells express low levels of CD11c ([354] and personal communication M Dahlgren and B Johansson-Lindbom), therefore repeated DTx injections will inevitably obliterate GC- or plasma cell responses in these mice. To circumvent this problem, we constructed bone marrow chimeras by reconstituting irradiated WT mice with a 4:1 mix of CD11c-DTR Tg:μMt and MHC-II$^{-/-}$ bone marrow donor cells. The resulting CD11c-DTR/MHC-II$^{B-/-}$ mice express MHC II and the DTR on 80% of the DC population; the remaining DCs are MHC II^{-}. Furthermore, all B cells lack MHC II expression [355]. Therefore, by adoptively transferring NP-specific GFP^{+} transgenic B cells that don't express the DTR, to the CD11c-DTR/MHC-II$^{B-/-}$ chimeras, repeated DTx injections could not affect the responding NP-specific GC- or plasma cells. This allowed us to give the mice repeated DTx injections in order to deplete DCs for longer periods of time, and also provided the possibility of accurately monitoring the NP-specific B cells directly via their GFP-expression.

Results

CTA1-DD localizes to follicular dendritic cells in a complement dependent manner

In order to gain a better understanding of how CTA1-DD augments immune responses, the *in vivo* distribution of the fusion protein following immunization was assessed. We sought to determine which cells the adjuvant targeted when using either iv. or in. routes. We found that the adjuvant localized in a distinct manner, accumulating within the B cell follicles of the spleen- or mediastinal lymph nodes respectively. Staining for Mfge8, a marker identifying FDCs [356], revealed that CTA1-DD co-localized to this population. FDCs express high amounts of the complement receptors CR1/CR2 and are known to trap antigen bound by complement [323]. Therefore, we analysed the ability of CTA1-DD to target FDCs in mice lacking complement C3 (C3-/-) or CR1/CR2 (Cr2-/-). Strikingly, CTA1-DD failed to bind FDCs in these mice, demonstrating that the accumulation of the fusion protein to FDCs was dependent on complement and complement receptors (Fig. 1).

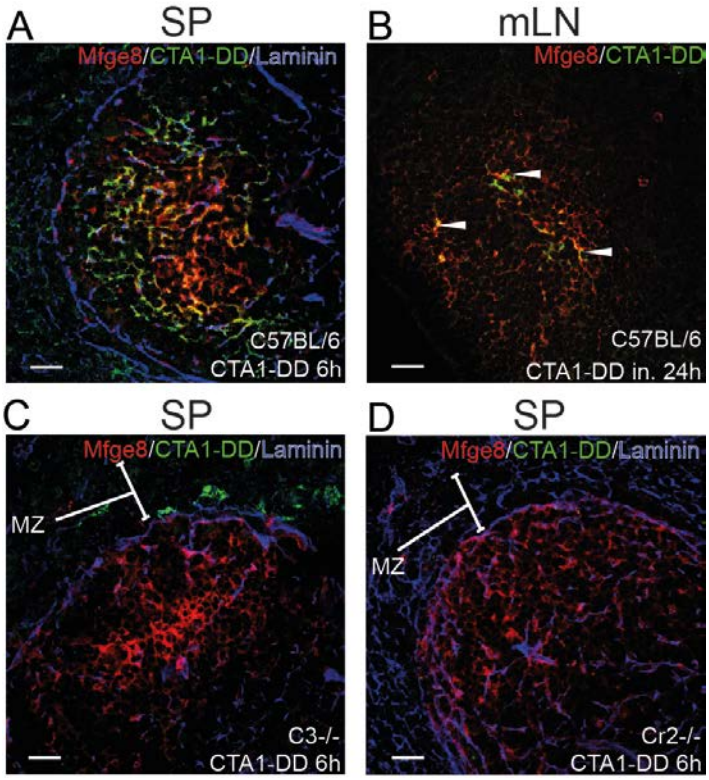

Figure 1. A-B. CTA1-DD localizes to the FDC network in the spleen (SP) or mediastinal lymph nodes (mLN) at indicated time points following iv. or in. immunizations respectively. **C-D.** Deposition of CTA1-DD to FDCs is dependent on complement as demonstrated by the lack of FDC localization in mice deficient in complement C3 or complement receptor 1 and 2. FDCs are labelled red with Mfge8, CTA1-DD is shown in green and laminin, demarking the marginal zone, in blue.

CTA1-DD is dependent on complement for full adjuvant function

The functional importance of the localization of CTA1-DD to the FDC network was assessed by immunizing C3-/- mice with CTA1-DD together with the antigen NP-CGG and evaluating antibody responses. NP-specific antibody titers were significantly reduced in the C3-/- mice as compared to wild type (WT) mice, demonstrating that the presence of complement is essential for the full immunoenhancing ability of CTA1-DD. However, despite the lack of complement and the resulting inability to trap CTA1-DD to FDCs in these mice, a residual

adjuvant effect was still present. To evaluate the nature of this remaining complement-independent adjuvant effect, we transferred NP-specific B cells that express the green fluorescent protein (GFP) into WT and C3-/- knockout mice. Recipient mice were then immunized with NP-CGG together with CTA1-DD and the phenotype of the expanding NP-specific B cells was evaluated. The frequency of GFP^+ B cells expressing the GC marker GL7 was completely abolished in the C3-/- mice, whereas the number of early plasma cells, identified by the marker CD138 remained unaffected. This demonstrates that the residual enhancement of the antibody response in the C3-/- mice is due to antibody secretion by extra follicular plasma cells whereas GC formation is critically dependent on the localization of CTA1-DD to the FDC network (Fig.2). Notably C3-/- mice do not lack the ability to form GCs as illustrated by the presence of GCs in unimmunized mice. Importantly, CTA1-DD was found to activate complement *in vitro*, establishing a mechanistic link to the *in vivo* findings.

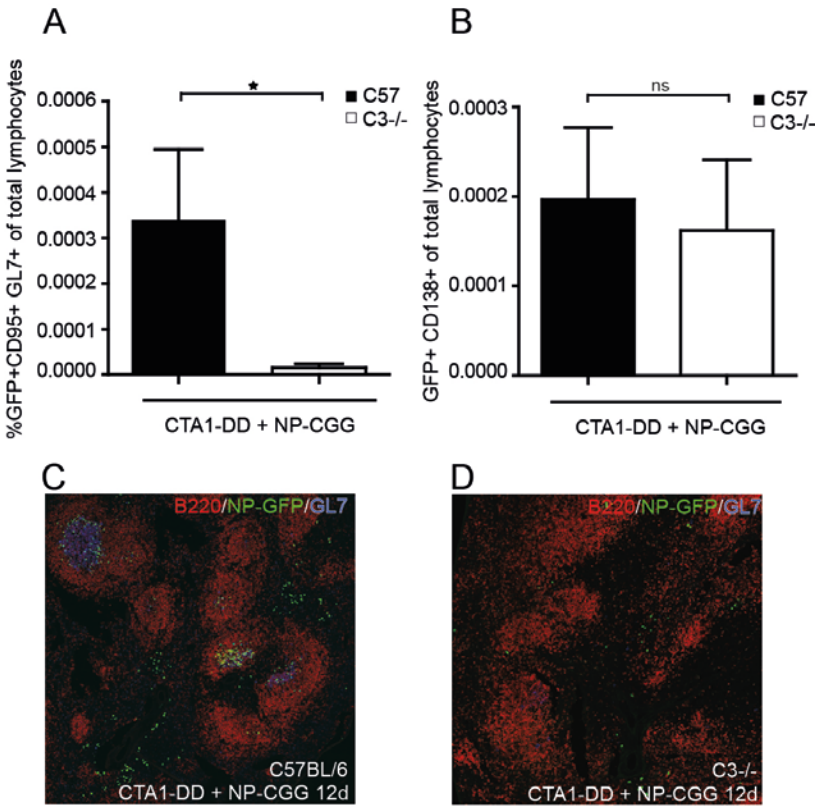

Figure 2. C3-/- mice fail to develop germinal centers following immunization with CTA1-DD, however extrafollicular B cell responses remain intact. **A-D.** C3-/- and WT mice were transferred with NP-specific GFP^+ B cells and immunized with CTA1-DD + NP-CGG. **A-B.** The frequency of NP-specific (GFP^+) germinal center B cells ($CD95^+GL7^+$) or plasma cells ($CD138^+$) was determined by FACS 12 days after immunization. **C-D.** Representative micrographs depicting GFP (green), the germinal center marker GL7 (blue) and B220 (red).

Complement C3 is a central component of the complement system. Thus, C3-/- mice completely lack the ability to activate complement. In order to more specifically address the role of the *in vivo* binding of CTA1-DD to FDCs via complement receptors, we assessed the adjuvant function of the fusion protein in Cr2-/- mice. Similar to the C3 gene knockout mice, Cr2-/- mice also displayed severely diminished antibody titers when using CTA1-DD as an adjuvant. Interestingly, WT mice responded with increased antibody titers when the adjuvant dose was increased from 1µg to 5µg, however this was not seen in the Cr2-/- mice. Instead we observed a lower level of adjuvant activity in the Cr2-/- mice that could not be further enhanced by increasing the adjuvant dose, this could indicate that augmentation of extrafollicular plasma cell levels require lower doses of the adjuvant (Fig. 3).

It is possible that the impaired ability of CTA1-DD to augment antibody responses in Cr2-/- mice was due to a diminished ability to prime $CD4^+$ T cells. To evaluate this possibility we transferred OVA specific transgenic DO.11.10 CD4 T cells to WT and Cr2-/- mice and immunized the mice with the CTA1-OVA-DD construct, which contains the immunodominant p323-339 OVA peptide. The proliferation of DO.11.10 T cells in the Cr2-/- mice was comparable to that of WT mice, demonstrating that the enhancing effect of CTA1-DD on $CD4^+$ T cell priming was unaffected by the lack of CR1/CR2. Furthermore, the decrease in antibody titers in Cr2-/- mice was not due to a general defect in their ability to respond to adjuvants, as illustrated by the unchanged ability to enhance antibody titers in the CR2-/- mice as compared to WT mice, when immunizing with the RIBI adjuvant (Fig. 3).

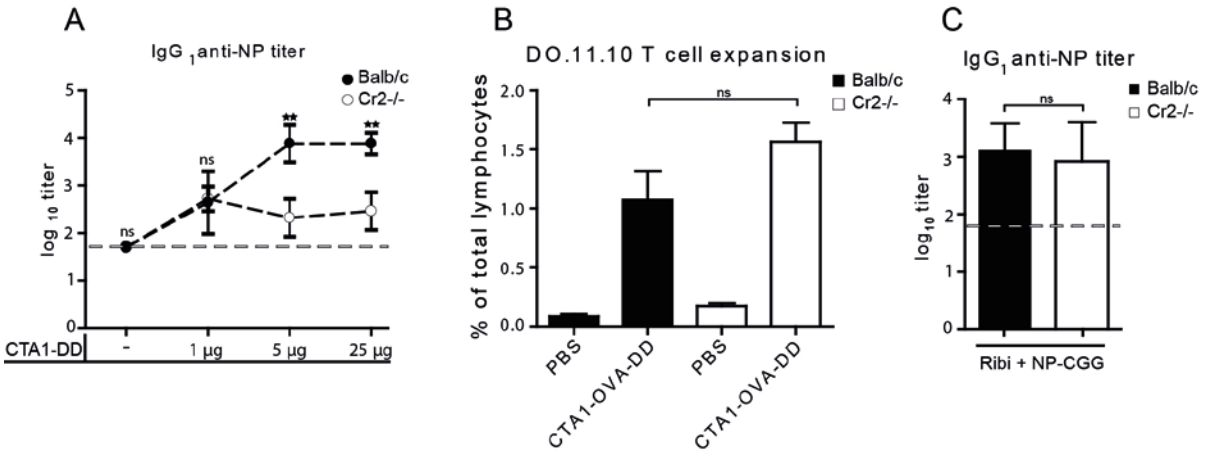

Figure 3. A. CTA1-DD exhibits a poor ability to enhance antibody responses, but retain the ability to promote CD4 T cell priming in Cr2-/- mice. In contrast the RIBI adjuvant promotes normal antibody responses in the Cr2-/- mice. **A, C.** Mice were given 1, 5, or 25 µg CTA1-DD. (A) or RIBI (C) admixed with NP-CGG followed by a booster dose with only NP-CGG. Serum was collected and anti-NP IgG_1 log $_{10}$ titers were determined. **B.** WT or $Cr2^{-/-}$ mice were adoptively transferred with DO.11.10 OVA-transgenic T cells, and immunized with 5 µg fusion protein that carried the p323 peptide from OVA inserted in the construct, CTA1-OVA-DD. Four days later, spleens were removed and the percentage of DO.11.10 T cells was determined by FACS.

The expression of complement receptors on FDCs mediates the adjuvant function of CTA1-DD

CR2 expressed on B cells associates with CD19 and CD81 to form a co-receptor complex that functions to enhance BCR signalling. Hence, simultaneous ligation of CR2 and the BCR results in a reduced threshold of activation in B cells [357, 358]. The inability to signal via this co-receptor complex could be the reason why we observed an impaired humoral immune response in C3-/- and Cr2-/-mice when immunizing with CTA1-DD. To be able to discriminate between the lack of complement receptors on B cells as compared to FDCs, we took advantage of the fact that FDCs, but not B cells, are radiation resistant. Cr2-/- mice were irradiated and reconstituted with WT bone marrow, the resulting chimeric mice expressed normal levels of CR2 on B cells, but completely lacked CR2 on FDCs. However, immunizations of chimeric mice with CTA1-DD resulted in a comparable defect in antibody titers as seen when using native Cr2-/- mice, demonstrating that the expression of CR2 on FDCs, and the resulting binding of CTA1-DD to this cell type, was required for full adjuvant function of the fusion protein (Fig. 4).

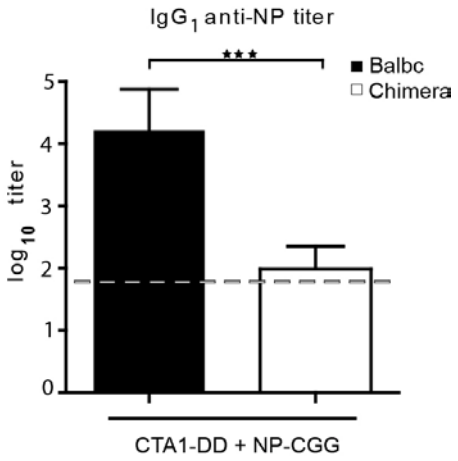

Figure 4. The adjuvant effect of CTA1-DD is impaired in chimeric mice specifically lacking CR1/CR2 on FDCs. WT and chimeric mice were given CTA1-DD + NP-CGG iv. followed by a booster dose with only NP-CGG. Serum was collected and anti-NP IgG_1 $\log_{10}$ titers were determined.

The role of classical dendritic cells in the CTA1-DD adjuvant effect

Many adjuvants modulate the immune response by activating DCs, either directly by binding to pattern recognition receptors (PRRs) or indirectly via the generation of DAMPs [359]. Previous studies have only indirectly addressed the role of DCs for the adjuvant function of CTA1-DD [126]. Therefore, we employed the CD11c-DTR mouse, in which $CD11c^+$ DCs express the human diphtheria toxin receptor (DTR), enabling a transient depletion of DCs by the injection of diphtheria toxin (DTx) [360] (Fig. 5). Using these mice we evaluated the importance of DCs in the generation of specific antibodies following immunizations with CTA1-DD together with NP-CGG. We found that the formation of antibody secreting cells (AFCs) in the spleens of immunized mice was significantly reduced in DC depleted- as compared to DC-competent mice. This was associated with a reduction in antibody titers, suggesting that DCs play a role in mediating optimal adjuvant functions of CTA1-DD. Next, we analysed the impact on $CD4^+$ T cell priming in the presence- or absence of DCs. To this end we employed mixed bone marrow chimeric DTR-mice in which the endogenous B cells lack MHC II, and therefore unable to act as APCs [355]. Following adoptive transfer of NP-specific B cells and OVA-specific OT-II $CD4^+$ T cells, the mice were immunized with CTA1-DD together with NP-OVA. Using this system, we could not detect a significant reduction of OT-II cells or NP-specific GC formation in DC-depleted mice (Fig 6).

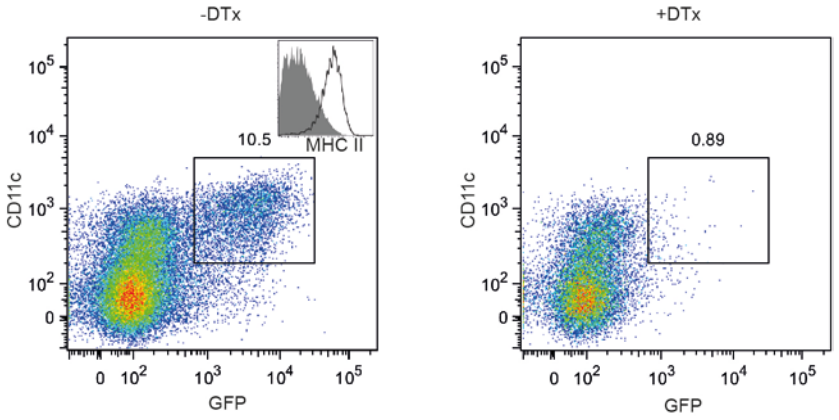

Figure 5. Administration of diphtheria toxin (DTx) to CD11c-DTR mice efficiently depletes CD11c DCs. CD11c-DTR mice were left untreated or given DTx and the spleen was analyzed for $CD11c^+$ dendritic cells by FACS 18h later. Live cells are gated as being $CD3^-$ $CD19^-$. CD11c-DTR mice co-expresses GFP with the DTR.

The fact that CTA1-DD promoted $CD4^+$ T cell priming in the absence of DCs prompted us to investigate this finding in more detail. Reasoning that CTA1-DD could have exerted adjuvant function by interacting with several cell types other than DCs when ample amounts of antigen was provided, we analyzed whether a limited access to antigen would change the outcome. To this end we used the CTA1-OVA-DD construct. Incorporating the immunodominant OVA peptide into the adjuvant represents an effective means of delivering antigen and adjuvant to the same cell, thus allowing for immunizations with a lower antigen dose. Nonetheless, CTA1-OVA-DD was still able to prime CD4 T cells in DC-depleted mice, indicating that CTA1-DD can compensate for the lack of DCs in the CD11c-DTR mice (Fig. 6). However, whereas DC-depleted mice, immunized with either CTA1-OVA-DD or CTA1-DD admixed with antigen, displayed no reduction in the frequency of OT-II T cells, a functional defect may still have been present in these cells, accounting for the reduced NP-specific antibody responses.

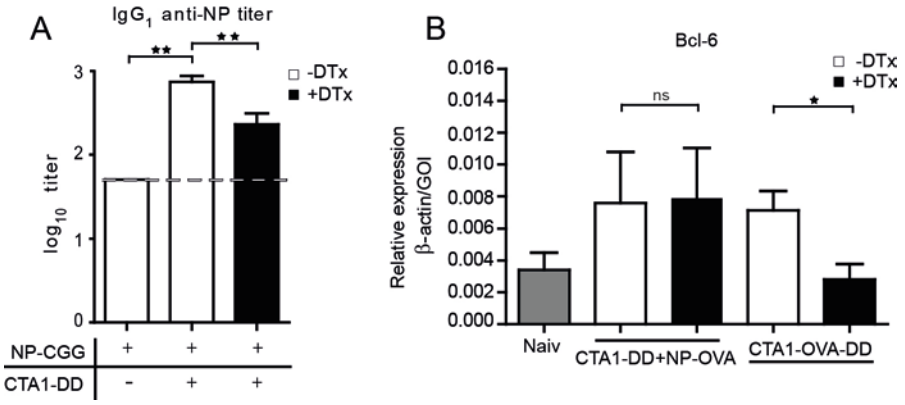

Figure 6. A. CD11c-DTR mice were untreated or given DTx , 18h later mice were immunized with NP-CGG together with CTA1-DD. Mice received a booster dose with only NP-CGG 10 d later, serum was collected and anti-NP IgG_1 $\log_{10}$ titers were determined. **B.** Reduced Bcl-6 expression in DC-depleted CD11c-DTR mice immunized with CTA1-OVA-DD but not in mice given CTA1-DD + NP-OVA. CFSE labeled $CD45.1^+$ OT-II T cells were adoptively transferred to CD11c-DTR ($CD45.2^+$) or CD11c-DTR/MHC-$II^{B-/-}$ chimeras ($CD45.2^+$) at day 1, the latter mice were also given NP-specific GFP^+ B cells. Mice were were untreated or given DTx. 18h later mice were immunized with CTA1-OVA-DD or CTA1-DD + NP-OVA respectively. Mice were sacrificed at day 9 and $CD45.1^+$ OT-II cells were sorted and their expression of Bcl-6 mRNA was determined by qRT-PCR.

Therefore we investigated the formation of OT-II Tfh cells. Tfh cells are characterized by their expression of the transcription factor Bcl-6 [291, 292]. We sorted OT-II T cells from DC-competent or DC-depleted mice that had been immunized with either CTA1-DD admixed with NP-OVA or the CTA1-OVA-DD construct. We found a significant increase in the Bcl-6 expression in immunized mice as compared to naïve controls. However, we detected no difference in Bcl-6 expression between untreated and DC-depleted mice that had been immunized with CTA1-DD admixed with NP-OVA. In contrast, we found a significant reduction of the Bcl-6 expression in DC-depleted mice immunized with CTA1-OVA-DD. This suggested that functional Tfh cells were generated in mice when ample amounts of antigen was provided, while mice immunized with CTA1-OVA-DD, and thus receiving a

limited dose of antigen, were dependent on DCs for an effective priming of OT-II Tfh cells. Interestingly, there was no significant difference in the frequency of GC B cells in these mice, arguing that even though the Bcl-6 expression in OT-II cells was reduced, CTA1-DD was able to compensate for this defect and still promote a strong GC-reaction (Fig. 6).

CTA1-DD directly activates FDCs

Despite the fact that DC-depleted mice fail to develop fully differentiated Tfh cells when immunized with CTA1-OVA-DD, these mice still developed normal GCs. We hypothesised that CTA1-DD employed a compensatory mechanism that allowed GC to form under these circumstances. For example, FDCs are known to express an alternative CD40 ligand, C4BP, and have been shown to be able to support GC-responses in the absence of T cell help [342-344, 346]. Given our previous results where the ability of CTA1-DD to bind to FDCs was correlated to the augmentation of antibody responses, we speculated that CTA1-DD could have activated FDCs and in this way supported GC formations. To investigate this possibility we sorted FDCs from naïve- or immunized mice and analysed their expression of a panel of genes that could potentially be involved in FDC maturation and GC formation. Strikingly, we found that mRNA expression of a number of genes was up-regulated in FDCs sorted from mice immunized with CTA1-DD as compared to FDCs from naïve controls. These genes included, Baff and IL-15, which are known to promote GC B cell survival, IL-6 which can drive isotype switching, VCAM-1 and ICAM-1 that are believed to facilitate the interactions between B cells and FDCs, CXCL13 that organizes B cell follicles and GCs and LTβR which is required for the survival of FDCs [270, 312, 319, 333-338, 341, 361, 362]. This observation may explain why CTA1-DD is dependent on CR1/CR2-mediated binding to FDCs in order to promote GCs. The activation of FDCs by the adjuvant could either be a result of direct effects of CTA1-DD on the FDCs themselves, or mediated indirectly via factors produced as a result of immunization. In order to address this issue, we used an FDCs cell line and incubated the cells with CTA1-DD. We found that cells incubated with CTA1-DD up-regulated some of the same genes that were also observed to be induced during the *in vivo* experiments, suggesting that CTA1-DD could indeed act directly on the FDCs to induce their activation. Future experiments will address which of these genes that are involved in the adjuvant function of CTA1-DD and, more specifically, in the formation and maintenance of GCs (Fig. 7).

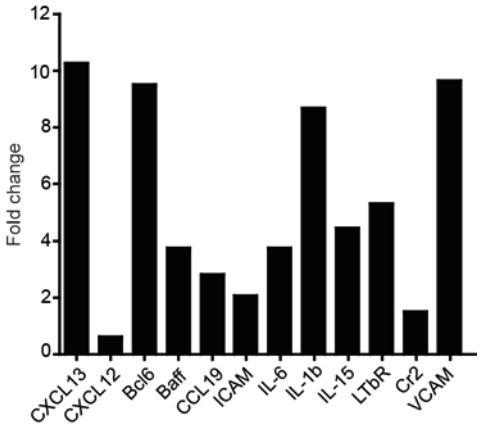

Figure 7. FDCs up-regulate genes involved in germinal center formation upon immunization with CTA1-DD. FDCs were isolated from irradiated naive- or CTA1-DD-immunized mice. Their expression of indicated genes was assessed using qRT-PCR. Expression was normalized to HPRT housekeeping genes, fold change is given as a ratio between naïve and immunized mice.

In conclusion these data suggests that CTA1-DD can enhance immune responses independently of DCs, but instead requires FDCs for full adjuvant function. This is in contrast to the native CT which has been suggested to function via the activation of DCs, highlighting the importance of the differential biding properties of these molecules [91-98]. Having demonstrated that CTA1-DD binds to FDCs in vivo, we wanted to explore if CT distributed in a similar manner.

Marginal zone macrophages are essential for accumulating CT in the marginal zone but dispensable for the induction of immune responses

While CTA1-DD activates complement, and subsequently allows the adjuvant to be localized to FDCs, the native cholera toxin employs a fundamentally different binding mechanism. The binding of CT to Gm1 gangliosides, enables the holotoxin to bind essentially all nucleated cells [62]. This promiscuous binding capacity of CT has complicated the identification of a principal target population by which CT exerts its adjuvant activity. In order to investigate whether CT is preferentially taken up by a specific cell type *in vivo*, we conjugated ovalbumin (OVA) to the holotoxin to allow us to detect the conjugate with anti-OVA antibodies and thus monitor the tissue distribution following administration. We immunized mice i.v with CT-OVA and found that CT accumulated in the MZM of the spleen. However, after we had depleted the MZM with chlodronate liposomes, we still had an intact adjuvant function of CT, suggesting that other cell types, such as DCs were responsible for the adjuvant function of CT. This assumption agrees well with earlier studies [91-98].

CT-immunization stimulates the generation of a mixed Th1/Th2/Th17 response

CT is widely regarded as a Th2-inducing adjuvant, a characteristic which was recently linked to the inhibition of the transcription factor IFR-8 which positively regulates IL-12 production and controls the differentiation of $CD8a^+$ DCs [104, 363]. Despite these reports, there are also several studies describing a potent Th1-inducing ability of the CT-adjuvant [78-80, 105-108]. In order to better determine the nature of CT-adjuvanted immune responses, we transferred OT-II cells into wild type recipient mice and immunized the mice with CT conjugated to ovalbumin. The cytokine profiles of re-stimulated splenocytes revealed a significant production of IFN-γ as well as IL-17A as compared to negligible amounts of these cytokines when mice were immunized with CTB-OVA or unconjugated OVA. In addition, CT-immunization generated equivalent levels of IgG_1 and IgG_{2c} anti-OVA antibodies, representing a mixed Th1/Th2 response. Given that CT has been shown to inhibit the production of IL-12, an ability that has been linked to impaired Th1 responses, we investigated its adjuvant function in IL12p40-/- mice. We found that CT was able to enhance OT-II T cell proliferation and IFN-γ secretion in the knockout mice comparable to that of WT controls. Thus, these results indicated that the ability of CT to prime $CD4^+$ T cells is independent of IL-12 and does not appear to be restricted to Th2 cells, but also involves Th1 and Th17 cells (Fig. 8).

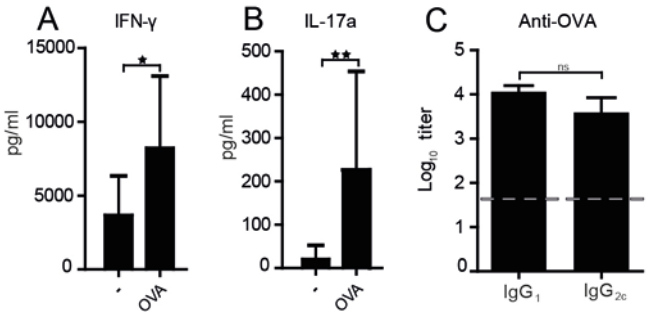

Figure 8. Cholera toxin induces a balanced Th1/Th2/Th17 response following immunization. **A-B.** Mice (CD45.2$^+$) were given CFSE labeled CD45.1$^+$ OT-II T cells at day 1. At day 2 mice were immunized with CT-OVA. Mice were sacrificed at day 9 and splenocytes were cultured with recall antigen OVA, supernatants were assessed for the production of IFN-γ(A) or IL-17A (B). **C** Mice were immunized using CT-OVA, 10 days later the mice received a booster with OVA. Serum was collected and OVA specific IgG_1 and IgG_{2c} log_{10} titers are given.

CT preferentially activates CD11b$^+$ DCs

CT has been shown to induce the maturation of DCs *in vivo*, in fact the presence of DCs have been reported to be essential in mediating the adjuvant effect of CT following oral immunization [95, 98]. To investigate the role of DCs during i.v immunizations we analysed the effect of CT on splenic DCs. By immunostaining spleen sections for CD11c and CD86, we observed a marked up-regulation of CD86 expression on CD11c$^+$ cells at 24h after CT-administration. Strikingly, activated DCs were induced to migrate into T cell areas. A corresponding FACS analysis revealed that the CD11b$^+$ DC subset was specifically induced to express CD86 whereas CD8α$^+$ DCs failed to mature in response to CT, indicating that the CD11b$^+$ population mediate the enhanced priming of CD4 T cells in mice immunized with CT, whereas CD8α$^+$ DCs are dispensable in this regard (Fig. 9).

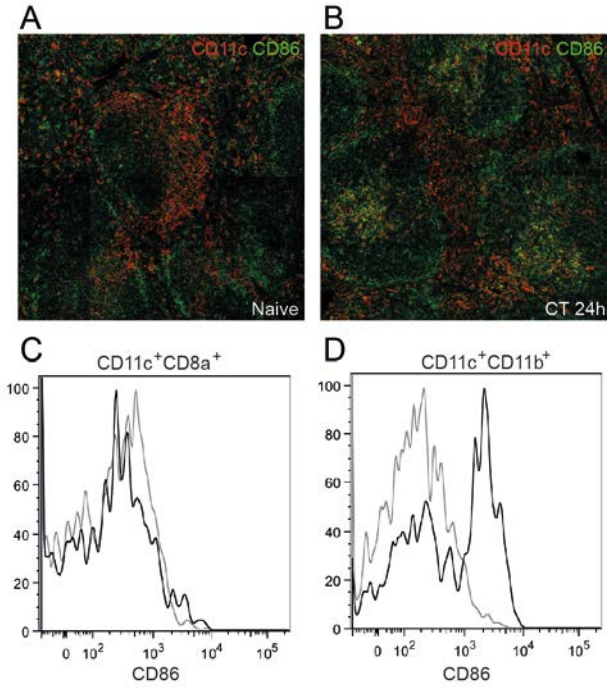

Figure 9. CT immunization induces the expression of CD86 on CD11b$^+$- but not CD8α$^+$ dendritic cells and activated cells migrates into T cell areas. Mice were given CT and were sacrificed after 24h. **A.** Spleen sections were stained using anti-CD11c (red) to visualize dendritic cells and anti-CD86 (green). **B.** FACS staining of CD86 expression on CD11b$^+$ and CD8α$^+$ splenocytes. Cells are gated on CD11c and MHC II. Grey lines indicate naïve- and black lines immunized mice.

CT pre-treatment abolishes the response to subsequent immunization

We observed a substantial reduction in total DC numbers after immunization with CT, initially the decrease was largely attributed to the loss of $CD8\alpha^+$ DCs, whereas the $CD11b^+$ population was largely unaffected. However at 5 days after CT immunization the latter population was also reduced. We analyzed whether this observation had any influence on the ability to respond with antibody production to a subsequent immunization. To this end we administered CT i.v and 24h later immunized the mice with CTA1-DD together with NP-CGG and examined the anti-NP response. CT pre-treatment completely abolished the ability to respond to CTA1-DD as demonstrated by the lack of NP-specific antibody titers in treated mice. This effect was not restricted to immunizations with CTA1-DD, the same result was seen when we used the RIBI adjuvant or LPS. Furthermore, the impact of CT pre-treatment on the ability to respond to subsequent immunizations was not limited to Th1-responses, as demonstrated by the loss of anti-NP antibody titers of both IgG_1 and IgG_{2c} isotypes. In addition to the effect on antibody responses, CT pre-treatment also blocked the ability to initiate $CD4^+$ T cell responses. We found that pre-treating mice that had received an adoptive transfer of OT-II T cells and then given CT after 24h failed to stimulate an OT-II T cell response to CTA1-OVA-DD. This indicated that the abrogation of immune responses by CT-pre-treatment was most probably due to a defect in an early priming event.

Given the marked reduction in DC numbers following CT-treatment we speculated that this was the reason for the impaired ability to respond to immunizations. To address this possibility we adoptively transferred highly enriched $CD11c^+$ DCs into CT-treated mice in order to restore DC numbers, and then immunized the mice with CTA1-DD together with NP-CGG. Strikingly, the transfer of DCs into pre-treated mice restored the ability to prime an NP-specific antibody response. Taken together, these results show that CT immunization severely impaired the ability to follow-up with additional immune responses due to the lack of functional DCs. Thus, we demonstrate that CT is a powerful adjuvant which consumes DC-functions completely, leaving the tissue with no ability to raise a priming response following a renewed immunization (Fig. 10). In contrast to previous reports, this effect was not restricted to a loss of IL-12 production as demonstrated by the impact on both Th1 and Th2 responses [104]. Furthermore we found that CT was able to efficiently promote the priming of Th1, Th2 and Th17 CD4 T cell responses.

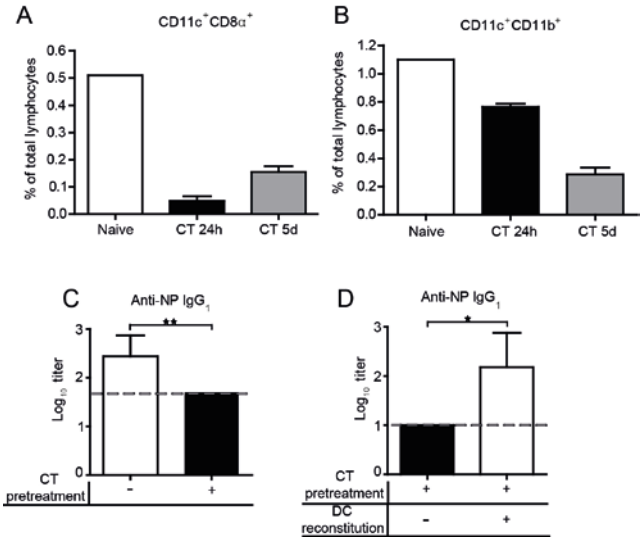

Figure 10. Treating mice with CT abolishes subsequent immune responses by inhibiting DC function. **A-B.** Mice were given CT and sacrificed after 24h or 5d, the numbers of $CD11b^+$ and $CD8a^+$ DCs in the spleen were determined by FACS. **C.** Mice were given CT and immunized with CTA1-DD + NP-CGG followed by a booster dose with only NP-CGG. Serum was collected and NP-specific IgG_1 antibody titers were determined. **D.** Mice were treated with CT and re-constituted with sorted $CD11c^+$ DCs followed by an immunization with CTA1-DD + NP-CGG and a booster dose of only NP-CGG. Serum was collected and NP-specific IgG_1 antibody titers were determined.

In conclusion, we found that CTA1-DD was able to enhance GC responses by activating FDCs, an ability that may compensate for the apparent lack of functional Tfh cells in DC-depleted mice. Furthermore, contrary to previous reports, we could demonstrate that the native CT is not only able to induce Th2 and Th17 responses but also induces Th1 cytokines independently of IL-12. The holotoxin selectively activated $CD11b^+$ DC, whereas the $CD8\alpha^+$ population was depleted. This correlated with the abolished ability to react to subsequent immunizations due to the loss of responsive DCs.

Discussion

This thesis addresses a significant knowledge gap in our understanding of how vaccine adjuvants modulate the immune response. While the identification of TLRs and other innate pathogen recognition receptors has provided new insights into the mechanism behind a number of immunostimulants, the mode of action of many adjuvants still remain unclear. For PRRs, there is a direct correlation between the receptor-ligand interaction and the resulting intracellular signaling events that lead to the activation of immune cells [364]. However many adjuvants, including the bacterial toxins CT and LT, lack a corresponding link between receptor binding and the immunostimulating effect. For example, CT can potentially bind to all nucleated cells in the body, but only a few of these cell types are likely to be involved in mediating the adjuvant effect. Thus, while CT binds to- and activates specific immune cells, it can also bind to other cells, such as enterocytes or nerve cells, which may cause toxic effects. Bell´s palsy is one of these side effects which forced the withdrawal of an LT-adjuvanted intranasal influenza vaccine from the market. CT and the closely related CTA1-DD adjuvant share the same dependence on the ADP-ribosylating enzymatic activity of the A1 subunit, however the different binding properties of CTA1-DD, as compared to the holotoxin, causes the molecule to be safe and non-toxic [114]. In the present thesis work, we hypothesized that the differential targeting of adjuvants can be exploited in order to understand how they modulate the immune response. Thus, the inherent qualities of CT and CTA1-DD provide a model that enabled us to evaluate the importance of cell targeting for their respective adjuvant effect. To this end we studied the distribution to- and the requirement of different cell types for the immunomodulating abilities of the two adjuvants.

In order to reliably deliver CT and CTA1-DD to their respective target populations we injected the adjuvants i.v, which allowed us to detect the adjuvants in the spleen. Interestingly we found that both CT and CTA1-DD initially localized to the MZM. This highly phagocytic macrophage population is ideally positioned in close contact with the slowly percolating blood of the MZ for the uptake of blood borne antigens. To assess the relative importance of this population for the adjuvant function of CT and CTA1-DD respectively, we selectively depleted the MZM by iv. administration of clodronate liposomes [174]. The liposomes are preferentially taken up by the macrophages, releasing clodronate intracellularly, thus killing the cells [365]. By depleting the MZM we found that this subset is dispensable for the adjuvant effect of both CT and CTA1-DD. In fact there was a clear trend towards a stronger T cell response in clodronate-treated mice that had been immunized with CT, as compared to mice with an intact MZM population. This suggests that the depletion of MZM might actually allow for a less restricted distribution of the adjuvant in the spleen, which would arguably increase accessibility to other cell types such as DCs. Whereas CT was mainly found to be associated with MZM, CTA1-DD also accumulated to the FDC network within the B cell follicles. This distribution was correlated to the ability of CTA1-DD to activate complement *in vitro* and to the requirement for complement C3 and complement receptors *in vivo*, as demonstrated by the lack of FDC-binding in C3-/- and Cr2-/- mice. Thus, it appears as though the MZM indiscriminately take up blood borne antigens, including CT and CTA1-DD,

whereas the deposition of CTA1-DD to the FDC network is more specific and critically dependent on complement activation, a notion that is further supported by the inability of CT to activate complement *in vitro*. Hence, the two adjuvants, while mechanistically dependent on ADP-ribosylation, appear to target different cell populations, which critically affect their adjuvant function *in vivo.*

Given that CTA1-DD does not appear to form immune complexes *in vivo* [122], we hypothesized that complement activation was mediated via the alternative activation pathway. In keeping with this hypothesis, CTA1-DD was normally distributed in C4-/- mice which are unable to activate the classical activation pathway [366]. This finding is in stark contrast to the complete abrogation of the FDC localization as seen in C3-/- or Cr2-/- mice. However *in vitro*, CTA1-DD activated complement by both the alternative- and the classical pathway suggesting that there may be a redundancy between the two pathways *in vivo*.

Antigens can be transported into splenic B cell follicles and deposited on FDCs via two different mechanisms, either by cellular transport or via the conduit system [325, 330]. Cellular transport of antigens is mediated by MZB cells, facilitated by their expression of the CR1/CR2 [325]. To be able to determine how CTA1-DD was transported to the FDCs we took advantage of the drug FTY720, which acts as a $S1P_1$ receptor agonist. Treating mice with FTY720 sequesters the MZB cells into the B cell follicles, rendering them unable to transport antigen from the MZ into the follicle [326, 367]. We found no defect in the ability of CTA1-DD to localize to the FDCs in mice treated with FTY720, which indicated that MZB cells were dispensable for the transport of CTA1-DD to the FDC network. Instead, the analysis of spleen sections from mice immunized with CTA1-DD revealed that CTA1-DD could be detected within conduits in the B cell follicle, indicating that the conduits were involved in the transport of CTA1-DD to FDCs. However, given that CTA1-DD activates complement, and hence should be able to bind complement receptors on MZB cells it was likely that the two mechanisms could act in a complementary fashion. In fact, a FACS analysis of splenocytes from mice injected with CTA1-DD i.v showed that a proportion of MZB cells had bound CTA1-DD *in vivo,* supporting the notion that MZB could still be involved in transporting CTA1-DD to the FDC (data not shown).

The inability of CTA1-DD to target FDCs in mice deficient in C3 or CR1/CR2 was associated with a significantly reduced adjuvant effect. Furthermore, chimeric mice that lack CR1/CR2 on FDCs, but retain normal expression of CR1/CR2 on B cells, displayed an identical inability to respond to CTA1-DD, demonstrating that the specific expression of complement receptors on FDCs appears to be required for full adjuvant effect. However, an alternative explanation to the reduced antibody responses in Cr2-/- and C3-/- mice could instead be an impaired ability of the NP-CGG antigen to localize to FDCs. Some studies have described a negative impact on the antibody response, including SHM, CSR and the generation of memory B cells, when antigen localization to FDCs have been impaired [320-323]. Potentially NP-CGG could have bound antibodies in a naïve animal, e.g. natural IgM, and formed complexes, which could have mediated binding to FDCs. Arguing against this interpretation, is the observation that no NP-CGG was deposited on FDCs in naïve animals.

By contrast, mice that had been previously immunized with NP-CGG were able to retain antigen on their FDC networks (Fig. 11). These findings are in agreement with a recent study that reported that soluble protein antigens are unable to locate to FDCs in the absence of pre-formed antibodies [327]. Therefore, we believe that when immunizing mice with CTA1-DD, the importance of complement activation in enhancing the immune response is rather to target the CTA1-DD adjuvant to FDCs, as opposed to the co-administered antigen. This notion is further supported by the finding that the RIBI adjuvant is able to promote comparable immune responses in Cr2-/- and WT mice, suggesting that the localization of antigen to FDCs is not a universal requirement for immune enhancement.

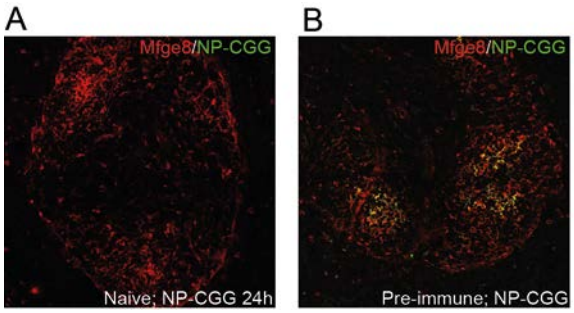

Figure 11. The antigen NP-CGG does not localize to FDCs in naïve mice. Naïve (A) or pre-immune (B) mice were given NP-CGG and spleens were removed 24h later. Spleen sections were stained for NP-CGG (green) or the FDC marker Mfge8 (red).

Even though antibody responses are significantly reduced in Cr2-/- and C3-/- mice when immunized with CTA1-DD, there is still a residual adjuvant effect as compared to mice that have been given antigen alone. By adoptively transferring NP-specific B cells into C3-/- hosts, this remaining adjuvant function of CTA1-DD could be attributed to the formation of early plasma cells. While the number of NP-specific GC B cells was completely abolished in the C3-/- mice, the frequency of early plasma cells was not significantly different from that in WT mice. Together with the fact that T cell proliferation- and cytokine secretion was unimpaired in Cr2-/- mice our study revealed that there is a specific defect in the ability of CTA1-DD to promote GC responses in these mice. Given that FDCs have a key role in the formation of GC reactions, and that they are known to up-regulate the expression of a number of genes that are involved in GC responses upon maturation, we speculated that CTA1-DD could enhance GC responses by activating FDCs [312, 315, 316, 333, 335, 338]. To experimentally address this hypothesis, we employed a method of isolating FDCs by FACS sorting in order to analyze their expression of GC-promoting genes. Indeed we found that the expression of a number of the genes were up-regulated in FDCs isolated from mice that had been immunized with CTA1-DD, indicating that the binding of CTA1-DD to FDCs could have induced their activation, promoting GC-formation. However, a critical aspect of this interpretation is undeniably the purity of the isolated FDCs. It should be emphasized that problems with isolating FDCs have hampered the study of FDC functions as well as their genetic and biochemical characteristics. This is in part due to their fragile nature and to their close association with B cells, which makes it difficult to isolate a viable and pure population. In addition, there is a lack of markers that are specifically expressed by FDC, complicating their identification. A combination of markers has previously been used to identify and isolate FDCs including Mfge8, CR1/CR2, FDC-M2 and ICAM-1 [352, 368]. In our experiments we

employed a protocol that was originally developed by Sukumar et al, in which mice are irradiated prior to analysis in order to remove contaminating lymphocytes. This step is followed by enzymatic digestion of spleens and subsequent cell sorting of CD45.2$^-$ Mfge8$^+$ CD21/CD35$^+$ cells [352]. We found that the genetic profile of the sorted cells was in agreement with previously published data, including the expression of ICAM-1, VCAM-1, Cr2, Baff and CXCL13 among other markers found on FDCs [333, 338]. However, to better control for contaminating cells we included mRNA expression of Bcl-6, as a marker for B cells. Unexpectedly, we found Bcl-6 mRNA expression to be up-regulated relative to that of FDC from untreated mice. This was surprising because the mice had been irradiated and lymphocytes were excluded from the sorting gate based on their small size. However, using an FDC cell line we could confirm the augmented expression of Bcl-6 expression following exposure to CTA1-DD, thus Bcl-6 appears to be endogenously expressed by FDCs and therefore not detected as a result of contaminating B cells. During the *in vitro* experiment, the cells were incubated with different concentrations of CTA1-DD in the presence of mouse serum, in order to provide complement components, and then analyzed for the same panel of genes that were used in the *in vivo* experiments. We found that some GC-promoting genes were up-regulated in the cell line upon incubation with CTA1-DD, indicating that the fusion protein directly induced the expression of GC-promoting genes. The enzymatically inactive construct, CTA1R7K-DD, which also effectively binds FDC via CR1/CR2, was unable to promote the enhanced gene expression as observed with CTA1-DD. This indicates that the activation of FDC by CTA1-DD was not merely a result of binding to the FDCs.

An important question is whether CTA1-DD directly activates FDCs or if the observed up-regulation of GC-genes is the result of secondary effects. TNFα and lymphotoxin secreted by GC B cells have been suggested to induce the activation of FDCs [340]. Therefore it is possible that the GC-promoting effect of CTA1-DD is only indirectly activating the FDCs. However, given that FDCs were isolated from mice that were immunized only 3-4 days before sorting, it is unlikely that any GCs could have formed at this time point. Also, our findings using the FDC cell line, argues against an indirect effect of CTA1-DD [341]. Future studies will aim to address this question.

Given that CTA1-DD was originally targeted to B cells, their ability to function as APCs in the priming of CD4 T cells when using the CTA1-DD adjuvant have been investigated in previous studies, which excluded their involvement [126]. However the requirement for DCs as APCs in the adjuvant function of CTA1-DD has not been fully investigated. Therefore we used the CD11c-DTR mouse to assess the ability of CTA1-DD to prime CD4 T cells and to promote antibody responses in the presence or absence of DCs. Unexpectedly, we found that CTA1-DD was able to enhance responses in DC-depleted mice. We observed only a moderate decrease in serum antibody titers in the absence of DCs, as compared to intact mice. While the DC-depleted mice exhibited a stronger reduction in the number of AFCs in the spleen following immunization, GC reactions and the antigen-specific CD4$^+$ T cell proliferation was comparable to that in DC-competent mice. Importantly, antibody titers in DC-depleted mice were clearly enhanced compared to titers recorded in mice immunized with antigen alone, demonstrating that CTA1-DD acted as an adjuvant even in the absence of DCs. In recent

years a number of pitfalls in the CD11c-DTR mouse model have been described. Thus, before definite conclusions could be made based on these results we need to better understand the CD11c-DTR mouse model. Firstly, DC depletion in these mice was not absolute, although we were consistently able to deplete 95% of the splenic DCs in our experiments. Whether the remaining 5% DCs were capable of priming CD4 T cells and to trigger antibody production cannot be completely excluded. Although, it appears unlikely that CTA1-DD could enhance the priming of T cells, almost to the same extent in unmanipulated mice, as in mice lacking 95% of the DC population. Secondly, DC depletion is transient and lasts for approximately 2 days following DTx administration [360]. Therefore, it is possible that replenishment with newly formed DCs, at an early time point following immunization, could have accounted for the observed adjuvant effect on T cell priming in DTx-treated animals. A simple solution to this problem would be to give repeated doses of DTx in order to prevent the recurrence of new DCs. However it has been shown that both plasma cells and GC B cells express low levels of CD11c and can therefore also be depleted by DTx injection ([354] and personal communication M Dahlgren and B Johansson-Lindbom). To overcome this problem we constructed chimeric CD11c-DTR mice where the endogenous B cells lack MHC II and consequently cannot function as APCs. We adoptively transferred NP-specific B cells and OT-II T cells and immunized the mice with NP-OVA. Since the transferred B cells do not express the DTR it was possible to give these mice multiple DTx injections. Again, we did not observe a significant defect in the ability to prime T cells or form GC responses in the DTx- treated mice. Therefore, we believe that the ability of CTA1-DD to enhance immune responses in DTx treated DTR mice were not due to the occurrence of newly formed DCs. Thus, a more likely possibility is that other APCs could compensate for the lack of DCs. This compensatory effect was not mediated by B cells since experiments in chimeric mice where B cells lack MHC II, exhibited the same adjuvant enhancement. Furthermore, both MZM and MMM are depleted in DTx treated DTR mice, while $CD11b^+$ macrophages are unaffected [369]. In addition, DTx treatment results in an influx of monocytes into the spleen, a population that is insensitive to DTx treatment, despite their expression of intermediate levels of CD11c [370]. Therefore, it will be important to confirm these results in a more well-defined DC-depletion model. Future experiments will be necessary in order to conclude whether CTA1-DD has a complete or partial requirement for DCs for the priming of CD4 T cells and antibody responses.

T cell dependent GC formation is critically dependent on the differentiation of Tfh cells [291-293]. To evaluate the impact on the induction of Tfh cells in DC-depleted mice, we sorted OT-II cells and analyzed their expression of CXCR5 and the Tfh cell transcription factor Bcl-6 at the mRNA level. We found that mice immunized with CTA1-DD together with NP-OVA exhibited Bcl-6 expression regardless of whether DCs had been depleted or not. In contrast, when using the CTA1-OVA-DD fusion protein, where the amount of antigen was severely limited and restricted to cells that had also taken up the adjuvant, the expression of Bcl-6 in sorted OT-II T cells was significantly reduced in DC-depleted mice. Despite this finding, we observed that the frequency of $CXCR5^+$ $PD\text{-}1^+$ OT-II T cells- as well as the mRNA levels of CXCR5 was unaffected. Whether these cells, with a dramatically reduced Bcl-6 expression, but still exhibiting a Tfh cell phenotype, were functionally comparable to their Bcl-6

expressing counterparts is currently unknown. We have attempted to address their functional characteristics by examining the expression IL-21 in sorted cells, however expression levels were below the detection limit. Ongoing studies are addressing this question and we hope to resolve whether these cells are functional in their ability to support GC formations. Although Bcl-6 expression in T cells has been shown to be essential in Tfh cell differentiation and GC formation, we could still detect normal GC formations in these mice [291-293]. Therefore we speculated that another factor might be able to compensate for this apparent defect in Tfh cell function. Given that CTA1-DD specifically targets FDCs, inducing their activation, and that FDCs are known to promote GC formation independently of T cells, we suggest that this ability could compensate for the lack of DCs in the DC-depleted mice.

Having explored the requirement of DCs in the adjuvant effect of CTA1-DD, we wanted to assess the impact of CT immunization on this subset. DCs are believed to play an important role in mediating the adjuvant activity of CT as demonstrated by the induced expression of CD80, CD86 and CD40 on DCs following CT immunization. [91-98]. However, in addition to enhancing the maturation state of DCs, CT has also been shown to deplete $CD8\alpha^+$ DCs [104]. This finding has been linked to the inhibited production of IL-12 and an inability to form Th1 responses in a study by la Sala et al [104]. We could confirm this observation and reported a significant depletion of $CD8\alpha^+$ DCs following CT treatment. Despite this effect, and contrary to many published studies, we were surprised to find a greatly enhanced production of IFN-γ, following immunization with CT-OVA. In fact, CT was able to enhance a balanced Th1, Th2 and Th17 response based on the cytokines produced upon *in vitro* re-stimulation of OVA specific T cells with recall antigen. This demonstrated that the adjuvant effect of CT was not simply skewing the response towards Th2, as has been claimed in the literature [81, 96, 99, 102, 103]. To investigate the notion that IL-12 plays a critical role for the immunomodualtory activity of CT, we tested CD4 T cell priming in IL-12-deficient (IL-12p40-/-) mice, adoptively transferred with OT-II CD4 T cells. We found that Th1 differentiation was achieved in IL-12p40-/- mice immunized with CT, suggesting that CT was able to promote Th1-differentiation independently of IL-12. We have, as of yet, not determined what factors that could be involved, but possible candidates include Notch ligands or CD70 expressed by DCs [197, 228]. In agreement with the report by la Sala et al [104], we found that immunizations with CT completely abrogated the ability to respond to a second immunization for a period of about 5 days. However this included both Th1 and Th2 responses and is therefore unlikely to be a result of reduced levels of IL-12, but instead due to a more extensive effect on immune functions. The effect on CD4 T cell proliferation, suggested that an early priming event was impaired in the CT-treated mice. In fact we could detect a significant impact on numerous cell types in the spleen following CT injection. These changes included the massive migration of $CD11c^+$ DCs into the T cell areas which was accompanied by their up-regulation of CD86, there was also an influx of monocytes and neutrophils and MZB cells were either depleted, or more likely, redistributed into the follicles. Interestingly, neither of these effects was seen when mice were immunized with CTA1-DD, despite the fact that the adjuvant effect of both CT and CTA1-DD rely on the ADP-ribosylating activity of the CTA1 domain (data not shown). This further emphasizes the importance of the differential binding properties of these two adjuvant molecules.

We speculated that the combination of a reduction in DC numbers- and the maturation of the remaining population may have caused an inability to respond in mice. To address this hypothesis we attempted to restore the DC population through an adoptive transfer of purified $CD11c^+$ DCs to CT- immunized mice and then immunized the mice with the CTA1-DD adjuvant. Strikingly the reconstituted mice were able to respond to immunization, suggesting that the lack of functional DCs in the pre-treated mice was causing the defect. Given that the results using the CD11c-DTR mice suggests that CTA1-DD is able to promote immune responses in the absence of DCs this result may seem contradictory. However the adjuvant function in CT treated DC-restored mice was not complete, since antibody titers remained lower as compared to untreated mice. Furthermore, while the CD11c-DTR model is specific for $CD11c^+$ cells, CT has a much more profound effect, not only restricted to $CD11c^+$ cells. It is therefore likely that CT affects other APCs in addition to DCs which could have resulted in a more complete abrogation of APC function as compared to the CD11c-DTR model. We have not experimentally addressed this hypothesis, however there are studies that have shown that CT can have a suppressive effect on e.g. macrophages, which supports this possibility.

Future studies will aim to address whether CT acts directly on DCs, or if the observed effects are indirectly mediated via factors produced by other cells. By constructing chimeric mice that lack Gm1 gangliosides on either hematipoetic or non-hematopoitic cells, we will be able to gain some insight into this question [371]. Furthermore, we are currently using qRT-PCR in order to identify the chemokines and cytokines that are induced upon CT immunization.

In conclusion we have identified a role for two cell populations with fundamentally different origin and function; the conventional dendritic cells and the follicular dendritic cells, in the adjuvant activity of CT and CTA1-DD respectively. This demonstrates the importance of differential cell targeting in terms of enhancing immune responses, while at the same time avoiding toxicity. This represents a critical aspect in the development of new effective and safe adjuvants that will be included in future vaccines.

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