The role of dendritic cells in adjuvant-induced immune responses

Tobias Gustafsson

UNIVERSITY OF GOTHENBURG

Department of Microbiology and Immunology
Institute of Biomedicine
University of Gothenburg
Sweden, 2013

ISBN: 978-91-628-8621-9
Electronic version at: http://hdl.handle.net/2077/31990

Printed by Kompendiet, Gothenburg, 2013

Till mamma och pappa

The role of dendritic cells in adjuvant-induced immune responses

Tobias Gustafsson
Department of Microbiology and Immunology, Institute of Biomedicine
University of Gothenburg, Sweden, 2013

Abstract

Dendritic cells (DCs) are sentinels of mucosal surfaces, residing directly under the epithelial layer. DCs are among the first cells that come in contact with pathogens and have the unique ability to activate T cells that subsequently can aid B cells to produce antibodies with high affinity. T and B cells constitute our immunological memory that protects us from reinfections – the basis for vaccination. Vaccines composed of purified antigens, confer high specificity but have low intrinsic immunogenicity, and require therefore an adjuvant that enhances the response. The most potent adjuvants are often toxic, and consequently a limited number of adjuvants are available for clinical use, mucosal adjuvants in particular. Therefore, a better understanding is needed concerning the interactions between adjuvants and DCs in order to unveil the mechanisms of adjuvanticity. Here we have *in vivo* studied the role of DCs and the characteristics of the immune response after immunization with different adjuvants.

Adenoviral (Ad) vaccine vectors inducing expression of ovalbumin (OVA) at different subcellular locations were used in a mouse model in which conventional DCs (cDCs) could be depleted. We show that cDCs are required for activation of T cells although a direct transduction of cDCs by Ad-vectors is not essential. Further we determine that secreted and membrane-anchored antigens are superior at activating antigen-specific $CD4^+$ and cytotoxic $CD8^+$ T lymphocytes as well as generating a serum IgG response compared to intracellularly expressed OVA.

Cholera toxin (CT) is one of the most potent mucosal adjuvants. CT binds the ubiquitously expressed ganglioside GM1 leading to efficient uptake that in epithelial cells results in secretion of fluid into the lumen. After oral immunization with OVA and CT we find that chimeric mice lacking GM1 on hematopoietic cells, and specifically GM1-expressing DCs, fail to induce adaptive immune responses to OVA. We conclude that CT does not require the toxic epithelial cell interaction for its adjuvant activity but is dependent on direct binding of GM1 on intestinal DCs.

To become plasma cells producing high affinity antibodies, B cells must undergo affinity maturation in the germinal center where they are dependent on the help of follicular helper T cells (Tfh). In DC-depleted mice, we show that immunization with the adjuvant poly(I:C) and non-limiting doses of OVA generates Tfh cells and germinal centers in absence of DCs. In contrast, B cell interactions are required for a fully differentiated Tfh phenotype and the activation of a Th1 mediated T cell response is totally dependent on DCs.

Strategies targeting vaccine antigens to DCs are becoming more promising as novel DC-specific receptors are being discovered. Taken together our results show great heterogeneity concerning the role of DCs in adjuvant-induced immune responses. How to modulate and take advantage of the interactions between adjuvants and DCs will be crucial knowledge in the construction of more effective and safe vaccines.

Keywords: Dendritic cells, adjuvant, mucosa, adenovirus vector, cholera toxin, T follicular helper cells

ISBN: 978-91-628-8621-9
http://hdl.handle.net/2077/31990

Original papers

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

I. **The subcellular location of antigen expressed by adenoviral vectors modifies adaptive immunity but not dependency on cross-presenting dendritic cells**
Henning P*, Gustafsson T*, Flach C-F, Hua Y-J, Strömbeck A, Holmgren J, Lindholm L, Yrlid U.
Eur. J. Immunol. 2011, 41: 2185-2196.

II. **Direct interaction between cholera toxin and dendritic cells is required for oral adjuvant activity**
Gustafsson T, Hua Y-J, Dahlgren M, Livingston M, Johansson-Lindbom B, Yrlid U.
Submitted manuscript

III. **T follicular helper cell development and germinal center formation in the absence of conventional dendritic cells**
Gustafsson T*, Dahlgren M*, Livingston M, Cucak H, Johansson-Lindbom B[#], Yrlid U[#].
In manuscript

* and [#] *These authors have contributed equally to this study.*

Papers not included in this thesis:

- **CD11c^{high} dendritic cells are essential for activation of CD4^{+} T cells and generation of specific antibodies following mucosal immunization**
  Fahlén-Yrlid L, Gustafsson T, Westlund J, Holmberg A, Strömbeck A, Blomquist M, MacPherson G. G, Holmgren J, Yrlid U.
  The Journal of Immunology, 2009, 183: 5032–5041**.**

- **Directed antigen targeting in vivo identifies a role for CD103^{+} dendritic cells in both tolerogenic and immunogenic T-cell responses**
  Semmrich M, Plantinga M, Svensson-Frej M, Uronen-Hansson H, Gustafsson T, Mowat AM, Yrlid U, Lambrecht BN, Agace WW.
  Mucosal Immunology, 2012, 5(2): 150-160.

Table of contents

Abbreviations

Ad	Adenovirus
APC	Antigen presenting cell
AT	Adoptive transfer
cDC	Conventional dendritic cell
CLN	Cervical lymph node
CT	Cholera toxin
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DTx	Diphtheria toxin
DTR	Diphtheria toxin receptor
GC	Germinal center
IEC	Intestinal epithelial cell
i.n.	Intranasal
i.p.	Intraperitoneal
IPV	Inactivated polio vaccine
LN	Lymph node
LPS	Lipopolysaccharide
MedLN	Mediastinal lymph node
MHC	Multihisotcompatibility complex
MLN	Mesenteric lymph node
MPL	Monophosphoryl lipid A
NALT	Nasal cavity associated lymphoid tissue
NCAC	Nasal cavity associated cells
NLR	NOD-like receptor
OPV	Oral polio vaccine
OVA	Ovalbumin
PDC	Plasmacytoid dendritic cell
p.o.	Peroral
PAMP	Pathogen associated molecular pattern
PP	Peyer's patch
PRR	Pattern recognition receptor
RA	Retinoic acid
SPL	Spleen
Tfh	T follicular helper cell
TLR	Toll-like receptor

Introduction

The role of our immune system is to protect us against invading pathogens and harmful agents. Conserved structural traits of pathogens are recognized by cells of the innate immune system, most often resulting in swift inflammatory responses and clearance of the threatening organisms. More challenging infections require the onset of the adaptive immune system, which has the ability to produce high affinity antibodies and induce memory responses. Activation of the adaptive immune system is most efficiently performed by antigen presenting cells (APCs), like the dendritic cells (DCs), which process and present antigen fragments on major histocompatibility complex (MHC) molecules to lymphocytes. Although the onset of the adaptive immune response is slower than the innate, it has a broader range of specificities, and provides the important memory cells. The adaptive immune system consists of T and B cells, for example cytotoxic T lymphocytes (CTL) which kill infected cells, and helper T cells that aid B cells to differentiate into plasma cells producing antibodies for clearance of extracellular pathogens. The induction of immunological memory provides a rapid protection against encountered pathogens, which is the essence of vaccination.

The innovation of vaccination is one of the most important landmarks of human health. Vaccination has resulted in that several infectious diseases have become controllable and in some cases even eradicated. Now, over two hundred years since Edward Jenner performed the first vaccination, vaccinations save millions of lives each year worldwide. Although old vaccines using killed, or live attenuated organisms have proven successful throughout history, variations in potency and safety have led to a demand for improvements. For some challenging diseases there is still no functional vaccine available. Several strategies to improve specificity and safety utilize purified pathogen associated subunits or surface proteins. Due to lack of intrinsic virulence these vaccines generally require administration together with immune stimulatory agents, i.e. adjuvants. Numerous agents with adjuvant function have been developed, however the majority of them are only suitable for animal use. Some function through defined receptor molecules and signaling pathways, but paradoxically, the few adjuvants licensed for clinical use are the ones, which we know the least about how they elicit their adjuvant function.

Development of future adjuvants will require a deeper knowledge of the interaction between adjuvants and the cells of the immune system. Depending on the composition of the adjuvant, the immune system can be tailored for optimal resistance, for example to produce antibodies against toxins and extracellular organisms or to initiate a cell-mediated defense to clear out virus or bacteria infected cells. Central to the role of adjuvants are the DCs, equipped with an array of pattern recognition receptors (PRR), they connect the innate and adaptive immune systems with the ability to induce immunological memory. Learning more about DCs and their interactions with adjuvants is crucial for constructing improved future vaccines.

This thesis is focused on the DCs and their involvement in immune responses induced by three different immunization systems.

We have investigated the role of DCs in the use of adenovirus vaccine vectors (Ad-vectors), and whether direct DC targeting is required. We also evaluated three different modes of Ad-vector mediated antigen expression, intracellular, secreted or membrane-bound. Furthermore, we studied the role of DCs in cholera toxin (CT)-induced immune responses. CT is known to be a highly effective adjuvant, but too toxic for human use. The precise mode-of-action for adjuvanticity and required target cells of the toxin still needs to be determined, in order to construct less toxic, safe variants. Finally we looked at the involvement of DCs in the priming of T follicular helper cells (Tfh) and the germinal center (GC) reaction driven by poly(I:C)-enhanced immunization. This is a crucial part of acquired immunity, important for production of high affinity antibodies and induction of memory responses.

In the following sections of the introduction I will present a general overview of the research area covered in this thesis, with emphasis on the parts that are central to the included papers.

Vaccination

Long before vaccination was introduced there was the practice of variolation where material from a patient was transferred to a non-infected individual to induce protection. This technique can be traced back to ancient China, where dried powder of smallpox crusts was inhaled through the nose. Variolation resulted in solid immunity however with variable efficacy, the disease could spread if uncontrolled and had a high mortality rate [1,2]. Edward Jenner realized that individuals working in close proximity to cows were spared from smallpox infection. In May 1796, Jenner immunized his first patient, an 8-year-old boy, with cowpox lesion material taken from the hand of a milkmaid, who had caught the disease after milking a Gloucestershire cow called Blossom. After a 2-week recovery from mild lesions, the boy was challenged by smallpox variolation on both arms. The result demonstrated solid immunity. Vaccination was defined as the way of inducing protection against a disease by inoculation with a different but related disease.

A lot has happened since Jenner's discovery. Smallpox is now considered to be eradicated, and polio reduced by 99%. In all, vaccines have brought at least ten major human diseases under some degree of control, including diphtheria, tetanus, yellow fever, pertussis, mumps, rubella, thyphoid fever, *Haemopilus Influenzae* type B, rabies and measles [3]. Still the need for new and improved vaccines is constantly present. We are fairly unprepared for pandemic threats. For instance, the worldwide influenza pandemic in 1918 killed in total more people than the World War I. Taking into account the growth of the human population, increasing traveling patterns, antibiotic resistance and global warming, that all are factors contributing to the spreading of epidemic infections. New ways to rapidly develop effective vaccines will therefore be of great need, should a new pandemic like the one in 1918 emerge. The vaccines presently in use and under development can be divided into different categories based on their

composition: (i) killed whole cell vaccines, (ii) live, attenuated vaccines, (iii) subunit vaccines and (iv) DNA and vector vaccines.

Killed whole cell vaccines

The method of using killed pathogens ensures that the organism is harmless yet still retain all its antigens. This only works with organisms that do not contain toxic substances. It has been successfully used to produce vaccines against polio (IPV), cholera (Dukoral®), typhoid fever, pertussis, plague, and influenza. Killed vaccines are unable to replicate or mutate and will not spread, thus are considered as rather safe. On the other hand, killed whole cell vaccines lose their immunogenicity and are generally administered together with an adjuvant like alum to enhance the effectiveness. In addition, vaccination with killed organisms commonly needs multiple immunizations in order to confer adequate immune responses. Killed vaccines raise good antibody response against surface antigens but are less a potent inducer of CTL responses due to low accessibility to antigens for major histocompatibility complex-I (MHC-I) presentation [4].

Live, attenuated vaccines

For successful vaccination results with live organisms it is important to be able to separate virulence from the ability to induce protective immunity. This can be achieved by weakening the pathogens through attenuation [4]. By cultivating pathogenic organisms in non-human cell cultures (like chicken cells) it is possible to induce mutations reducing their virulence towards humans. Nevertheless, there is always an underlying risk that the attenuated organisms reverts back to a virulent state, as has been seen with the oral polio vaccine (OPV). In OPV vaccinated patients, the virus will replicate for some time in the gut, which could lead to a loss of the attenuating mutation [5]. Due to the close resemblance to a real infection, attenuated vaccines are potent inducers of both humoral and cell-mediated immunity. This technique has been used to produce vaccines against measles, mumps, rubella, varicella and the Bacille Calmette–Guérin (BCG) tuberculosis vaccine [3].

Subunit vaccines

Vaccine development involving live or killed whole organisms is referred to as empirical, that is, relying much on observation and experience. A lot of emphasis is now instead put on rational vaccine design using purified subunit antigens, like toxoids, recombinant proteins, and purified lipopolysaccharides (LPS) [6]. Examples of subunit vaccines are the toxoid vaccines against diphtheria and tetanus, and carbohydrate vaccine against pneumococcus. Purified antigens benefit from being specific and safe, yet suffer from low inherent immunogenicity. Consequently, subunit vaccines generally contain immune enhancing agents and may also require specific delivery systems. High specificity may also have implications on a large population scale; genetic diversity can cause a vaccine to be functional in only some individuals. Furthermore, rapidly mutating pathogens (e.g. human immunodeficiency virus -HIV) and pathogens with many serotypes (e.g. dengue virus) pose as particularly hard to target [7].

DNA and vector vaccines

Vaccines based on DNA do not contain any antigens, instead they provide the genetic material for antigen production by the host cell. In a way this mimics a viral infection, inducing antigen-expression by host cells ensuring optimal MHC-I presentation and CTL response [8]. DNA vaccines also manage to induce antibody response against expressed antigens, probably via antigen presenting cells (APCs) processing material from dead cells. Although praised for their simplicity, naked DNA vaccines suffer from very low transduction percentage. DNA vaccines have been mixed with lipid complexes, microparticulates and the adjuvant alum, in order to enhance delivery to target cells and recruitment of APCs. Empty virus particles, devoid of their genomic material and ability to replicate have been explored as adjuvants during immunization with subunit antigens. These virus-like-particles (VLPs) have the ability to assemble spontaneously, even in cell free cultures. VLPs have been demonstrated to mediate strong CTL-responses without additional adjuvant [9]. Conversely, strong antibody responses instead of CTLs, result from conjugation with alum or in oil emulsion [10]. VLPs are used in several human vaccines, like the hepatitis B vaccine FENDrix® and the human papillomavirus (HPV) vaccines Cervarix® and Gardasil® [11,12].

Adenovirus vaccine vectors

Attenuated live viruses provided a new delivery system with good transduction efficiency for antigen expression in target cells. The most extensively studied virus vectors are constructed of adenoviruses (Ad). Ad belongs to the family *Adenoviridae*, a large group of DNA viruses that infects a multitude of species including humans. There are approximately 50 different human Ad serotypes causing different clinical manifestations such as respiratory illness, gastro intestinal infections and keratoconjunctivitis. Most studies using Ad as vaccine vectors have used serotype 5 (Ad5). Ad5 typically cause mild respiratory illness but may also be fatal in patients with impaired immune system. A live oral vaccine against Ad4/Ad7 causing acute respiratory illness has been developed and used successfully by the U.S. military for decades [13,14]. The virus particle binds the coxsackie adenoviral receptor (CAR) and is taken up by the cell through receptor-mediated endocytosis, with subsequent insertion of viral DNA into the host nucleus [15]. Expression of viral early genes mediates the production of proteins capable of hi-jacking the control over the hosts DNA-transcription and protein synthesis leading to suppression of the cells defenses and the subsequent assembly of new virus particles. By rendering the virus replication-incompetent it is possible to use the particle to transport DNA into target cells. This technique was initially developed for treatment of genetic disorders, but in recent years the use as a potential vaccine carrier has been studied extensively [16-19]. Ad-vectors are made replication-incompetent through the deletion of the early (E1) gene segments and replacing them with genes of interest. This enables the Ad-vectors to deliver target DNA to the nucleus for antigen expression while the assembly of new virus particles is inhibited.

Although Ad-vectors are not adjuvants *per se,* the particle backbone possesses an intrinsic adjuvanticity. It is not exactly clear how adenoviruses provide an adjuvant

effect, but they induce a rapid innate response with TNF-α, IL-6, MIP-1, and MIP-2 production by alveolar macrophages, resulting in infiltration of neutrophils, NK cells and more macrophages [20]. Furthermore, vector-DNA clearance up to 90% has been measured in spleen already 24h after i.v. administration of Ad-vectors [21]. Given the preference for mucosal epithelial cells, Ad-vectors are more prone to induce long lasting $CD8^+$ memory responses when administered mucosally rather than parenterally [22,23]. Vaccination with Ad-vectors has in animal models resulted in good protection against various diseases including Ebola, respiratory syncytial virus, tuberculosis, herpes and botulism [23-27]. Ad-vectors transduce proliferating as well as non-proliferating cells. They are easy to grow and to purify in high titers. The capacity to carry large size gene segments, safe handling, ease to modify and their natural tropism for epithelial cells makes Ad-vectors a promising tool for future vaccine techniques. One caveat with the use of Ad-vectors, is the possible existence of neutralizing antibodies that may impair the effect of immunization. However, this may be overcome by increased or repeated vector doses, alternating Ad serotypes or alternating immunization routes [28].

Adjuvants

Adjuvants have acquired their name from the Latin ‘adjuvare’, which means ‘to help’, due to their ability to enhance both the quality and durability of immune responses. Adjuvants were first introduced by Gaston Ramon, while he was developing the tetanus vaccine. With formalin and heat treatment, Ramon was able to make an attenuated version of the tetanus toxin, later known as a ‘toxoid’. As the toxoid became harmless, it also became devoid of some of its immune stimulatory properties. Gaston discovered that the addition of aluminium hydroxide (alum) to the vaccine formulation was able to boost the immune response. Alum is now the most prevalently used adjuvant in vaccine formulations worldwide.

All through evolution, the immune system has encountered different organisms and managed to develop in order to withstand new threats. It became possible to recognize foreign and potentially dangerous objects through pattern-recognition receptors (PRRs) that can recognize various pathogen-associated molecular patterns (PAMPs). Of these, the toll-like receptors (TLR) are most studied. TLRs are specific for various conserved PAMPs: LPS by TLR-4, flagellin by TLR-5, microbial DNA and RNA by TLR-9, 3, 7, and 8. Many adjuvants possess known features of PAMPs, enabling them to be recognized by PRRs of the innate immune system, while the functions of other adjuvants remain unclear. Main targets of most adjuvants are the APC, mainly DCs. DCs are cells equipped with a large array of receptors and the key cells for induction of acquired immunity. During the years, many adjuvants have been developed, yet only a handful has made it to clinical use. The major impediment is that the potency of an adjuvant generally correlates with its toxicity, making their use often hampered by side effects like inflammation and fever. Currently, there are only four adjuvants that are licensed for human applications; alum, MF59 (oil emulsion), adjuvant system 03 (AS03) (squalene based), and AS04 (monophosphoryl lipid A (MPL) + alum).

Alum

Over the past 70 years, immunizations has relied on **alum** to enhance less effective vaccine formulations and it is still the most commonly used adjuvant for human use. Aluminium hydroxide and related aluminium salts, generally referred to as 'alum', are very potent inducers of Th2 response and produce a strong antibody response against extracellular pathogens or toxins like diphtheria, tetanus and hepatitis [29], but does not convey any Th1 induction [30,31]. Conversely, activation of cell-mediated immunity or responses against peptides is rather poor [32]. Alum is considered to have three main features that may endorse adjuvanticity; formation of a depot that slowly releases antigen over a longer time period, induction of inflammation with subsequent recruitment of APCs, and ability to conform soluble antigen into particulate form, facilitating uptake [33]. The mechanism underlying the effects of alum has for long been enigmatic. Reports have shown that alum-induced antibody responses occur in mice lacking the adaptor proteins MyD88 and TRIF, suggesting that the immunostimulation is independent of TLR-signaling [34]. Others propose an inflammasome-dependent pathway activated by uric acid, as a requirement for NLRP3 has been observed *in vitro* [30,31,35]. Alum have long been believed to act on DCs, but *in vitro* studies has also shown that driving differentiation of monocytes to DC phenotype seems to be central for alum function [36]. An *in vivo* study shows how alum can induce muscle tissue to release chemokines, attracting MCH-II$^+$ and CD11b$^+$ cells from the blood into the peripheral tissue surrounding the injection site [37].

Oil-in-water emulsions

Oil-in-water emulsions like **MF59** are potent adjuvants with a good safety record in influenza vaccines [38]. Though its mode of function is not known, it is demonstrated to be phagocytosed by DCs and is thus not providing a depot-mediated adjuvant effect [39]. Similarly to alum, it has been shown to mediate activation of innate immunity at the injection site when given intra muscularly [37]. Squalene is a precursor to cholesterol added to some adjuvant emulsions, like MF59 and AS03. It is naturally available in both plants and animals, produced in the human liver and circulates in the blood stream. By itself, squalene is not an adjuvant but enhances the immune stimulatory properties of adjuvant emulsions. It is also included in an adjuvant formulation with QS21 (AS02A). QS21 is a saponin compound that binds cholesterol and punches holes in the lipid bilayers of cell membranes. This makes them fairly toxic, and for use in adjuvant formulations, cholesterols like squalene are added to quench their toxic pore-forming effect [40].

TLR agonists

TLR agonists are strong inducers of immune responses and have been widely studied as adjuvants. The TLR-4 ligand LPS for example, is a constituent of cell membranes of Gram-negative bacteria. LPS confers very strong immune responses although its tendency to cause septic shock makes it too hazardous for clinical applications. An attenuated derivative of LPS, monophosphoryl lipid A (**MPL**) is now available, which retains immune stimulatory properties yet is safe enough to use in clinical settings. MPL

is the only TLR agonist allowed for use in human vaccines, as part of the adjuvant formulation AS04 together with alum [40]. Intravenous injection of MPL in mice induces migration of $CD11c^+$ cells from splenic marginal zone to the T cell area, with subsequent up-regulation of CD86 and MHC-II [41], a similar response as with LPS [42]. Combinations using MPL with alum (MPLA) can be further modified depending on the desired immunogenic outcome. Antibody responses are induced when MPLA is used in water formulations, while oil formulations skew the response towards cellular immunity. In general, adjuvants based on oil in water emulsions (for example MF59) result in Th2 responses, but when combined with TLR ligands the response tilts towards Th1. Even miniscule amounts of MPL will suffice. [40]

Poly(I:C)

Detection of intruding viruses is mainly mediated through the recognition of viral DNA or RNA by endosomal TLRs. The adjuvant polyinosinic:polycytidylic acid (poly(I:C)) is a synthetic double-stranded (ds) RNA comprising a polyinosinic strain annealed to a polycytidylic strain. By mimicking viral RNA, poly(I:C) becomes a potent adjuvant capable of eliciting both Th1 and humoral immune responses [43]. One important feature of its strong adjuvanticity is the ability to induce the production of type-I IFN from both hematopoietic and stromal cells [44,45]. Analogous to dsRNA, poly(I:C) is recognized by TLR3, which results in type-I IFN production, through signaling involving transcription factors IRF-3, IRF-7, AP-1, and nuclear factor kappa B (NF-κB). Furthermore, poly(I:C) also bind to the cytoplasmic RNA helicase, melanoma differentiation-associated gene-5 (MDA5), mediating a TLR-independent type-I IFN production, by activating IRF-3 [46,47]. Type-I IFNs are important for both innate and adaptive immunity, and well known for their antiviral properties [48], activating a set of genes that interfere with viral gene translation and assembly [49]. Type-I IFNs stimulate Th1 responses, on one hand by inducing IFN-γ production, and on the other by inhibiting production of IL-4 and IL-5 [50,51]. In addition, cross-presentation of extracellular antigen to $CD8^+$ T cells by DCs is facilitated by type-I IFN stimulation [52], but also DC maturation and $CD4^+$ responses are dependent on type-I IFNs after immunization with poly(I:C) [44,53].

Mucosal adjuvants

The majority of vaccines today are given through parenteral administration, i.e. intradermal, subcutaneous, and intramuscular injections. These generally provide good systemic IgG production. Pathogens are constantly being encountered at our mucosal surfaces, which is probably why almost 80% of the immune system cells can be found here. However, most mucosal tissues are impermeable to IgG, which thus provides low mucosal protection, especially against non-invasive pathogens like *V. cholerae* or ETEC [54]. The main antibody at mucosal surfaces is secretory IgA, which crosses the epithelial layer via transport by the polymeric Ig receptor through the epithelial cells. In the mucus, secretory IgA can inhibit attachment and colonization by bacteria as well as neutralize toxins [55]. Mucosal immunity is poorly induced by parenteral immunization, and usually requires local administration. Mucosal vaccines can stimulate local IgA and

systemic IgG as well as cellular immune responses. Conversely, as mucosal tissues like the intestine must accept harmless nutrients, the immune response is preset on tolerance. This makes oral vaccination quite a challenge and explains why there are only a handful of mucosal vaccines available at present [56].

Enterotoxins

Enterotoxins are powerful immunostimulators and have great potential as mucosal adjuvants if their toxicity can be diminished. The most extensively studied enterotoxins are cholera toxin from *Vibrio cholerae* and heat labile toxins (LT) of enterotoxigenic *Escherichia coli* (ETEC), which have good adjuvant effects when co-administered with antigens after mucosal immunization [57-60]. Features that have been implicated to contribute to this potent adjuvanticity are increased epithelial permeabilization facilitating antigen uptake, improved antigen presentation by DCs and other APCs, enhanced B cell differentiation into IgA-producing plasma cells, as well as direct effect on T cell expansion and cytokine production [61].

Many enterotoxins produced by intestinal bacterial species are composed as holotoxins, i.e. combined by different protein subunits. AB-toxins consists of a catalytically active A-subunit and a B-subunit possessing the receptor-binding capacity. A subgroup of AB-toxins is the AB_5-toxins, where the B-subunit is a ring-shaped pentameric moiety. Examples of well-known AB_5-toxins are CT, LT-I and LT-II, shiga toxin of *Shigella dysenteriae* and Shiga toxigenic *E.coli*, pertussis toxin, and the enterotoxin of *Campylobacter jejuni* [62].

The AB_5-toxins have their differences but share many basic features, like binding through the B-subunit to ganglioside surface molecules for entry into host cells, internalization of the holotoxin, detachment of the A_1-subunit that disrupts essential functions of the host cell. The intracellular route of AB_5-toxins will be described more in detail in the next section, exemplified by the pathway of CT. Even though the AB_5-toxins have large structure homology and share the same B-subunit binding fold, they differ in their receptor specificity of glycan receptors, where CT only has one known receptor in the GM1 ganglioside [63], LT-I binds preferentially to GM1, but it has ability to also bind GM2, Gd2, and GD1b [64]. The LT-II toxin, which is subdivided into LT-IIa, LT-IIb, and LT-IIc, has binding affinity for a large selection of gangliosides including GD1a-b, GM1, GM2, and GM3 to mention a few. Most STs are specific for glycosphingolipid Gb3, which is found mainly on the microvascular endothelium but also in the tubular epithelium of the kidney [65]. AB_5-toxins also differ in their catalytic specificity. While the A_1-subunits of CT and the LT species exerts ADP-ribosylation of Gsα proteins to activate adenylate cyclase, pertussis toxin negatively regulates adenylate cyclase through activation of the inhibitory G protein Giα. Moreover, the subspecies SubAB toxin from Shiga toxigenic *E. coli* (STEC), does not involve G proteins at all, but abrogates the function of the chaperone protein BiP in the endoplasmatic reticulum (ER), killing the cell through disruption of the ER homeostasis [66].

Recombinant versions of both CT and LT have been created aiming at abrogating toxicity while retaining adjuvanticity. A fusion protein was made combining CTA_1 and a D-fragment dimer from *Staphylococcus aureus* protein A. The resulting

CTA_1-DD adjuvant was shown to be devoid of the toxic effects of the CTB containing holotoxin [67]. At the same time, it was successfully targeted to APCs and B cells through its DD-domain, binding complement-activating antibodies, which in turn results in follicular dendritic cell (FDC) binding by complement receptor Cr2 [68]. Immunization with CTA1-DD strongly enhances GC formation, with systemic IgG and mucosal IgA responses to co-administered T cell-dependent antigens [67,68]. An altered version of LT has also been made, were point mutations in R192G and L211A results in a trypsin resistant double mutant (dmLT) unable to release its A_1-subunit [69]. This nontoxic mutant has been shown to generate effective protection in several challenge models and has also recently been included in human clinical trails [70-72].

Cholera toxin

CT is a virulence factor and the causative agent of disease produced by the bacterium *V. cholera,* which infects the proximal small intestine causing severe diarrhea, and is responsible for many deaths worldwide each year. Consequently, CT is one of the most powerful toxins known. CT belongs to the group of AB_5 toxins, it is composed of a pentameric B-subunit (CTB) and an enzymatically active A-subunit (CTA), that is in turn composed of one A_1 and one A_2-subunit linked together by a disulfide bond. CTB mediates the interaction with enterocytes by binding to GM1, leading to endocytotic entry into the cell [63,73,74]. GM1 is generally expressed on all nucleated cells. Once inside the cell, the holotoxin passes the golgi apparatus through retrograde transport to the endoplasmatic reticulum (ER). Here the A_1-subunit is detached by enzymatic cleavage of the disulfide bond. Free CTA_1 is actively transported out into the cytosol by the ER-associated degradation (ERAD) pathway, usually used to shuttle misfolded proteins to proteasomal degradation [75,76]. As CTA_1 manages to refold without being ubiquitinated, it evades degradation and gains access to the cytosol. Here it mediates ADP-ribosylation of G-protein Gsα at the cell membrane, leading to constitutive activation of adenylate cyclase. The net result of this is elevated levels of cyclic-AMP and export of Cl^- ions followed by large amounts of water entering the intestinal lumen [77]. As little as 0.5μg purified CT is enough to cause diarrhea in humans and can rapidly lead to dehydration and death as water loss may reach rates of 1.0-1.25 liters/h [78]. CT provides a strong adjuvant effect when administered at several mucosal routes, e.g. oral, nasal, vaginal, and sub lingual, and generated protection in infection models like cholera, influenza, and helicobacter pylori [57-60,79-83]. The extremely effective reabsorption of the mouse colon permits safe immunizations with high concentrations of CT in order to study the adjuvant effects [84]. But, for use of CT as adjuvant in human vaccinations, there must first be means to separate its immunostimulatory ability from its toxic properties.

The immune system

Treatment and prevention of many infectious diseases are still performed in an empirical fashion. For more effective and tailored vaccines, we need a better understanding of how to deliver the vaccines to the immune system in the best way. The oldest and most

preserved part of the immune system is the innate immune system. It has been preserved throughout evolution and can be found in insects and some plants even. DCs and macrophages of innate immunity reside in tissues whereas neutrophils, eosinophil and monocytes circulate in the blood stream ready for rapid deployment. Both immune cells and non-immune cells have PRRs capable of recognizing conserved structures prevalently found on pathogens, like mannose, lectins, and LPS. PRR activation induces secretion of pro-inflammatory cytokines and chemokine that interfere with spreading pathogens and recruits immune cells. The response of innate immunity is fast reactive, however it has limited variability and lacks the ability to confer long-term protection. Milder infections can be controlled by innate immunity and may pass un-noticed. More persistent infections require involvement of the humoral and cellular response of the adaptive immune system. Pathogens taken up by DCs are broken down to fragments that are presented as antigens on MHC molecules. In this way DCs are able to prime $CD4^+$ and $CD8^+$ T cells with the corresponding antigen specificity. Priming of naïve T cells takes place in the T cell zone of lymphoid organs, in close proximity to the B cell follicles. Activated $CD8^+$ and some $CD4^+$ T cells exit the lymph node into the blood circulation, and by expression of chemokine receptors, travel to the site of infection. Activated $CD4^+$ T cells that remain in the lymph node may become follicular helper T cells that migrate towards the B cell follicle, where they assist B cells in forming germinal centers (GC), the site where maturation and selection of high affinity and isotype switched antibody-producing plasma cells and memory B cells take place.

The two arms of the immune system use different receptors in order to convey specificity. While innate immunity relies on broad spectrum receptors recognizing conserved pathogen structures, the cells of the adaptive immune system goes through extreme selection processes to refine their T or B cell receptors to fit a certain motif. Sensors of innate immunity include, TLRs, nucleotide oligomerization domain (NOD)-like receptors, retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) + some members of the C-type lectin family.

The Lymphoid organs

The human body is a large organism that can be confronted with pathogenic infiltration at a variety of target areas. Due to the incredible diversity of lymphocyte specificity, the chance of a pathogen encountering the right lymphocyte at the right time and in the right tissue seems fairly unlikely. However, this match making is facilitated by the organization of specialized lymphoid tissues, where circulating lymphocytes are gathered. Tissues like the spleen and LNs, are known as secondary lymphoid organs and serve as surveillance checkpoints, filtering antigens circulating the blood or lymphatic fluids respectively. The primary lymphoid organs comprise the bone marrow and the thymus, where B and T lymphocytes, respectively, are matured from progenitor cells.

LNs are connected to a distinct organ or tissue from where interstitial fluid is retrieved. For example, the cervical LN (CLN) drain the nasal tissues, the mediastinal LN (MedLN) drain the lungs, and the mesenteric LN (MLN) drain the small intestine. The Peyer's Patches are specialized lymphoid follicles lining the epithelium of the small

intestine, orchestrating responses to luminal antigens gathered by microfold cells (M cells). The LNs are highly compartmentalized organs forming structures for optimal interactions between lymphocyte populations, antigens, and APCs. In LNs, B cells gather in follicles together with macrophages and follicular DCs that, unlike conventional DCs, are derived from stromal cells called ubiquitous perivascular precursors (preFDC) [85]. FDCs produce follicle-associated chemokines and using Fc-receptors and complement receptors, they entrap soluble antigens percolating the node [86,87]. As FDCs lack antigen-presenting functions, they solely provide B cells with a display of membrane bound intact antigens. After antigen activation, B cells form GCs within the follicles where further proliferation and differentiation into effector B cells occur. The T cell zone, or paracortex, lays adjacent to the B cell follicles and is the area where T cells and APCs reside. This is also where the inlet from the blood system is connected in the form of high endothelial venules (HEV), through which lymphocytes arrive to the node. Lymphatic fluid is led into the LN by the afferent lymph vessel, subsequently passed through the subcapsular sinus, surrounding the node, further through the paracortex, collected in the medulla, and finally exiting through the efferent lymph vessel [88]. From incoming lymph passing the subcapsular sinus, low-molecular mass antigens are able to diffuse into the follicles and larger antigens are captured by follicle-associated macrophages beneath the sinus [89,90].

The spleen is not connected to any afferent lymphatic vessels, instead it is specialized in dealing with antigens circulating in the blood, removal of old erythrocytes and iron recycling [91]. It resembles the LNs as it contains T and B cell areas in what is called the white pulp. Outside of the white pulp is the blood-enriched red pulp that harbors many APCs, lymphocytes and plasma cells. Between the red and white pulp lies the marginal zone. Blood enters from the afferent artery into the marginal sinus where it continues to the red pulp, either directly or via the marginal zone or through a conduit network to the white pulp. The marginal zone holds a high number of macrophages and DCs entrapping blood-borne antigens. In addition, the marginal zone contains a population of non-recirculating B cells important in early immune responses against both T dependent and independent antigens. These MZ B cells can recognize conserved microbial patterns using BCR with non-mutated immunoglobulin variable (IgV) genes [92]. After microbial detection, MZ B cells can rapidly differentiate into extrafollicular plasmablasts, producing high levels of IgM and early IgG, and are also able to differentiate in a follicular pathway involving T cell help [93,94].

Immune cells central to this thesis

Dendritic cells

DCs were first discovered in the microscope of Ralph Steinman in 1973, and proclaimed as a novel cell type based on its protruding morphology [95]. DCs soon became identified as important ‘accessory cells’ with strong ability to activate antigen specific T lymphocytes [96]. Today DCs are considered to be the most potent APCs and an

essential link between the innate and adaptive immunity [97,98]. The discovery of DCs and their important role in directing the quality of the immune response rewarded Steinman with the Nobel Prize in 2011. Driving the differentiation of a naïve T cell into a certain effector subset can be the difference between inflammation and tolerance. The common nominator of several agents with adjuvant activity, like LPS, MPL, endotoxins, MF59, flagellin, and poly(I:C) is that they stimulate immune responses by inducing maturation of DCs [33,41,42,44,99-101].

Throughout the years, many subtypes of DCs with similar functions and appearance have emerged, and a clear distinction based on cell surface markers is hard to maintain due to phenotypic changes depending on maturation status and tissue location [102]. Currently DCs can be divided into four main categories: conventional DCs (cDC), plasmacytoid interferon-producing DCs (pDC), Langerhans cells and monocyte-derived DCs. DCs are derived from blood-circulating precursors, originating from the bone marrow, and differentiate in peripheral tissue [103]. In the bone marrow, hematopoietic stem cells (HSC) give rise to lymphoid (LP) and myeloid progenitors (MP). The MPs are further developed into monocyte, macrophage and DC precursors (MDP), which in turn diverges into common DC precursors (CDP), monocytes, and some macrophages (Fig. 1). The transcription factor Flt3 has been shown to be required for differentiation of bone marrow DC progenitors and maintenance of DCs in peripheral lymphoid tissues [104,105]. Out of the CDP comes both pDCs and pre-conventional DCs (pre-DC) that leaves the bone marrow to enter the blood circulation [106]. Pre-DC goes through their final differentiation when they leave the blood and enter the target tissue, to become either $CD8\alpha^+$ or $CD11b^+$ cDCs in lymphoid tissues, or $CD103^+$ cDCs in the non-lymphoid tissues of the intestine, skin, and lungs [106-108]. MDP-derived monocytes enter the blood as $Ly6C^{hi}$ or $Ly6C^{lo}$ populations. In the tissue, $Ly6C^{hi}$ monocytes can give rise to CX_3CR1^+ lamina propria DCs (lpDC), and 'inflammatory monocytes'. The $Ly6c^{lo}$ cells are called 'patrolling monocytes' that may become macrophages [103,109].

Subtypes

Conventional DCs are located where antigens are most likely to first be encountered, e.g. dermal tissues, and mucosal surfaces of the nasal cavity, lungs and intestine. Upon antigen encounter, cDCs become migratory and move through lymphatic vessels to interact with T cells in the draining LNs. In humans, cDCs are also found in blood but are not common in the circulation of mice. Expression of the β integrin CD11c and MHC-II is generally used to define cDCs in mice. As immature cells, cDCs have low expression of surface MHC molecules and co-stimulatory molecules, however, they posses high phagocytic activity and an abundance of PRRs, for example TLRs, FcRs, and C-type lectin receptors [110]. Besides direct stimulation through PRRs, DCs can also be activated indirectly as a result of tissue damage when DCs take up damage associated molecular pattern agents (DAMP) like uric acid, adenosine triphosphate (ATP), or High-mobility group protein B1 (HMGB-1) from dying cells [33]. Encountered antigens are internalized by endocytosis, which initiates the maturation process of the antigen presenting functions. Following endocytosis, antigens are

disassembled in lysosomes, subsequently loaded on MHC molecules and transported to the cell surface for easy access by T lymphocytes. At the same time, all phagocytic activity is halted and the cell initiates expression of co-stimulatory molecules CD80 and CD86 [110]. Another feature of DC maturation is the expression of chemokine receptor CCR7, which enables a gradient-dependent migration of DCs towards the chemokines CCL21 and CCL19, produced in the T cell zone of lymphoid tissues. Just recently, migration of peripheral cDCs to nearby lymphatic vessels was demonstrated to be guided by an immobilized CCL21-gradient bound to extracellular matrix [111].

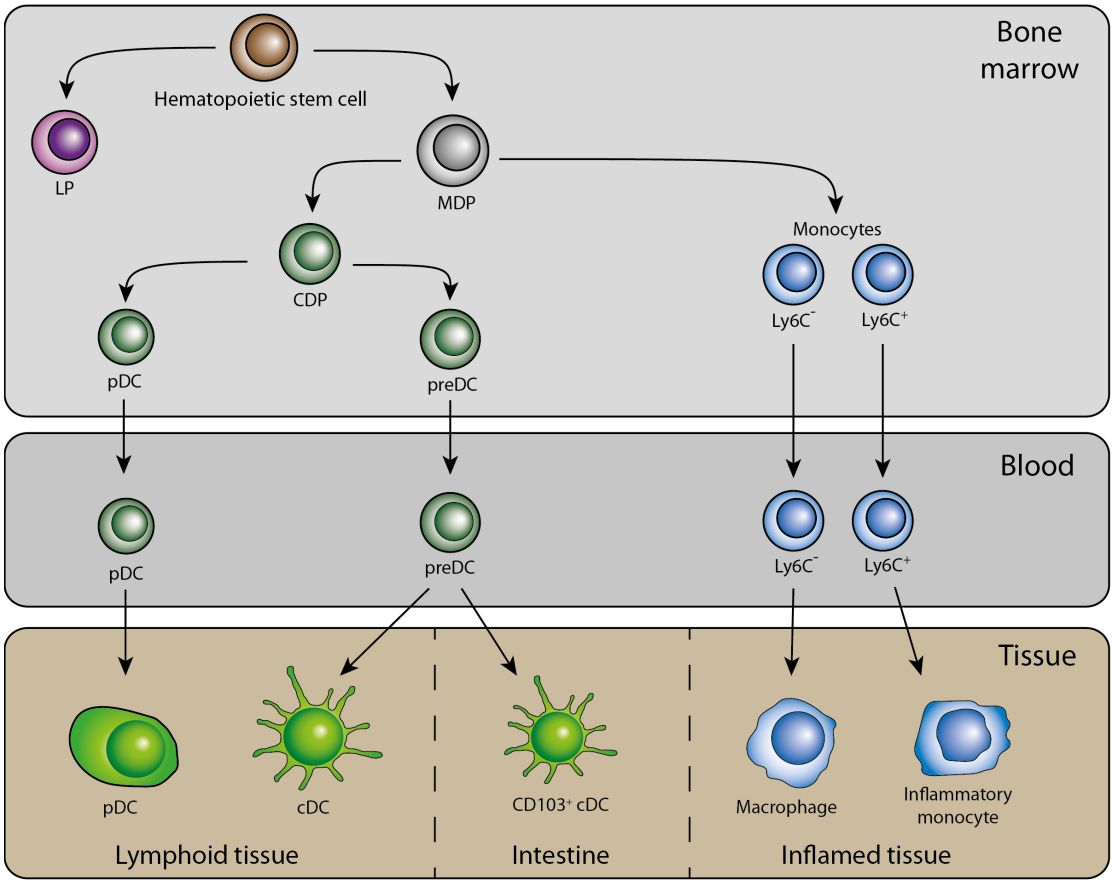

Figure 1. Differentiation of dendritic cells from bone marrow precursors.

Early studies in mice revealed heterogeneity in the splenic $CD11c^+MHC\text{-}II^+$ cDC population, which was related to their functional properties. Splenic cDCs subsets could be distinguished by their surface expression of either $CD8\alpha^+CD11b^-CD4^-$ or $CD8\alpha^-CD11b^+CD4^+$, referred to as $CD8\alpha^+$ and $CD11b^+$ DC respectively. In addition, a double negative $CD8\alpha^-CD11b^-CD4^-$ has also been identified. $CD8\alpha^+$ DCs are highly efficient at cross-presenting extracellular antigens on MHC-I to naïve $CD8^+$ T cells [112,113], whereas $CD11b^+$ DCs are the most potent cells at processing and presenting antigens to $CD4^+$ T cells on MHC-II [114] (Fig. 2). These subsets also possess distinct cytokine profiles as $CD8\alpha^+$ DCs produce IL-12, to induce a Th1 response with IFN-γ secreting

$CD4^+$ T cells, whereas $CD11b^+$ DCs stimulate secretion of IL-4 by Th2 cells [115-117]. These lymphoid-resident DCs are blood-derived but are also accompanied in lymph nodes by migratory cDC subsets arriving from peripheral tissues through afferent lymph vessels.

While $CD11b^+$ cDCs are readily found in intestinal tissue, $CD11b^-CD8\alpha^+$ cDCs have been more enigmatic to find. However a dendritic cell with similar capabilities can be identified by the integrin $\alpha_E\beta_7$ (CD103) [118]. Interestingly, recent studies have shown that both $CD11b^+CD8\alpha^-$ and $CD11b^-CD103^+$ DCs migrate in intestinal lymph [119]. $CD103^+$ cDCs have been shown to have a central role in tolerance by induction of regulatory T cells. $CD103^+$ cDCs are mostly found in the mucosal tissue of the lamina propria, dermis and the lung. The relationship between blood derived $CD8^+$ and $CD103^+$ cDCs extends beyond functional similarities as they also share the same transcription factor requirements. Both *batf3*$^{-/-}$ and *irf8*$^{-/-}$ mice has been shown to be deficient of $CD8\alpha^+$ as well as $CD103^+$ populations in peripheral tissues [120,121]. Mutations in IRF8 has also been reported to result in deficiency of DC populations in humans [122].

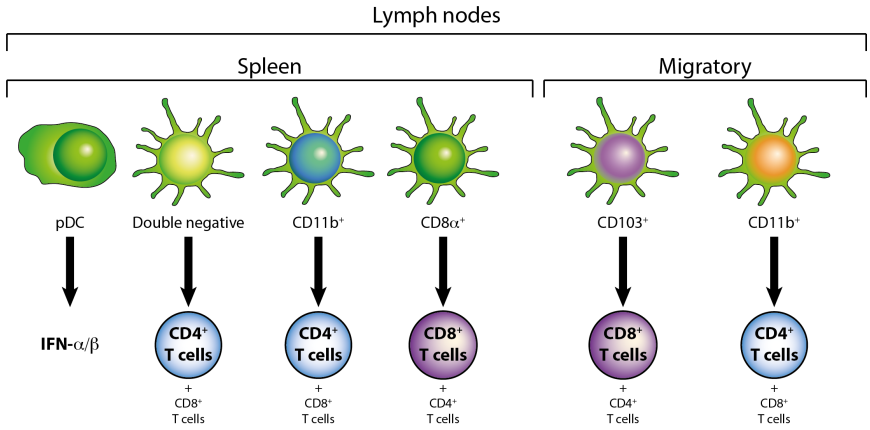

Figure 2. Generalized picture of DC subsets and their functions.

A second subtype of DCs can be found in the lamina propria identified by the expression of the chemokine receptor CX_3CR1, but not CD103. These cells are residing close to the epithelial layer of the lamina propria and have abilities to push their dendrites through the tight junctions of the intestinal epithelial cells (IEC) to sample the luminal contents [123]. However, $CD103^+$ but not CX_3CR1^+ cells are migrating to the MLN after antigen encounter in the small intestine, and CX_3CR1^+ cells have weak antigen presenting properties [124]. Although $CD103^+$ and $CD103^-$ DC were found equally potent of inducing proliferation and IFN-γ production of $CD8^+$ T cells, only $CD103^+$ DCs induced expression of the gut homing receptors CCR9 and α4β7. Accordingly, oral but not i.p. immunization mediated gut homing $CCR9^+$ and $\alpha4\beta7^+$ $CD8^+$ T cells [125][126,127]. Furthermore, gut homing imprinting by DCs has been shown to be dependent on retinoic acid (RA)[128], also nicely demonstrated by treating skin-draining DCs with RA, which then were able to drive gut-homing T cell responses

[129]. DCs have been shown to also covey commensal bacteria to the MLN in order to induce local IgA production. This is believed to keep the commensal flora at a comfortable distance without inducing local inflammation [130]. Furthermore apoptotic epithelial cells are also transported to MLN to maintain self-tolerance [131].

Plasmacytoid DCs are morphologically different from cDCs. As cDCs have extending dendrites, pDCs are more spherically formed. In mice these cells can be identified by the expression of B220, Ly6C, PDCA1 and siglec-H. At the same time they lack CD11b and only express low levels of CD11c. pDCs express MHC-II but are in contrast to cDCs non-migratory. Instead they use the blood circulation as transport and can be found in spleen, lymph nodes, liver and the bone marrow[105]. pDCs are mainly activated by TLR7 and TLR9, resulting in production of extremely high levels of type I interferons, important in the protection against viral infections [132]. pDCs have been shown to prime naïve $CD4^+$ T cells in LNs, but not $CD8^+$ T cells or $CD4^+$ T cells in the spleen [133].

Langerhans cells, which are found in ectodermal tissue and dermis, differ from other DC subtypes in that they have self-renewal abilities in tissue, due to resident and not blood-borne progenitor cells. This has been seen in human transplanted limbs, which retain their own local populations of Langerhans cells several years post surgery [134]. Upon antigen recognition, Langerhans cells migrate to the skin-draining lymph nodes for antigen presentation.

Monocyte-derived DCs stems from the macrophage and DC precursor (MDP) in the bone marrow but are separated from the common DC precursor (CDP) pathway. The monocytes are either of the $Ly6C^{lo}$ $CCR2^{lo}$ 'patrolling' subtype or the $Ly6C^{hi}$ $CCR2^{hi}$ 'inflammatory monocyte' subtype. The patrolling monocytes can be found in the intestinal lamina propria and has been defined as CX_3CR1^{hi} DCs. Although, the fact that these CX_3CR1^{hi} cells are $CD103^-$ $CD11b^+$ $F4/80^+$ $CD69^+$, and don't prime naïve T cells or migrate to the MLN, suggests that they in fact are more closely related to macrophages than DCs [135]. Under inflammatory conditions, $Ly6C^{hi}$ monocytes can differentiate into 'TNF and inducible nitric oxide synthase-producing inflammatory DC' (Tip-DC). Although they express CD11c and MHC-II, they are not dependent on GM-CSF and have gene-expression differences linking them more to activated monocytes than cDCs [109].

Antigen processing and presentation to T cells

As mentioned above, DCs are excellent APCs and have a well-described superior capacity compared to other APCs to activate naïve T cells. In addition to DCs, B cells and macrophages are also APCs. B cells can present antigens on MHC-II, but they have poor antigen endocytic properties unless mediated through Ig receptors. Conversely, macrophages are excellent at internalization through a variety of receptors, but are weaker presenters due to a lysosome-dominant endocytic pathway being more suited for degradation than MHC-loading [136].

Antigens presented on MHC-II are generally from extracellular origin, taken up by receptor-mediated or unspecific endocytosis, but some cytosolic proteins can be loaded as well [137]. MHC-II leaves the ER/Golgi stabilized by the invariant chain protein bound to the antigen-binding groove, which is also contain an endosomal transport signal. After localizing to the endosomes, the invariant chain is proteolytically degraded to a CLIP fragment, which is subsequently exchanged by an antigenic peptide in endocytic compartments during transport to the cell surface [138].

MHC-I is expressed on all nucleated cells. Antigens loaded on MHC-I originates from the cytosol where most of them are degraded in the proteasome, transported into the ER by transporter associated with antigen processing (TAP), loaded on MHC-I, and further routed via the Golgi apparatus to finally be presented on the cell surface. $CD8\alpha^+$ and $CD103^+$ DCs are able to take advantage of this pathway for "cross-presentation" of extracellular antigens from damaged or infected cells to $CD8^+$ T cells. This is the way naïve $CD8^+$ T cells are primed to become cytotoxic T lymphocytes (CTL) that recognize cells with intracellular organisms, the most important protection against virus infections. In addition, some extracellular antigens can be routed through a vacuolar TAP-independent pathway, being processed by the protease cathepsin S instead of the proteasome [139]. Even though some other APCs may have some cross-presenting function *in vitro*, DCs are considered to be the cells that mainly perform this process *in vivo* [140].

T cells

T lymphocytes express the T cell receptor (TCR), enabling recognition of their cognate antigen peptide presented on major histocompatibility complex (MHC) molecules. TCRs are heterodimers composed of an α and a β chain, but the less common γδ heterodimers are also produced. The structure of the TCR is related to Fab fragmets of immunoglobulins (Ig), containing Ig-like variable and constant regions. Also like the Ig, the TCR retains its specificity through recombination of the variable (V), diverse (D), and joining (J) –gene segments of the β-chain antigen binding loops, and V-J recombination of the α-chain. Resulting in immense receptor diversity. In the TCR complex, the α and a β chains confer the antigen recognition, while the intracellular signal transduction is mediated through accessory molecules CD3ε, δ and γ, and a CD247 (ζ) homodimer [141]. In addition, T cell-expressed co-receptors augments the ligation sensitivity by binding directly to the MHC molecules, and are linked to an intracellular lck tyrosine kinase that contributes to the signaling transduction. Peptide-MHC-I complexes are recognized by T cells expressing co-receptor CD8, while CD4-expresing T cells recognize peptide-MHC-II complexes [142].

Activated $CD8^+$ T cells, become CTLs with the ability to recognize damaged apoptotic cells and infected cells. These target cells display DAMPs or pathogenic antigen fragments on MHC-I for CTL recognition. $CD8^+$ T cells do not lyse target cells unless first primed by a DC. CTLs secrete the protein perforin, which creates pores in the cell membrane, enabling the delivery of apoptosis-inducing granzymes to the cytoplasm of the target cell [143]. $CD8^+$ T cells have also been reported to inhibit the

spread of HSV-1 virus in neurons without inducing apoptosis. In this case, granzymes appears to focus their protease effect on the viral proteins [144]. In addition to granzymes, CTLs secrete interferons and TNFs that are able to synergize to enhance antiviral properties [145].

Activation of $CD4^+$ T cells by APCs, and their differentiation into helper cells, is a pivotal step for full-featured adaptive immune response. Both antibody and CTL responses are suggested to be able to be initiated without $CD4^+$ T helper cells [146]. However, the generation of high-affinity antibodies and expansion of cytotoxic memory cells require activation of helper T cell assistance [147]. Cross-presentation of exogenous antigens on MHC-I have even been reported to require interaction of CTL and $CD4^+$ helper T cell with the same APC [148], an interaction mediated through CD40-CD40L signaling [149]. For activation of T helper cell responses, it is essential that CD80 /CD86 on APCs delivers co-stimulatory signals to CD28 on $CD4^+$ T cells [150]. In vitro studies have demonstrated that antigen-stimulation in absence of co-stimulation inhibits proliferation, leading to an unresponsive state, or clonal anergy [151]. Generation of mature effector $CD4^+$ T cells are dependent on three activation signals; first, antigen recognition by the MHC complex, second, co-stimulation trough CD28, and the third is mediated trough secreted cytokines.

$CD4^+$ T helper subsets

Upon activation by APCs, naïve $CD4^+$ T cells (Th0) can differentiate along several phenotypically defined pathways. Depending on the type of antigen, DC subset, innate stimuli and surroundings of the interaction, the naïve T cell can differentiate into subsets with different effector functions, for example Th1, Th2, Th17, Treg, or Tfh [152] (Fig. 3). Type 1 helper T cells (**Th1**) are characterized by T-bet expression and production of the pro-inflammatory cytokines interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), central cytokines for macrophage activation and the cellular defense against intracellular pathogens. Th1 differentiation requires CD28 stimulation together with cytokines IL-12 and/or IL-18 [153]. Production of IFN-γ and development of Th1 cells is governed by transcription factor T-bet and further maintained by IL-12 and STAT4 signaling. Mice deficient of STAT4 signaling fail in Th1 differentiation and are skewed to Th2 [154-156]. A quite recently discovery is the IL-17 producing T cells, called **Th17** cells. They are defined by their ability to produce IL-17, but also produce TNF-α and IL-22, pro-inflammatory cytokines important in the response against bacteria, parasites and fungi [157-159]. Activation of Th17-stimulating cytokine expression is predominantly driven by members of the C-type lectin family of receptors, producing IL-23, IL-1, IL-6, and TNF-α [160]. Th17 differentiation is dependent on IL-23, STAT3, and expression of the transcription factor ROR-γt [161]. TGF-β together with IL-6 and IL-21 synergize to drive STAT3 activation leading to ROR-γt, which regulates expression of the IL-23 receptor required for Th17 development [162]. Conversely, too high levels of TGF-β and IL-6 will induce IL-10 production and possibly inhibit the effector functions of Th17 cells. Th17 cells play an important role in the mobilization of defense against microbial threats, but are also involved in

autoimmune inflammatory diseases [163]. Stimulation with cytokines IL-4 and IL-33 will induce differentiation of a type 2 helper T cell phenotype (**Th2**) [164]. Additional co-receptor signaling through OX40-OX40L by TSLP-induced APCs also contributes to Th2 induction [165]. These T cells are regulated by the transcription factor GATA-3 and secrete predominantly IL-4, IL-5 and IL-13. This cytokine repertoire is important in the extracellular defense against helminth, or eukaryotic parasites. The response to such organisms is production of IgE antibodies and activation of mast cells and basophils. In allergy, a dysfunctional Th2 regulation results in IgE-mediated mast cell activation and release of histamine in the affected tissue. In mice, Th2 responses can also be characterized by production of IgG1 antibodies. Naturally occurring regulatory $CD4^+$ $CD25^+$ T cells (**Treg**) have matured already as they leave the thymus and perform important negative regulation of immune responses to control inflammation, autoimmunity, and allergy. Tregs are defined by expression of the transcription factor FOXP3 and cytokine transforming growth factor beta (TGF-β). IL-10 producing regulatory T cells (**Tr1**) is an inducible subtype differentiated from Th0 cells in the periphery in an IL-10 dependent manner. Tr1 cells regulate adaptive immunity trough cytokine mediated suppression of effector T cells. In addition to IL-10, they also produce high amounts of TGF-β and IL-5 [166,167].

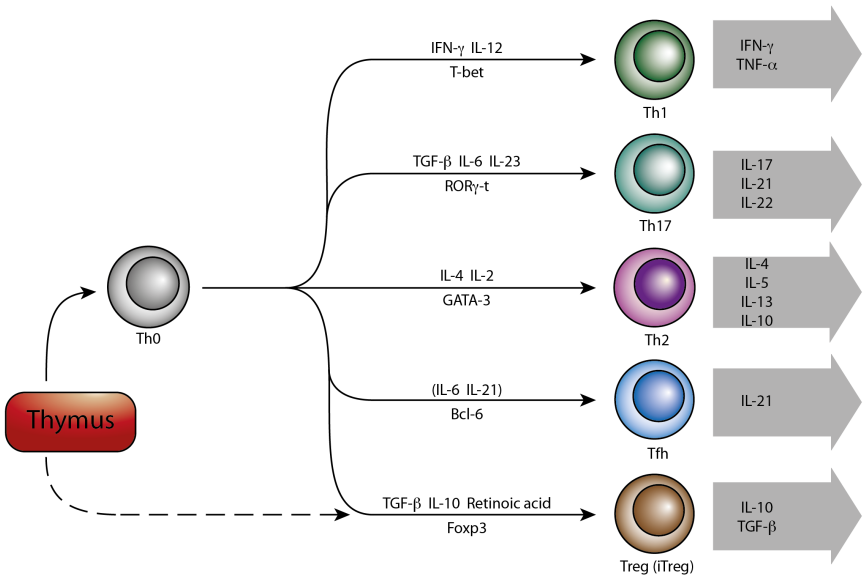

Figure 3. Differentiation of $CD4^+$ T cell subsets and their effector cytokines.

Follicular helper T cells (**Tfh**) are $CD4^+$ T cell specialized at providing assistance to GC B cells (Fig. 4). Formation of GCs, with generation of Ag-specific memory cells and plasma cells, is totally dependent on T cells [168-170]. Equally, differentiation of Tfh cells is also dependent on interaction with B cells, as has been demonstrated in several reports. For example, Tfh cell differentiation was impaired in B cell deficient μMT mice and mice with unrelated BCR-specificity (MD4, hen egg lysozyme specific) following viral infection, parasite infection, and protein immunization [171-173]. In addition,

CD19$^{-/-}$ mice, with normal T and B cell compartments but without ability to form GCs, were also unable to differentiate Tfh cells [171]. However, T cells with constitutively induced retroviral expression of the transcription factor Bcl-6, managed to develop Tfh phenotype with antigen-unspecific B cells and even in the absence of B cells [172]. This finding has led to that Bcl-6 is considered a master regulator of Tfh differentiation. Bcl-6 induces IL-21 production and sustains expression of CXCR5. Bcl6 is also a transcriptional repressor, as it inhibits the function of T-bet, Gata-3, and ROR-γt, the respective transcription factors of Th1, Th2, and Th17 responses.

DCs have been reported to have a role in the initial priming of Tfh cells, inducing up-regulation of CXCR5, Bcl6, and ICOS, although they fail to mediate signals for full Tfh cell differentiation including expression of PD-1 and IL-21 production [174]. B cells have proven required for Tfh cell survival but not their priming, and although DCs are effective inducers it is not known whether they are irreplaceable for Tfh cell differentiation.

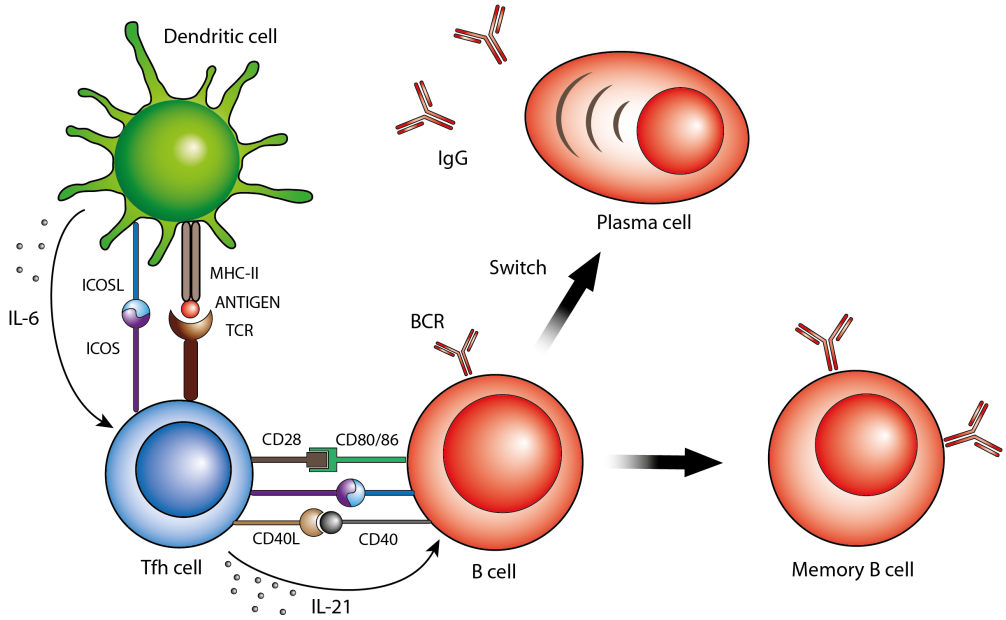

Figure 4. Important interactions of Tfh cell differentiation and GC B cell activation.

The canonical marker for Tfh cells is the chemokine receptor CXCR5 [171]. It's ligand, CXCL13, is expressed by stromal cells, and follicular DCs in the B cell follicle. Positioning Tfh cells at the border of the B cell follicle is mediated by up-regulation of CXCR5 synchronized with CCR7 down-regulation [171]. Likewise, activated B cells down-regulate CXCR5 and up-regulate CCR7 in order to meet up at the border. The T/B cell interaction is initiated by antigen/MHC-II-recognition by cognate TCR. Signaling through co-stimulatory molecules ICOS-ICOSL and CD40-CD40L enhances the activation and intensifies the interaction [175,176]. Expression of ICOSL by B cells has been shown to be redundant for initial priming but necessary for the survival of Tfh cells [177]. SAP expressed on Tfh cells mediates prolonged cell-contact trough binding to SLAM, and SLAM family receptors on B cells. Mice lacking SAP have an aberrant IL-21 production by their Tfh cells, consequently these mice are unable to establish B cell

help and form GCs [178,179]. However, SAP-deficiency does not affect the interaction between Tfh cells and DCs [178,180], and early Tfh cell differentiation still occurs in mice with SAP deficient T cells [177] SLAM-SLAM mediated signaling induces Th2-independent IL-4 production by Tfh cells, providing signals for GC B cell survival as well as class switch recombination [181].

Second only to the BCR, CD40 is one of the most important surface molecules on B cells providing signals for activation, proliferation, and survival. Both CD40- and CD40L-deficient mice fail to form GCs and produce class switched antibodies in response to T-cell dependent antigens [182,183]. CD40L and IL-4 mediates essential survival signals to GC B cells, that otherwise are highly prone to initiate apoptosis, indicated by the high expression of FAS [184,185]. IL-21 together with CD40L can also provide maintenance of GC B cell proliferation. At the same time, CD40L inhibits plasma cell differentiation, clearly demonstrated by CD40L removal while still providing IL-21 (or IL-10), which instantly converts GC B cells into plasma cells [186-188]. IL-21 is the most efficient cytokine to promote differentiation of plasma cells, and stimulates both Bcl-6 and Blimp-1 expression in B cells [189]. Il-21 is the prime cytokine produced by activated Tfh cells, through IL-21, Tfh cells support GC formation and Ig isotype switching [190,191]. There are many different models describing putative pathways of Tfh differentiation, one current view, by S. Crotty, propose a multistage and multifactorial pathway where Th0 cells are primed to early Tfh phenotype by DCs and further differentiated by cognate B cell interactions. This model makes a distinction between $CXCR5^{+}PD\text{-}1^{+}$ Tfh cells at the border of the T/B cell zone, and $CXCR5^{++}PD\text{-}1^{++}$ Tfh cells (GC Tfh) localized in the light zone of the GC [188].

B cells

B lymphocytes can be found circulating in the blood but are most abundantly found in special follicles of LN and the spleen. Activation of B cells requires BCR stimulation either by soluble antigen or antigen delivered on DCs, macrophages and follicular DCs. Some antigens can activate B cells in a T cell-independent manner. Crosslinking BCRs by multiple polyclonal or repeated antigenic epitopes, can provide enough stimuli for the B cell to be activated. Extrafollicular T cell-independent responses rapidly generate neutralizing antibodies within 2-3 days, but does not result affinity maturation or memory B cell responses [192-194].

A T cell-dependent antigen attached to the BCR is processed and presented on MHC-II molecules for subsequent $CD4^{+}$ T cell interaction [195]. B cells express high levels of MHC-II and possess antigen presenting functions, although inferior to DCs since antigen internalization by B cells is very inefficient except by their Ig receptors. B cells up-regulate CCR7 and down-regulate CXCR5, initiating migration towards the boundary of the T/B cell zones. The subsequent Tfh cell interaction initiates proliferation and formation of GCs [196-198].

Bcl-6 is not only important for Tfh cell differentiation but also an essential regulator of activated GC B cells. Bcl-6 inhibits the anti-apoptotic protein Bcl-2, placing the B cell in an apoptose-predisposed state. Pro-apoptotic B cell are dependent on

affinity-based survival signals to make it through clonal selection, facilitating exclusion of low-affinity and self-reactive B cells. Bcl-6 also silences Blimp-1, the transcription factor regulating differentiation of plasma cells, thus keeping the B cell in the GC reaction [199]. Up-regulation of Blimp-1 drives GC B cells to become either short-lived plasma cells that reside in secondary lymphoid tissue or long-lived plasma cells found in the bone marrow [200]. The stimulation required for memory B cell differentiation is not clearly understood.

B cells in germinal centers

Naïve B cells gathers in lymphoid organ follicles and awaits incoming antigen. Upon antigen exposure, the B cells start to proliferate and form a GC within the follicle. It takes about one week to create a visible GC [201], which is polarized into two distinct areas, the dark zone (DZ) close to the T cell zone, and the light zone (LZ) facing the subcapsular sinus in lymph nodes and the marginal zone in the spleen (Fig. 5). The DZ is filled with large mitotic B cells, whereas the LZ in addition to B cells contain a large number of FDCs, hence the brighter histological appearance of the LZ. Also Tfh cells, cDCs and tingible-body macrophages (TBMs) populate the LZ of GC, though in smaller numbers than B cells and FDCs. CXCL13 is expressed by FDCs, retaining B cells and attracting Tfh cells expressing CXCR5 to the GC [202]. Further localization to the DZ is guided by CXCR4 on GC B cells, attracted by CXCL12/SDF-1 which is more strongly present in the DZ than in the LZ [203]. The two areas have separate functions. The DZ is where GC B cells proliferate and goes through the process of somatic hyper mutation and class switch recombination. The LZ is the site for clonal selection through recognition of antigen-bearing FDCs and interactions with Tfh cells. These are the processes leading to memory B cells and plasma cells producing high affinity antibodies, which is the goal of most vaccinations [196,197,204]. The location and activity of GC B cells can be monitored by their surface molecule repertoire, DZ B cells are $CXCR4^{hi}$ $CD83^{lo}$ $CD86^{lo}$, while LZ B cells are $CXCR4^{lo}$ $CD83^{hi}$ $CD86^{hi}$ [205].

Somatic hypermutation is initiated by the expression of the enzyme activation-induced cytidine deaminase (AID), which introduces single-strand breaks in the V(D)J-section of the Ig genes, causing mutations induced by the DNA-repair machinery. These somatic hypermutations alters antibody affinity and induces class switch recombination. Class switch recombination changes the constant region of the Ig heavy chain from IgD or IgM to IgG, IgA, or IgE [206], which is a crucial step in humoral immunity owing to the different effector functions associated to each class and isotype of Igs. In mice, IgG2 is powerful against virus, IgG1 and IgE are effective against bacteria and parasites respectively, while IgA is secreted to neutralize organisms and toxins at mucosal surfaces. Although the mechanism underlying the clonal selection of matured B cells is not completely understood, it appears that selection for survival signals is based on their ability to present antigens to Tfh cells rather than solely competing for antigens on FDCs [205]. The out-competed B cells that don't receive any survival signals will die from apoptosis and be disposed of by macrophages. After interaction with Tfh cells, B cells can either be programmed to become memory B cells, or plasma cells producing high affinity antibodies.

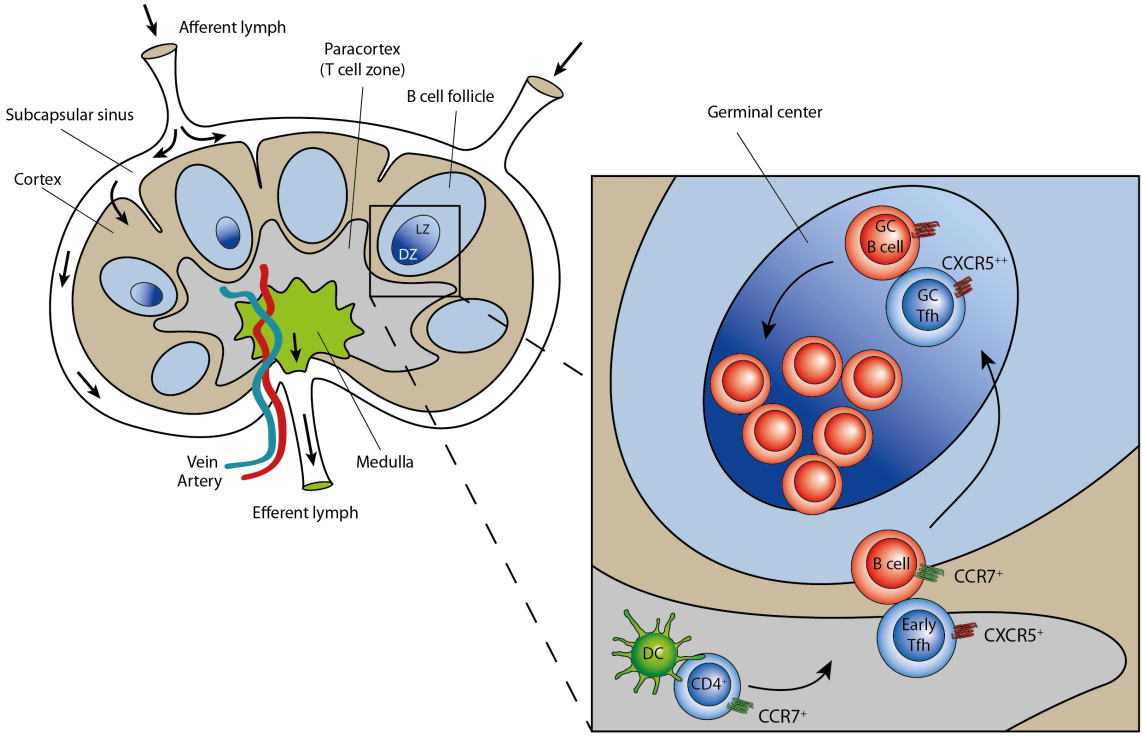

Figure 5. Schematics of lymph node architecture, with enlarged view showing interactions of germinal center formation.

Aims

The overall aim of this thesis was to investigate the dependency on DCs in immune responses induced by three different adjuvant systems: Ad vaccine vectors, CT and poly(I:C).

Specific aims of the thesis:

- To compare the quality of the adaptive immune response generated by differential subcellular antigen expression mediated by Ad-vectors.
- To determine if DCs are required for Ad-vector mediated immune responses and if so - Do they need to be directly transduced?
- To study the cellular requirements for the oral adjuvant function of CT.
- To determine whether intestinal DCs are activated directly by ingested CT or through factors secreted by other cells.
- To determine if DCs send unique signals for Tfh cell differentiation and subsequent GC formation.

Key methodologies

Mice

The immune system is all about interactions, and as a whole cannot be studied *in vitro*. Mice are suitable model animals that are easy to handle, have a rapid reproduction cycle, and are available in numerous genetic variants. In this thesis, the well-known breed C57Bl/6 was used as the wild type (WT) control. All transgenic mice used in this thesis are bred on a C57Bl/6 background. CD11c-DTR transgenic mice (B6.FVB-Tg Itax-DTR/GFP 57Lan/J) are fitted with a gene encoding the diphtheria toxin receptor (DTR) under the control of the CD11c promoter. Mice do not naturally express DTR, so the presence of DTR solely on CD11c-expressing cells enables DTx-mediated conditional ablation of DCs. In addition, the gene for GFP is inserted together with the DTR gene, enabling detection through fluorescence [140]. OT-II TCR transgenic mice [207] and OT-I TCR transgenic mice [208] have TCR receptors specific for OVA peptide sequences binding MHC-II and MHC-I respectively. These mice were used to study the antigen-specific T cell activation. By crossing these OT-I and OT-II mice with C57Bl/6-CD45.1 mice we created mice with T cells that were be easily traced after adoptive transfer into allogeneic $CD45.2^+$ recipients, using anti-CD45.1 antibodies. Mice genetically deficient for β1,4-N-acetylgalactosaminyltransferase have a complete lack of complex gangliosides, including GM1 [209], thus allowing an assessment of the cellular dependence of CT. We took advantage of this to explore more precisely the role of non-hematopoietic and hematopoietic cells in CT-induced adjuvant responses.

Flow cytometry

Flow cytometry, or Fluorescence Activated Cell Sorter (FACS), is a technique that enables the characterization of cells within a diverse population. By pressing a cell suspension through a nozzle, it is possible to analyze the properties of individual cells. The cells are passed through multiple lasers on a single line and the scattered light is collected by sensitive detectors. The scattered light gives information of the size and granularity of the cell. Other characteristics are detected by using fluorochrome-conjugated antibodies specific for a certain cellular epitope. Lasers excite the fluorochromes, which then emits light in a detectable wavelength. These antibodies can be used to study expression of both cell-surface and intracellular proteins. This technique has been extensively used in all three papers included in this thesis, with up to twelve different fluorochromes used at one point. These analyzes were performed on an LSR-II flow cytometer from BD bioscience.

Gene expression

To study gene-expression during immune responses, we collected the cells of interest and measured mRNA expression by quantitative Real-Time PCR (qPCR). To study the gene-expression of OT-II cells, we prepared cells from spleen and enriched them for $CD4^+$ T cells by magnetic depletion of $CD19^+$ cells on an AutoMACS (Miltenyi

Biotech). The cells were further sorted on FACS Aria (BD Bioscience) based on a $CFSE^-$ $CD4^+$ $CD45.1^+$ $B220^-$ $7AAD^-$ profile. Sorted OT-II cells were collected in RLT buffer with β-mercaptoethanol. The cells were further lysed and extracted RNA reversely transcribed to cDNA using RNeasy Micro Kit (Qiagen) and Superscript III First-Strand cDNA kit (Invitrogen) respectively. Gene expression was further analyzed by quantitative polymerase chain reaction (qPCR) using SYBR Green qPCR SuperMix (Invitrogen) and recorded on an iCycler (Bio-Rad). The expression of target genes was normalized against expression of housekeeping gene β-actin. In paper I we analyzed gene-expression of OVA in nasal cavity associated cells. These were retrieved by removing the palate, together with epithelial cells and NALT, which was immediately vortexed in PBS to detach cells into the suspension. After centrifugation, the cell pellet was resuspended in RNALater until further homogenized in RLT buffer (Qiagen).

Antibody assays

Antibody response was measured in two different ways, either as a measure of the actual specific antibody titer from blood or tissue samples, or as a measure of the number of specific antibody-producing cells. The antibody titer was measured using enzyme-linked immunosorbent assay (ELISA). In this assay the specific antigen was used to coat the bottom of the analysis wells, enabling the antibodies to adhere. After washing, surrounding surfaces not covered by antibodies were blocked with BSA to prevent unspecific binding. A secondary, horseradish peroxidase (HRP)-conjugated antibody, with specificity for mouse IgG (+subclass) or IgA was then added (Southern Biothechnology). An o-phenylene-diamine dihydrochloride (OPD) substrate then finally activates the HRP, leading to a detectable color change. The color intensity is proportional to the antibody titer and can be measured optically at a wavelength of 490nm. Materials for ELISA was taken from serum, lung, vaginal, fecal and intestinal samples

The enzyme-linked immunosorbent spot (ELISPOT) assay was used to measure the number of B cells secreting antigen-specific antibodies. A single cell suspension of spleen cells were incubated in antigen-coated wells for four hours. After washing and BSA blocking, HRP-conjugated antibodies were added and incubated over night in 4^oC. The following day development using AEC peroxidase substrate kit (Vector Laboratories Inc.) induced a HRP-mediated color change. The resulting spots on the floor of the wells, representing single antigen-specific B cells, were then counted using an ImmunoSpot Series I Analyzer (Cellular Technology)

In vivo cytotoxicity assay

The effector function of $CD8^+$ T cells is to kill cells presenting danger- or pathogen-associated antigens on MHC-I. In order to study the cytotoxic properties of endogenous $CD8^+$ T cells, we assessed the ability of immunized C57Bl/6 mice to kill peptide-loaded target cells. Spleenocytes pulsed with OT-I-specific peptide $OVA_{257-264}$ (SINFEKL, 2μg/ml) were stained with high concentration CFSE and mixed at ratio 1:1 with non-pulsed cells stained with a low concentration CFSE. Stained cells were transferred i.v.

one week after immunization with Ad-vectors expressing OVA protein. Following day, presence of CFSE-stained cells in spleen was analyzed by flow cytometry. The ratio between peptide-pulsed and non-pulsed cells was used to calculate CTL-activity.

Bone marrow chimeras

Transplantation of bone marrow is a technique frequently used throughout this thesis. DTx-treatment of CD11c-DTR mice efficiently depletes DCs *in vivo* [140]. However, the treatment is fatal after approximately 72 hours mainly due to CD11c-expression in unidentified stromal cells [210]. To circumvent this problem, we transferred CD11c-DTR bone marrow to irradiated C57Bl/6 (WT) mice with the outcome that DTx only affects the hematopoietic cell compartment. In addition, by constructing bone marrow chimeras we were also able to direct the presence of GM1-expression to either hematopoietic cells or non-hematopoietic cells. Furthermore we transplanted mice with bone marrow from both CD11c-DTR and $GM1^{-/-}$ -donors at a 1:1 ratio. After DTx-injection, these mice were left with only $GM1^{-/-}$ cDCs, whereas the remaining hematopoietic cells were expressing GM1.

In addition to DCs and plasma cells, also GC B cells were found to express enough levels of CD11c to be depleted by DTx in the CD11c-DTR mouse model. Therefore, we constructed bone marrow chimeric mice that enabled the depletion of $CD11c^{+}$ DCs without affecting GC B cells. First, CD11c-DTR mice were cross-bred with B cell-deficient μMT mice [211], resulting in the offspring CD11c-DTR/μMT. Bone marrow from CD11c-DTR/μMT mice were then mixed at a 1:4 ratio with bone marrow from MHC-II-deficient mice [212], and injected into C57Bl/6 recipients. The resulting $B^{MHC\text{-}II\text{-}/\text{-}}$CD11c-DTR mice thus contain DTx-sensitive DCs and MHC-II-deficient B cells.

Adoptive transfers

For effective studies of antigen-specific T cell activation, we used cells from OT-I and OT-II TCR transgenic mice. These T cells have TCRs that are specific for OVA-epitopes binding to MHC-I and MHC-II respectively. Cells were taken from LNs and spleen of donor mice. OT-I and OT-II cells were positively enriched by immunomagnetic capturing of $CD8^{+}$ or $CD4^{+}$ cells respectively on an AutoMACS (Miltenyi Biothech). For proliferation assays, purified T cells were stained with CFSE (Invitrogen) before injection with 2-5 x 10^{6} cells into recipient mice. CFSE is retained within cells for a long time and provides a way to visualize proliferation, as the intensity of CFSE is reduced by half for every cell division.

Results

Functionality of Ad-vectors

Adenovirus as a vaccine vector mediates an efficient delivery of expressed antigens and in addition comprises an intrinsic adjuvant effect. Most adjuvants activate DCs through TLRs or other specific receptors. The precise adjuvant function of Ad-vectors is not entirely established and therefore we were intrigued to investigate the role of DCs during an Ad-vector mediated immune response, and the preferred localization of expressed antigen for optimal presentation to the adaptive immune system. For this purpose we generated three candidate Ad-vectors all expressing ovalbumin but at different subcellular locations: intracellular (Ad/iOVA), membrane bound (Ad/mOVA), or as a secreted protein (Ad/sOVA).

First, we established that the transduction capacity and OVA expression were functional. For this we used an *in vitro* assay, transducing lung epithelial A549 cells with the vector constructs. Analysis by western blot confirmed that the protein was expressed and at the right size. sOVA, being the native form was comparable to the control recombinant OVA, whereas iOVA, lacking signaling sequence for extracellular transport gave a slightly smaller protein, and finally, mOVA that was linked to a trans-membrane sequence, resulted in the largest protein. Optical quantification showed that the amounts of expressed OVA were comparable between iOVA and sOVA, at 16.5 and 23.5 arbitrary units (AU), while mOVA were expressed at 124.0 AU. In addition, through immunocytochemistry the subcellular location of the expressed OVA was confirmed. All vector-transduced cells stained for intracellular OVA, but only Ad/mOVA-transduced cells stained for membrane-bound OVA (Fig. 6). OVA could also be detected by ELISA in the culture medium of Ad/sOVA-transduced cells.

Transduction *in vivo* was confirmed by visualization of OVA and GFP in epithelial cells of the nasal cavity, following intra nasal (i.n.) administration. The level of vector-mediated expression of OVA mRNA was comparable of all three vectors in nasal cavity associated cells (NCAC). However, transduction could not be detected in the nasal associated lymphoid tissue (NALT), indicating that Ad-vectors mainly infect epithelial cells.

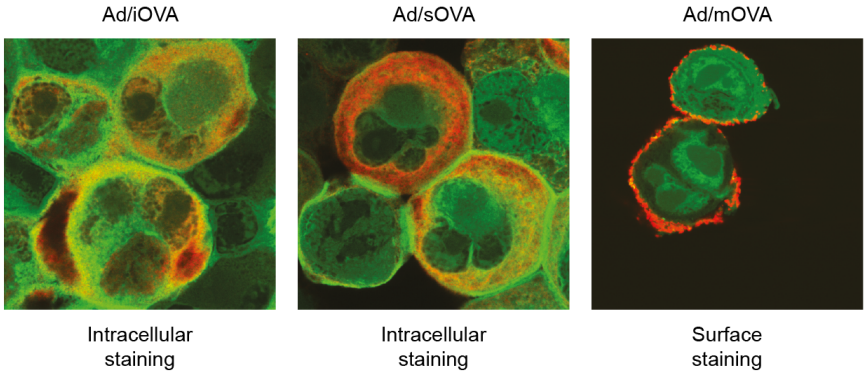

Figure 6. Targeted OVA expression by Ad-vectors. Confocal microscopy of OVA (red) and GFP (green) expression by A549 cells transduced *in vitro* with Ad/iOVA, Ad/sOVA or Ad/mOVA. Pictures where taken using a 60x objective lens in BioRad Radiance 2000 confocal microscope.

Induction of cytotoxic T cells

After confirming that the transduction and OVA-expression was functional, we set out to test the immune-stimulatory properties *in vivo* of these three constructs. We began by investigating the activation of $CD8^+$ T cells, since CTL activity is a good measure of Th1 response and important defense against viral infections. Therefore, induced CTL activity was studied by immunizing mice i.n. with the Ad-vectors and one week later measure their ability to kill OVA-peptide-loaded cells *in vivo*. We found that Ad/mOVA and Ad/sOVA immunized mice displayed very efficient CTL activity, with almost complete target cell elimination in some animals, whereas the Ad/iOVA vector only resulted in minor clearance of target cells (Fig. 7a).

Antibody response to Ad-vector immunization

The lower CTL response in Ad/iOVA was an intriguing notion that induced speculations whether also other immune responses were affected. Thus, we assesed the activation of B cells through their antibody production against Ad-vector-expressed OVA after nasal administration. Serum samples were taken 12 days after a single immunization, which measured high titers of IgG in both Ad/mOVA and Ad/sOVA-immunized mice (Fig. 7b). Ad/iOVA on the other hand was not able to raise any significant IgG response, even after receiving a ten times higher dose or after a second immunization. In addition, both Ad/mOVA and Ad/sOVA mediated significant IgG titers in vaginal washes but only Ad/sOVA resulted in measureable titers of IgA in the lungs.

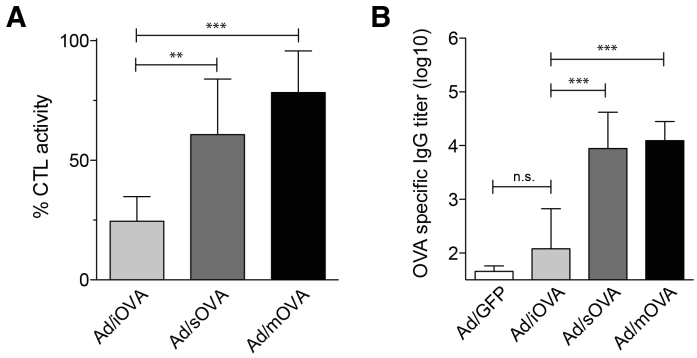

Figure 7. Potent CTL activity and IgG response induced by Ad/mOVA and Ad/sOVA, but not Ad/iOVA. **A**, One week post immunization with 1 x 10^9 pp of Ad/GFP or Ad/OVA vectors, mice were injected with splenocytes pulsed with OVA peptide plus a high dose of CFSE mixed 1:1 with splenocytes pulsed with a low dose of CFSE without peptide. One day later, splenocytes were analyzed for CFSE expression by flow cytometry. CTL activity calculated as the loss of the peptide-pulsed $CFSE^{high}$ population compared with the unpulsed $CFSE^{low}$ population. **B**, Anti-OVA IgG titers measured by ELISA twelve days post immunization with 5 x 10^9 pp of Ad/OVA vectors. Error bars show SD.

T cell proliferation

Given that Ad/iOVA failed to induce both $CD8^+$ cytotoxic responses as well as $CD4^+$ T cell-dependent antibody responses, we speculated whether variations in CTL and B cell activation might be due to altered antigen-presentation on MHC molecules. Therefore we chose to study the activation of $CD8^+$ and $CD4^+$ cells more closely. For this study we used $CD8^+$ (OT-I) and $CD4^+$ (OT-II) T cells that have pre-arranged OVA-specific TCRs. Transferred OT-I and OT-II cells labeled with the CFSE were analyzed for proliferation five days after i.n. immunization with Ad-vectors. Extensive expansion of OT-I cells was seen in all three groups and also of OT-II cells for Ad/mOVA and Ad/sOVA. Strikingly, Ad/iOVA immunized mice induced high levels of proliferation in OT-I cells but totally failed to stimulate expansion of OT-II cells (Fig. 8).

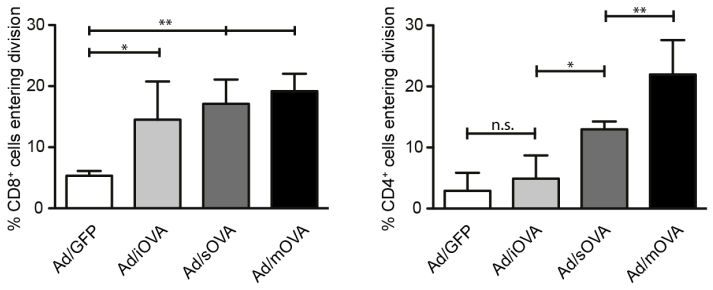

Figure 8. OT-I T-cell proliferation induced by all three Ad-vectors, but only Ad/sOVA and Ad/mOVA induce proliferation of OT-II T cells. FACS analysis showing frequency of transferred T cells entering division five days after intranasal immunization of 1 x 10^9 pp Ad-vectors. Mice were adoptively transferred with 5 x 10^6 OT-I and OT-II cells. Error bars show SD.

cDCs are necessary for T cell expansion

It is established that $CD11c^+$ cDCs are required to mediate activation $CD8^+$ T cell activation by cross-presentation during bacterial and parasite infections [140], as well as to induce $CD4^+$ T cell proliferation and T cell-dependent antibody responses to mucosal immunization with OVA and CT [213]. To further investigate whether the role of DCs is equally central during Ad-vector immunizations, we used the CD11c-DTR mouse model. These mice express the human diphtheria toxin receptor (DTR) under the control of the CD11c-promoter, which enables conditional ablation of $CD11c^+$ cDCs through injection of diphtheria toxin (DTx) [140]. Unfortunately, these mice die after a few days due to collateral elimination of unidentified stromal cells, obstructing repeated DTx-injections for long-time depletion [210]. To overcome this predicament, we constructed chimeric mice where only the hematopoietic compartment expressed the DTR gene. cDC-depleted mice were immunized with the Ad-vectors and analyzed for proliferation of transferred OT-I and OT-II cells. Without cDCs the proliferation of OT-I and OT-II cells were totally abolished compared to immunized WT mice (Fig. 9).

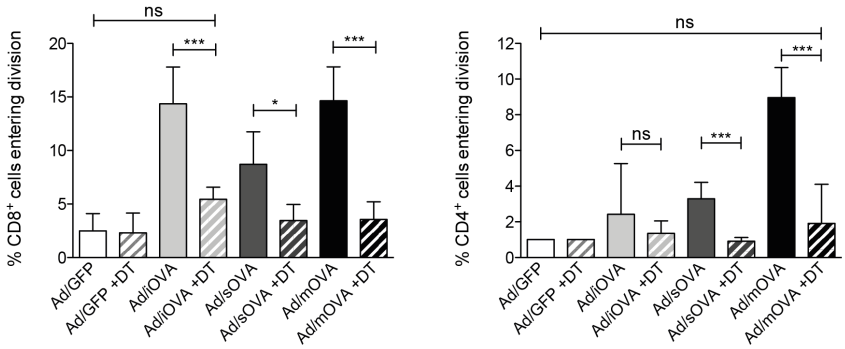

Figure 9. Proliferation of Ad/OVA vector-induced T cells is abolished in mice depleted of cDCs. Division of transferred OT-I and OT-II cells in the CLN. Mice were immunized with 1 x 10^9 pp of the indicated vectors the day after T-cell transfer and sacrificed five days later. Percentages in the bar graphs indicate the frequency of transferred cells entering division.

Transduction of cDCs not required for vector-mediated T cell activation

Adenoviruses are known to have a natural tropism for epithelial cells of mucosal surfaces but do in fact possess the ability to transduce all nucleated cell types. The apparent requirement for cDCs in Ad-vector-mediated immune responses raised the question whether the adenovirus needs to directly transduce a DC or if DCs may acquire activation through other cells. The difference in life span of cDCs (3-8 days) and airway epithelial cells (approx. 100 days) made it possible for us to measure the activation of transferred T cells introduced at a time point when any transduced DCs would be dead. OT-I and OT-II cells were therefore transferred into mice 1, 7 or 14 days after immunization with Ad/mOVA or Ad/sOVA. Neither OT-I nor OT-II cell proliferation was significantly affected when introduced 7 days after immunization. Even transfer 14 days after immunization resulted in proliferation although slightly reduced in mice immunized with Ad/mOVA (Fig. 10a and b).

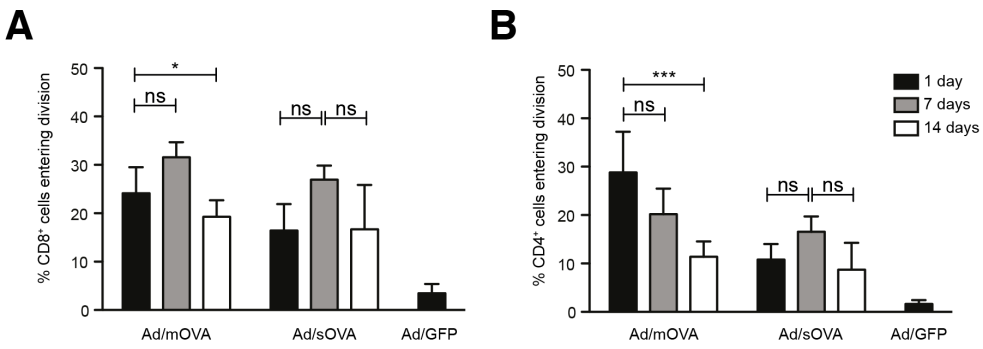

Figure 10. Activation of OVA-specific T cells does not require transduction of cDCs. OT-I and OT-II cells were co-transferred to C57BL/6 mice 1, 7, or 14 days following Ad/OVA vector immunization (1 x 10^9 pp). Proliferation of OT-I (A) and OT-II (B) cells was analyzed by FACS 5 days after transfer. Error bars show SD.

To further confirm these results, Ad/mOVA was this time given to DC-depleted mice, to ensure evasion of cDC-transduction, followed by T cell transfer two weeks later, which also resulted in significant proliferation of both OT-I and OT-II cells (Fig. 11a and b). These experiments clearly demonstrate that direct Ad-vector transduction of short-lived cells like cDCs is not required for T cell activation.

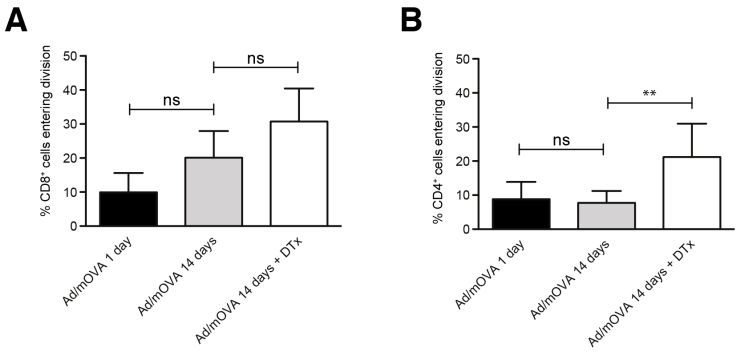

Figure 11. Redundancy of cDC-transduction confirmed by cDC-depletion. CD11cDTR/WT mice with or without DTx-injection were immunized 18 hours later with Ad vectors. Either 1 or 14 days later, OT-I and OT-II cells were transferred. OT-I (**A**) and OT-II T (**B**) cell proliferation was analyzed 5 days after transfer. Error bars show SD.

GM1 on epithelial cells is redundant for intestinal IgA

Images of DCs extending dendrites through the intestinal epithelial cells (IEC) of the gut mucosa [123] shifted the view of microfold cells (M cells) as the sole entry point where immune cells could acquire luminal pathogens. Even though gut DCs actively participates in bacterial uptake, these have been characterized as CX_3CR1^+, which lack the ability to migrate to lymph nodes and activate naïve T cells. Hence, the majority of DCs most likely acquire their antigens in other ways. DC maturation, defined by up-regulation of co-stimulatory molecule CD86, can be induced by bystander cells in contact with LPS or flagellin, without DCs interacting with the actual pathogen [214,215] [216,217]. Based on this information we wanted to examine whether the potent oral adjuvant effect of CT acts through DCs or through IECs. CT mediates target cell entry through binding of GM1, the only known receptor for CT [218]. Hence we constructed chimeric mice where either the non-hematopoietic (WT→GM1-KO) or the hematopoietic cell populations (GM1-KO→WT) lacked GM1 and fed them with OVA and CT. Strikingly, we found that mice with no GM1-expression on IECs were fully competent of producing intestinal IgA (Fig. 12). In contrast, mice with GM1-defective hematopoietic cells, including cDCs, had a significantly decreased IgA response, comparable to GM1-KO mice lacking GM1 on all cells.

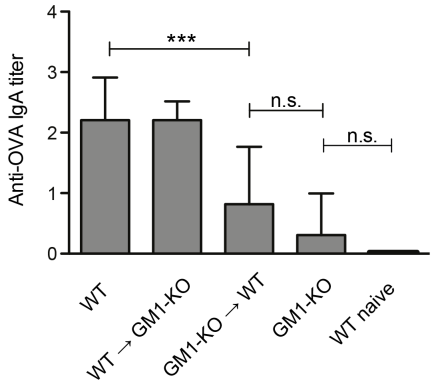

Figure 12. Intestinal IgA response after oral OVA and CT immunisations. OVA and CT was fed three times to WT, GM1-KO and GM1 chimeric mice at ten day intervals. Fecal pellets was analysed for Anti-OVA IgA by ELISA 7 days after the last immunisation. Error bars show SD.

Hematopoietic GM1-expression required for cDC maturation

CT is a strong inducer of immune responses, but requires the ganglioside GM1 for entry into target cells [63]. We have previously shown that cDCs are essential for activation of CD4+ T cells following oral immunization of OVA + CT [213]. Here, we investigated the requirement of GM1 for maturation of cDCs. Not surprisingly, after feeding CT, cDCs in MLN and PP from WT mice up-regulated CD86 significantly whereas cDCs in GM1-KO mice did not. To further narrow down the cellular requirement, we addressed whether CT interacts directly with hematopoietic cells. We found that chimeric mice with GM1-defective hematopoietic cells failed to up-regulate CD86 on cDCs in both MLN and PP, after being fed CT, indicating that DCs are not being matured properly in this system. These results show that CT does not induce cDC maturation through activating IEC, that then secrete factors increasing CD86 expression by cDCs.

T cell responses requires GM1 on hematopoietic cells

Knowing that production of high-affinity antibodies requires help from Tfh cells during GC formation, we continued to investigate if Tfh-cell differentiation is affected by GM1 in response to CT. First of all, we found that transferred OT-II cells, retrieved from MLN or PP of chimeric mice deficient of hematopoietic GM1-expression showed a significantly lower level of proliferation compared to mice transferred with WT bone marrow (Fig. 13a). Furthermore, only mice with GM1-competent hematopoietic cells were able to induce Tfh cell specific markers CXCR5, PD-1 and Bcl-6 in the transferred OT-II cells. CT had no effect in (GM1-KO→WT) mice, as the level of proliferation and Tfh cell specific markers was comparable to that of mice fed only OVA (Fig. 13b and c).

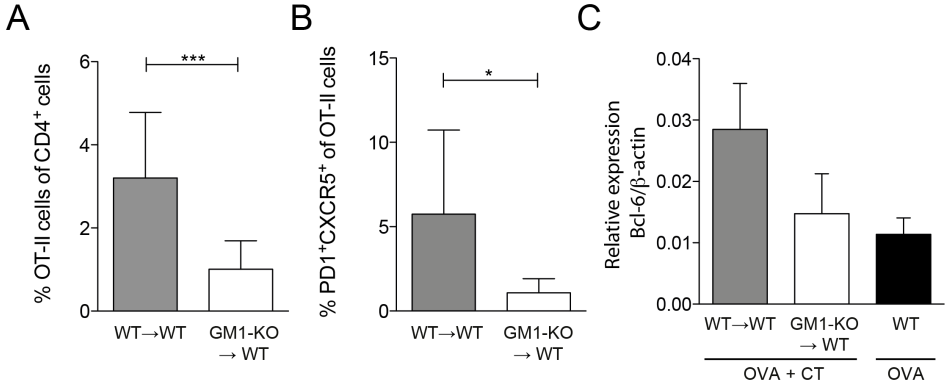

Figure 13. Expansion of OT-II T cells and Tfh phenotype following oral immunisation with CT is dependent on GM1 expression on hematopoietic cells. **A**, Frequency of congenic OT-II cells among $CD4^+$ cells in MLNs eight days after immunisation with OVA and CT. **B**, Graphs show the frequency of PD-$1^+$$CXCR5^+$ among transferred OT-II T cells in WT→WT and GM1-KO→WT mice. **C**, Levels of Bcl-6 mRNA in relation to β-actin expression in transferred OT-II cells

Expansion of OT-II cells requires direct contact between CT and cDCs

Our results have shown that GM1 needs to be present on hematopoietic cells and not IECs to mediate adjuvant effect by orally administered CT. Taken into account also that

cDCs are essential for $CD4^+$ T cell activation [213], we further investigated whether DCs are required to express the ganglioside themselves or if secondary interactions through accessory cells are sufficient. We thus created bone marrow chimeric mice that contained two equally large subsets of DCs, one that lacked GM1 and one that had GM1 but also expressed DTR on $CD11c^+$ cDCs, i.e. from the CD11c-DTR mouse described earlier (CD11c-DTR/GM1-KO→WT). After DTx-treatment of these mice, only cDCs lacking GM1 would be present, whereas 50% of the remaining hematopoietic cells will express GM1 (Fig. 14a). Oral immunization of untreated CD11c-DTR/GM1-KO→WT and CD11c-DTR/WT→WT controls resulted in equal OT-II proliferation, despite a 50% reduction of the GM1-expressing hematopoietic population in CD11c-DTR/GM1-KO→WT mice (Fig. 14b). Strikingly though, DTx-injection of CD11c-DTR/GM1-KO→WT mice, leaving only GM1-KO cDCs, displayed significantly decreased levels of OT-II cell expansion compared to DTx-treated CD11c-DTR/WT→WT mice, demonstrating that CT requires direct interaction with GM1-expressing cDCs to induce T cell expansion.

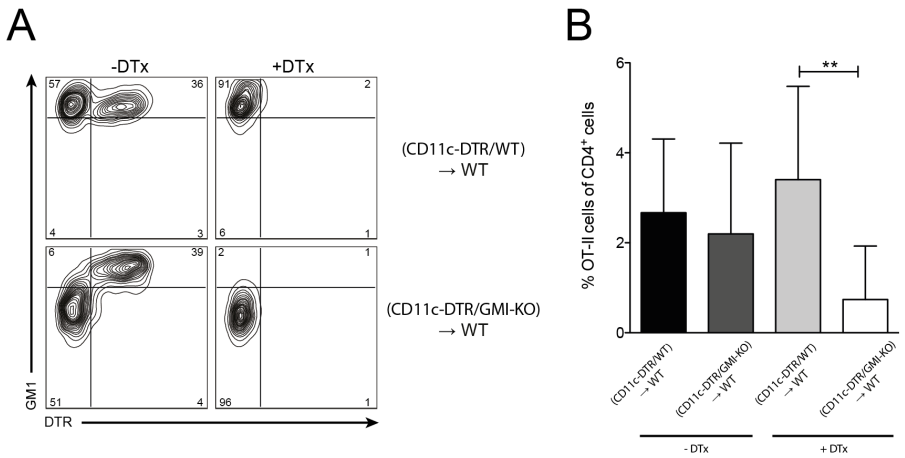

Figure 14. GM1-expressing cDCs are essential for OT-II T cell expansion in mice fed OVA + CT. Mice were treated with or without DTx 18 hours prior to immunization and then at 48h intervals until analysis eight days later. **A**, FACS analysis of GM1 expression on gated $CD11c^+MHC\text{-}II^{high}$ splenic DCs from (CD11c-DTR/WT)→WT and (CD11c-DTR/GM1-KO)→WT mice 18 hrs after DTx-injection, i.e. at the time of oral immunisation. **B**, Frequency of OT-II cells among $CD4^+$ T cells in MLNs.

Dependence of cDCs for Th1 response, but not Tfh cells and GC development

cDCs are by far the most potent cells when it comes to presenting antigens and activating naïve T cells [110]. Accordingly we have seen that in cDC-depleted mice after mucosal immunization with limiting amounts of antigen, DCs are indispensible for T cell activation and antigen production [213]. In contrast, during exposure to high amounts of antigen, the dependence of cDCs could be overridden. Based on these results and the fact that Tfh cells are completely required for GC development [172,219,220], we set out to investigate the actual role of cDCs in Tfh cell priming and GC formation. For this purpose, we used the synthetic RNA analog poly(I:C) as adjuvant, a strong

inducer of Th1 response and GC development, and employed our OT-II adoptive transfer system.

During initial experiments when we were evaluating our model system, we confirmed the potent adjuvant effect of poly(I:C), which induced expansion of OT-II cells, down-regulation of CD62L and up-regulation of CXCR5 expression three days after immunization. Mice receiving only OVA, or cDC-depleted mice receiving OVA and poly(I:C), had significantly less OT-II cell expansion, CXCR5-expression and did not down-regulate CD62L. Interestingly, by administration of a ten-fold higher dose antigen, we could rescue OT-II cell proliferation (Fig. 15a) as well as CXCR5 expression. Although CXCR5 is the canonical marker for Tfh cells, it does not define a true Tfh phenotype by it self. The transcription factor Bcl-6 is the master regulator of Tfh cells and is believed to be up-regulated, together with PD-1, upon interactions GC B cells. Therefore we assessed and found high PD-1 expression on the detected $CXCR5^{hi}$ cells, 14 days after immunization, and in sorted OT-II cells we could also detect high Bcl-6 expression when cDCs were depleted but the access to OVA was not limiting. As a further confirmation of functionality, high antigen dose was also able to rescue formation of GCs (Fig. 15b). Even if Tfh differentiation and GC formation was rescued by the high antigen dose, we observed that IFN-γ production and T-bet mRNA expression, characteristic cytokine and transcription factor of the Th1 response, were still abrogated in OT-II cells of cDC-depleted mice even when antigen was limiting (Fig. 15c).

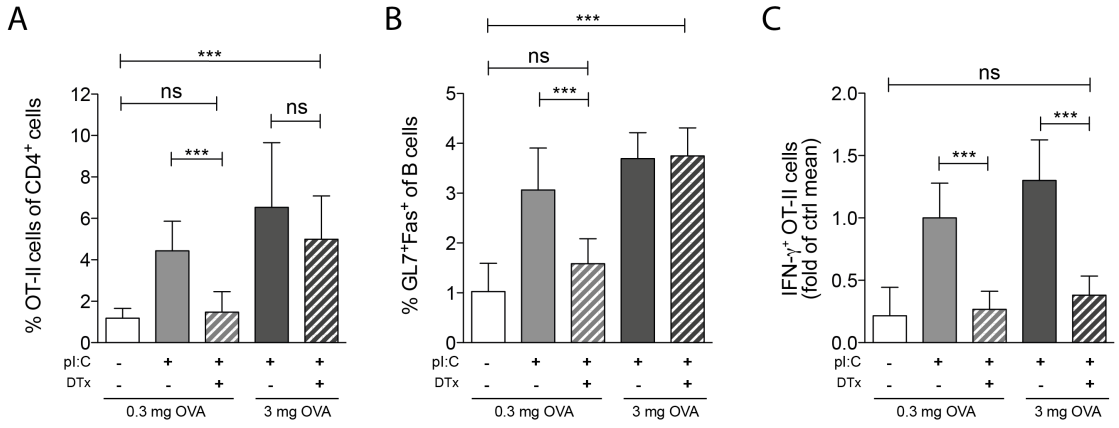

Figure 15. Efficient GC formation, but lack of IFNγ-producing $CD4^+$ T cells induced by an increased dose of antigen in cDC-depleted mice. CFSE-labeled $CD45.1^+$ OT-II cells were transferred into CD45.2 CD11c-DTR/B6 recipients. Splenocytes were analyzed by FACS fourteen days post i.p. immunization with OVA alone or with poly(I:C). Graphs show frequency of $CD45.1^+$ OT-II cells (A) and $GL7^+Fas^+$ GC B cells (B). **C**, IFN-γ production by transferred $CD45.1^+$ OT-II cells analyzed by intracellular staining after stimulation with PMA and ionomycin, shown as fold increase compared to control mean.

Partial Tfh phenotype without cDCs and GC B cells.

The CD11c-DTR/WT chimeric mouse model enables efficient conditional depletion of cDCs. However, it has been shown that plasmablasts up-regulate CD11c upon activation, impairing the use of these mice to study antibody responses that require

multiple immunizations [221]. We discovered that also GC B cells up-regulated CD11c and hence were depleted by DTx-treatment (Fig. 16). Accordingly, we found that DTx-treatment of CD11c-DTR/WT mice, even seven days after immunization with OVA and poly(I:C), completely ablated GCs. Differentiation of Tfh cells requires an initial priming signal, presumably by DCs or some other APC, with subsequent activation through prolonged interactions with B cells. Early stage Tfh cells can up-regulate CXCR5, but are believed incapable of expressing Bcl-6, or IL-21 in the absence of B cell contact. [177,178,222]. Repeated DTx-injections, one every other day during the experiment, provided us with a system with ablation of both cDCs and GC B cells. During these conditions, we found that $CXCR5^{+}PD\text{-}1^{+}$ early Tfh cells could be developed. However, these Tfh cells did not express Bcl-6 and IL-21, essential factors for the effector functions of Tfh cells.

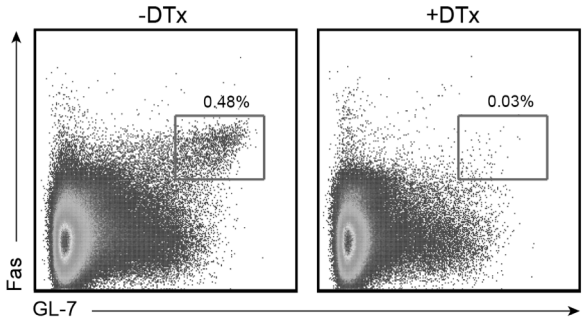

Figure 16. Efficient depletion of GC B cells in addition to DCs, following DTx injection. FACS analysis of $GL7^{+}Fas^{+}$ GC B cells from CD11c-DTR/B6 mice five days after immunization with OVA + poly(I:C) with or without DTx-injection one day before sacrifice.

Tfh cell activation is not dependent on PMN recruitment during cDC-depletion

An influx of polymorphonuclear cells (PMN) can generally be observed in lymphoid tissues, blood, kidneys and lungs after depletion of cDCs [213]. This effect is believed not to be a direct cause of DTx, but possibly because of an abundance of FLT3L that otherwise would be consumed by DCs, in combination with elevated levels of CXCL1 and CXCL2-mediated PMN recruitment from the bone marrow [223,224]. We investigated the role of PMN influx to the spleen in Tfh priming by injecting mice with an anti-Ly6C/G monoclonal antibody (clone RB6-8C5). This antibody has been extensively used for *in vivo* PMN-depletion experiments [225-227]. Injecting DTx-treated mice with anti-Ly6C/G antibody successfully hindered the aforementioned influx of PMNs to the spleen. When DTx + anti-Ly6C/G-treated mice were immunized, we observed unperturbed OT-II cell expansion, CXCR5 expression, and GC formation. The result that these mice displayed T cell and B cell responses comparable to untreated mice indicates that recruited PMN cells are not responsible for Tfh cell activation in absence of cDCs.

Tfh phenotype requires cognate B cell interaction, but not cDCs

In order to deplete cDCs without affecting GC B cells, we constructed chimeric mice by transfer of bone marrow from CD11c-DTR/μMT mice and MHC-$II^{-/-}$, at an 4:1 ratio, into C57Bl/6 recipients. In these $B^{MHC\text{-}II\text{-}/\text{-}}$CD11c-DTR mice, all B cells were MHC-$II^{-/-}$ and the DCs could be depleted by DTx-injection. To study Tfh cell activation and GC

formation, we transferred OT-II cells and GFP-expressing NP-specific B1-8^{hi} cells to these mice one day before immunization with OVA conjugated to the hapten NP (OVA-NP) and poly(I:C). Eight days after immunization, we observed efficient GC formation in DTx-treated mice, but only in mice that received B1-8^{hi} cells (Fig. 17a). In addition, frequencies of IgG1 producing GC B cells and CD138^{+} GFP^{+} plasma cells did not differ significantly between mice with B1-8^{hi} cells given DTx or not. Furthermore, mice with B1-8^{hi} cells induced significantly higher frequency of CXCR5^{+} PD-1^{+} OT-II cells and Bcl-6 expression than mice with only MHC-II deficient B cells (Fig. 17b and c). These results confirm the importance of cognate B cell interaction for complete Tfh cell development and establishes the redundancy of cDCs when antigen is not limiting.

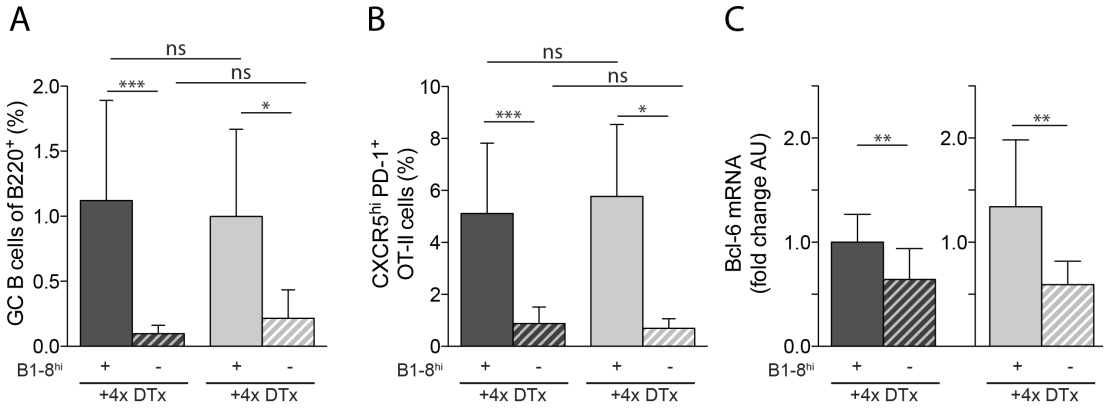

Figure 17. T-B cell interactions are required for development of Tfh phenotype and GC formation. B$^{MHC-II-/-}$ CD11c-DTR mice were given NP-specific B1-8^{hi} GFP^{+} B cells together with OT-II cells and DTx. The mice were immunized with OVA and poly(I:C) 20 hours after transfer. 4 DTx-injections were given during an eight day period to DC-depleted mice. **A**, GC formation in DTx- or untreated mice, with or without NP-specific B cells. Plot shows presence of GC B cells expressing GL-7 and Fas. **B**, FACS analysis of CXCR5^{+}PD-1^{+} cells of viable transferred OT-II cells. **C**, Bcl-6 mRNA expression in transferred OT-II cells analyzed by qPCR.

cDCs fail to induce CXCR5 expression by CD4^{+} T cells *in vitro*

Thus far, it appears that signals given to prime an early Tfh phenotype is not exclusively provided by cDCs. Interestingly, even in absence of cDCs and MHC-II competent B cells, we could observe intermediate levels of CXCR5 on OT-II cells. Expression of Bcl-6 is a hallmark of Tfh phenotype and likely regulates the full induction of CXCR5. Johnston et al. addressed this hypothesis by transducing T cells with retroviral Bcl-6 and were able to drive CXCR5 expression and Tfh development without B cells *in vivo* [172]. There are conflicting reports regarding the ability of cytokines to induce expression of Bcl-6 or CXCR5 in vitro [190,219,228,229]. In an effort to activate CXCR5 expression in vitro, we used transduced OT-II cells with retroviral Bcl-6 for constitutive expression, together with antigen pulsed cDCs. Even though successfully transduced, as confirmed by GFP expression, cDCs were not able to drive CXCR5 expression by OT-II cells *in vitro*. Addition of IL-6, or a cytokine cocktail previously reported to induce differentiation of "Tfh-like" cells *in vitro* [219,230], or even B cells did not rescue CXCR5 expression. However, OT-II cells from mice immunized with the OVA-pulsed cDCs were able to express high levels of CXCR5, comparable to mice immunized with OVA and poly(I:C). Thus, cDCs appear not to provide enough

stimulatory signals to drive CXCR5 expression or Tfh differentiation in vitro and require additional in vivo derived activation factors.

Functional antibody memory response but impaired Th1 isotype switch in cDC-depleted mice

Given that Tfh cells and GC formation are readily induced even in absence of cDCs, we speculated whether a functional antibody response could be generated. A potent memory response should be able to initiate a rapid production of antibodies of high affinity to combat a second pathogen encounter. We immunized cDC-depleted mice with poly(I:C) and OVA-NP. The mice were given a booster injection with OVA-NP without adjuvant after 14 weeks. At this time point we would minimize extrafollicular plasma cell responses. One week after booster injection, we found that mice without cDCs at the time of immunization had a total NP-specific total serum IgG comparable to untreated mice. Strikingly, DTx-treated mice showed severely abrogated isotype switching to the IgG2c isotype, but produced highly elevated levels of IgG1 (Fig. 18a and b). Production of complement-fixing IgG2c is considered to be the canonical antibody for Th1 response, and isotype switching to IgG2c is stimulated by IFN-γ, which we previously observed to be poorly produced in absence of cDCs. Also during this booster experiment, we found cDC-depleted mice unable to up-regulate IFN-γ production in $CD4^+$ T cells re-stimulated with OVA (Fig. 17c). Despite the deficiency of inducing Th1 phenotype in absence of cDCs, we measured a competent IgG1 response with as high affinity as the untreated mice. Furthermore, the frequency of total antibody secreting cells remained equal regardless of cDC-depletion or not. Taken together, these results show that a functional GC with somatic hyper mutation and class switch recombination can be formed without presence of cDCs. These mice were also able to mobilize a potent memory response with high affinity IgG1 antibodies. Conversely, poor expression IFN-γ and lack of IgG2c antibody production demonstrates a total dependence of cDCs for Th1 phenotype development. Finally, we did not observe up-regulation of GATA-3, indicating that increased IgG1 levels are not due to a triggered Th2 response, rather a result of the endogenous IL-4 production by activated Tfh cells.

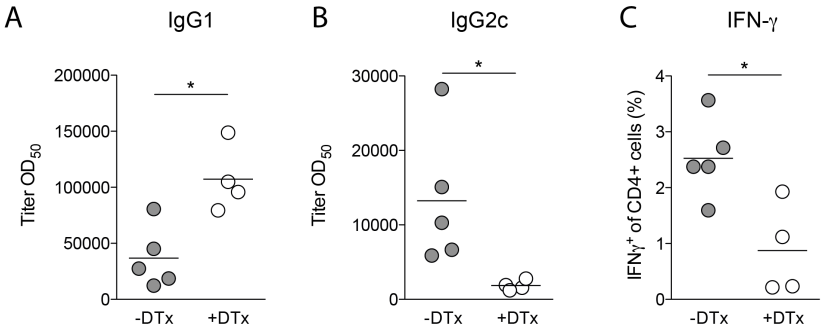

Figure 18. Functional memory response, but impaired Th1 phenotype in cDC depleted mice. DTx- or untreated CD11c-DTR mice were immunized with NP-OVA and poly(I:C), boosted with NP-OVA after 14 weeks and analyzed one week later. Generation of NP-specific IgG1 (A) and IgG2c (B) antibodies analyzed by ELISA. **C**, Frequency of IFN-γ producing cells of total $CD4^+$ T cells re-stimulated with OVA peptide in vitro.

Discussion

Generation of a potent adaptive immunological memory is dependent on efficient antigen presentation. In this regard, the DC is the most potent activator of adaptive immunity. Studies using different microbial infection models or antigen with adjuvant immunizations have found DCs to be indispensable for a functional adaptive immune response. Yet the mechanisms of many potent adjuvants in use or in development today are still puzzling. How DCs acquire immunostimulatory signals in different settings is the focus of several ongoing research projects. The results in this thesis bring some light to the heterogeneity of DC functions and their activation requirements during immunizations with variable adjuvants and administration routes.

A central issue of inducing protection is how antigens are best delivered to DCs for optimal engagement of adaptive immunity. We have addressed this question using Ad-vectors expressing OVA at three different subcellular locations, intracellular, membrane-bound, and as a secreted protein. In previous studies using adenovirus vectors, the location of the expressed protein has generally been decided by its intrinsic sorting sequence. Judging by our results, showing an apparent defect in cross-presentation and CTL activation in response to a cytosolic antigen, secreted or membrane-bound antigens are more likely to generate a protective immune response.

Stimulation of cytotoxic $CD8^+$ T cells with viral antigens from transduced cells generally requires cross-presentation, or 'cross-priming' [231]. Vector based delivery is known to stimulate cellular immunity with strong $CD8^+$ cytotoxic activity [22,232]. Using an *in vivo* CTL assay we found that antigen expressed as a membrane-fused protein induced the most effective cytotoxic killing of target cells. Secreted OVA mediated almost as good CTL-activity as the membrane-bound, whereas only weak CTL-induction was shown in response to the intracellular antigen. This system provided a pure view of the cytotoxic effector functions, as we used the endogenous $CD8^+$ cells for evaluating CTL-activity. In addition, our system also contained endogenous $CD4^+$ T cells, unlike many other studies with Ad-vectors, using predominantly $CD4^+$ T cell-deficient mice.

The natural response to intracellular virus infections is to present foreign cytosolic peptides on MHC-I for targeting by CTLs. This implies that Ad/iOVA would be the easiest of the tree antigens to be presented on MHC-I. In sharp contrast, Ad/iOVA mediated the lowest levels of CTL activity and antibody response. It has been reported that cytosolic expression of OVA can result in impaired protein stability [233], which could reduce MHC-I presentation. The difference in efficacy between Ad/iOVA and the other two constructs could hence be due to lower antigen levels. However, as proliferation of $CD8^+$ T cells actually was fully functional, the cross-presentation cannot be dysfunctional *per se*. Cytosolic iOVA induced proliferation of transferred OT-I cells at a level comparable to secreted or membrane-anchored OVA, even after a 100-fold titration of the vectors. Also, although the level of expressed iOVA in transduced cells

was similar to sOVA, the CTL activity in Ad/sOVA transduced mice was considerably higher.

The observation that Ad/iOVA induced poor $CD4^+$ T cell proliferation made us speculate that cDCs could induce $CD8^+$ T cells to proliferate, albeit not develop effector functions without $CD4^+$ T cell help. In fact, the poor $CD4^+$ T cell priming could be explained by the decreased protein stability of iOVA. It has been shown that less digestible proteins are favored for MHC-II presentation, resulting in efficient $CD4^+$ T cell priming and antibody responses [234]. This may be why iOVA is efficiently presented on MHC-I but not on MHC-II. Whether or not CTL-differentiation requires $CD4^+$ T cell help is still an open question [235]. It has been reported that helper T cells are necessary for cross-presentation of antigens from apoptotic cells, and furthermore, that $CD4^+$ and $CD8^+$ T cells must interact simultaneously with the same APC [148]. Others have proposed that virus infections can substitute $CD4^+$ T cell help and induce co-stimulatory activity of APCs [146,236], possibly by TLR-activation and expression of inflammatory cytokines like TNF-α or IFN-α/β, thus mediating $CD4^+$-independent CTL-activation. Some suggests that a primary CTL response can be generated in $CD4^+$ T cell deficient mice, but without a resulting $CD8^+$ T cell memory response. However, we made an attempt to rescue CTL activity by transferring activated $CD4^+$ T cells into the immunized mice. But, despite additional T cell help, these mice were unable to generate a significant cytotoxic response.

Ad/iOVA also generated poor serum IgG production, most likely due to the lack of helper T cell activation, as T cells are necessary for GC formation. Even a 100-fold higher viral dose did not result in IgG levels significantly higher than the GFP control vector. Surprisingly, the secreted OVA showed improved IgA response in the lungs compared to the membrane-bound. If secreted OVA can travel from the nasal cavity to the lungs better than membrane bound, it would do so without of the viral capsid. As OVA alone would not be able to stimulate enough immune response, it is more likely that equal amounts of virus particles reach the lungs but the additional expressed secreted OVA getting there could give an advantage against membrane-anchored OVA. Despite the use of small dosage volumes, it cannot be ruled out that some spillover might occur during immunization that also bring the Ad-vectors to the lungs and reach the medLNs [237]. The fact that distant mucosal tissues are immunologically linked is clearly demonstrated in our study, as nasal immunization provides vaginal IgG responses [54]. Although being a mucosal tissue, the most prevalent antibody in the vagina is IgG instead of IgA [238]. Given that Ad/sOVA and Ad/mOVA generated equal levels of vaginal IgG suggest that the difference in the lung IgA would be owing to the advantage of additional antigen reaching the medLNs of Ad/sOVA immunized mice.

This study also showed a total requirement of cDCs for activation of antigen-specific T cells after intra nasal immunization with Ad-vectors. Mice depleted of cDCs were completely unable to induce proliferation of $CD4^+$ and $CD8^+$ T cells. Furthermore we found that antigen acquired from transfected neighboring cells was sufficient for DCs to induce T cell activation, demonstrating that a direct transduction of DCs by Ad-vectors was not required. We observed that transfer of OT-II cells two weeks after

immunization with Ad-vectors had basically no effect on T cell proliferation. At this time point no directly transduced DC would be left alive, considering the turnover of a DC to be 3-5 days [239]. We confirmed these results in a similar experiment by immunizing cDC-depleted CD11c-DTR/B6 mice, which also induced proliferation of OT-II cells transferred two weeks afterwards. As also macrophages in the spleen and lymph nodes are depleted, and takes longer than DCs to repopulate tissues, they are probably not accountable for activating T cells in cDC-depleted animals [140,240]. Also the possibility that lingering Ad-vectors might infect later DC populations is unlikely due to effective vector clearance by innate immunity within only a few days [21].

Our results indicate that the proteins most accessible to APCs are the secreted and the membrane-bound. Intracellular antigens thus seems harder to reach by the APCs but may be accessed through direct uptake of debris from dead transfected cells. DCs have been shown to also take up antigen from PMNs that have endocytoced apoptotic cells [241]. There are also reports of both monocyte-derived and plasmacytoid DCs able to retrieve antigens from MHC molecules of live neighboring cells, a process called "nibbling" [242,243]. Even if that is the case, the amount of antigens acquired by this route must be inferior to retrieving proteins that are secreted or anchored to cell surfaces.

The intestine is a heavily regulated environment, with demands of being able to keep the microbiota of the lumen under control and at the same time tolerate the uptake of food nutrients. Because the default local immune response in the intestine is set to tolerance, inducing immunity through oral vaccination has proven to be a challenge. Purified CT has shown to confer strong adjuvant effect through mucosal as well as parenteral administration. From previous studies, we know that DCs are needed to activate adaptive immunity in response to oral CT stimulation, unless high doses of antigen were delivered together with CT [213]. Still, the mechanism used by CT to activate immune responses through cDCs remains largely unknown.

We show that interaction with IECs and other non-hematopoietic cells is not sufficient to generate intestinal IgA or induce proliferation of antigen-specific $CD4^+$ T cells. Although normally highly affected by the toxic effects of CT, enterocytes did not convey any immunostimulatory signals, demonstrating that toxicity and adjuvanticity by CT are two separate pathways. We further show that maturation of cDCs was aberrant in mice where GM1 was restricted to non-hematopoietic cells. cDC maturation is crucial as the activation state of cDCs during MHC/TCR interactions will influence if the T cell will develop effector functions, be silenced, or deleted. These experiments provide evidence that CT must interact with hematopoietic cells to confer immunostimulatory effects. These results are in sharp contrast to experiments using TLR agonists for mucosal immunization. TLR-4 deficient cDCs was shown to acquire sufficient maturation signals from lung epithelial cells after LPS inhalation. [214,215]. Also parenteral immunizations with flagellin mediated maturation of $MyD88^{-/-}$ cDC via bystander cell activation [216,217]. However, Spörri and Reis e Sousa found that even if DCs were able to receive sufficient maturation stimulation indirect from other cells, direct interactions were required for complete DC activation, e.g. the ability to induce T cell effector functions, [244].

Having observed a dependency on CT to bind GM1 on hematopoietic cells for cDC maturation, we speculated whether this occurs by direct CT-cDC interaction or via another hematopoietic cell. It has previously been shown that DCs need to express GM1 to express CD80/86 and to reactivate T cells in vitro [73], but whether this is also the case in vivo was still unknown. To address this question, we used a chimeric mouse system where $GM1^+$ cDCs were depleted with DTx, leaving only cDCs lacking GM1 expression. We found that cDCs were unable to induce $CD4^+$ T cell activation without direct interaction with CT. Despite the fact that these mice contained a sufficient amount of GM1-competent hematopoietic cells, they were unable to activate $CD4^+$ T cells.

Our findings demonstrate that cDCs are able to retain intact toxin that can traverse the epithelial barrier from the intestinal lumen. As GM1 today is the only known receptor for CT, transport across the barrier may well be receptor-independent. Transport through M cells in the follicle associated epithelium has long been considered the only gateway between the intestinal lumen and the lamina propria, transporting luminal pathogens to DCs residing underneath in the subepithelial dome. Recruitment of DCs to the follicle associated epithelium has been reported within two hours following intra gastric immunization with CT, indicating that this can possibly be the main distribution channel of CT to DCs [245,246]. Enterocytes have been reported to possess endocytic abilities and manage to internalize *E.coli* in a TLR-4-dependent fashion. Whether they also perform receptor-independent endocytosis is not known [247]. Recent studies have even discovered that goblet cells, which were known to only expel mucus into the lumen, may in fact also have a role in taking up luminal particles for delivery to $CD103^+$ DCs [248]. DCs have in addition been found able to interfere with IECs and extend protrusions out through their tight junctions and capture pathogenic bacteria by themselves [123,249]. These sampling DCs were found to be $CX3CR1^+$ mononuclear phagocytes that don't migrate to MLN or prime naïve T cells. A future study might reveal whether $CX3CR1^+$ DCs can bystander activate other DCs, e.g. $CD103^+$ DCs.

Whether bystander activation between cDCs occurs was not addressed in our settings. A possible experiment to investigate this would be to transfer equal amounts of bone marrow from $CD45.2^+$GM1-KO and $CD45.1^+$ WT mice into a recipient mouse, and then observe if $CD45.2^+$ cDCs are able to up-regulate CD86. A similar experiment has been performed investigating the dependence for the type-I IFN receptor [44]. If DCs acquire CT by sampling the lumen, from M cells, or by any other way possible, remains to be discovered. Knowing how to best deliver CT to DCs will greatly simplify the study of the immunostimulatory effects of CT and hopefully lead to a future application as adjuvant in mucosal vaccines.

The signaling pathways of TLRs leading to production of proinflammatory cytokines are extensively studied and largely defined. As for CT, the precise immunostimulatory pathway is still not explained. TLR-signaling is not needed for immunostimulation by CT, as epicutaneos injection with OVA and CT was shown to be MyD88/TRIF-independent, but required Batf3 DCs and type-I IFN signaling to mediate adjuvanticity [80]. It is known that adjuvanticity is mediated without CTB. Vesicular transport associated with internalization through CTB-GM1 binding is not necessary for the catalytic activity of CTA_1, as demonstrated by the function of targeted CTA_1

expression in plasmid-based transfection systems, as well as the function of the CTA1-DD construct [67,250]. ADP-ribosylation is central for the adjuvant effect by CT, LT and CTA_1-DD, indicating that enzymatic activity of the A_1-subunit is of importance. A non-toxic version of LT (mLT), containing one amino acid substitution in the A_1-subunit, totally lacked ADP-ribosyltransferase activity, and also did not provide immunostimulatory functions [251]. Even though the mutant CDA_1-DD only performs 25% of ADP-ribosyltransferase activity compared to CT it still provides strong adjuvanticity [67]. In preliminary studies not presented in this thesis, we have also seen that CD11c-Gsαflox mice, lacking Gsα in CD11c-expressing cells, are unable to activate CD86-expression on cDCs following oral immunization with CT. This demonstrates that $CD11c^-$ hematopoietic cells cannot bystander activate cDCs. CT also had a severely decreased adjuvant effect in these mice. Whether the adjuvant effect is a direct cause of cAMP-levels has not yet been confirmed. Recent reports indicate that the NLRP3-inflammasome can be triggered by osmotic alteration of the cell volume. Osmotic stress caused caspase 1 activation and secretion of IL-1β by macrophages [252]. If the adjuvant effect of CT is mediated through NLRP3-inflammasome activation remains to discover, but taken the perturbating effect CT has on ion-channels, this pathway could be worth investigating.

Tfh cells are essential for the generation of memory B cells and an adaptive antibody response by aiding the B cells in the GC reaction. Although DCs have been found to take part in early Tfh differentiation through ICOS-signaling, little is known whether DCs provide unique polarizing signals for Tfh, as for Th1 and Th2 responses [177,188]. In our previous findings, we showed that cDCs are required for induction of $CD4^+$ T cell proliferation and humoral response, but the dependency of cDCs could be overridden by administration of a higher dose of antigen [213]. Using the CD11c-DTR/B6 mouse model with OT-II transfer, we sought to investigate the involvement of cDCs in Tfh cell development and GC formation. For this purpose we took advantage of the strong Th1 and GC inducing properties of the adjuvant poly(I:C), the synthetic dsRNA analogue binding to TLR-3, but also to the cytosolic RNA helicase MDA5. Accordingly, we found that differentiation of Tfh cells and germinal center formation was possible in cDC depleted mice, if rescued by a high antigen dose. These mice induced high proliferation of transferred OT-II cells, together with expression of the hallmark Tfh markers CXCR5 and Bcl-6. The functional properties of these Tfh cells were strengthened by the generation of $CD95^+$ $GL\text{-}7^+$ GC B cells. Surprisingly, the high antigen dose did not rescue polarization of Th1 phenotype as OT-II cells lacked the ability to produce the inflammatory cytokine IFN-γ and the transcription factor T-bet.

Knowing that cDCs repopulate mice around 3-5 days after DTx-injection, and the fact that GCs are developed by day 8, we sought to ensure efficient cDC-depletion during the complete duration of the study. We thus employed a strategy involving repeated DTx-injections. However, also plasmablasts up-regulate CD11c and DTR, and are therefore effectively depleted by DTx-treatment of the CD11c-DTR/B6 mice. As our interest was in the GC B cells we analyzed them for GFP-expression, which is co-

expressed with the DTR gene, and susceptibility to DTx. We found that GC B cells became GFP^+ and were directly ablated by DTx-treatment. Thus, repeated DTx-injections provided measures to study Tfh development when both cDCs and GC B cells are depleted. Transferred OT-II cells induced CXCR5 and PD-1-expression equally well in mice with or without 4x DTx-injections. In contrast, only OT-II cells in the mice without DTx-injection were expressing Bcl-6 and IL-21. The OT-II cells of mice receiving 4x DTx-injections failed to do so, and consequently did not adopt full Tfh differentiation. These results demonstrate a clear boundary between early ($CXCR5^+PD\text{-}1^+$) and late ($Bcl\text{-}6^+IL\text{-}21^+$) Tfh phenotypes, as primed Tfh cells require prolonged cognate GC B cell interactions and ICOS-mediated maturation signals for full differentiation.

In order to study Tfh cell development in a system without cDCs but with intact GCs, we constructed a chimeric mouse where bone marrow from CD11c-DTR/μMT mice and MHC-II-deficient mice were transferred, at a 4:1 ratio, into C57Bl/6 mice. This $B^{MHC\text{-}II\text{-}/\text{-}}$CD11c-DTR mouse then offered a system where cDCs could be depleted with repeated DTx-injections while the B cell compartments remained unharmed but incapable to provide MHC-II presentation. To assess the GC formation and Tfh cell development we transferred OT-II cells with or without NP-specific $B1\text{-}8^{hi}$ cells to the mice before immunization. Mice that did not receive $B1\text{-}8^{hi}$ cells could not form GCs or develop $CXCR5^{hi}PD\text{-}1^{hi}$ OT-II cells. In contrast, mice with transferred $B1\text{-}8^{hi}$ cells showed efficient GC formation and expansion of $CXCR5^{hi}PD\text{-}1^{hi}$ OT-II cells regardless of DTx-treatment or not. These results contribute to earlier studies and demonstrate that differentiation of a functional Tfh phenotype requires cognate GC B cell interactions [177,178,181]. Interestingly, OT-II cells did not induce PD-1 expression, and just intermediate levels of CXCR5 in cDC-depleted mice with MCH-II-deficient B cells. PD-1 is an inhibitory receptor up-regulated on T cells during prolonged TCR interactions, preventing excessive proliferation of Tfh cells in the GC [188,253]. In contrast, $CXCR5^+PD\text{-}1^+$ OT-II cells was developed in our system with depleted cDCs and GC B cells, suggesting that cognate interaction was made between T cells and GC B cell in those mice, and that CD11c is up-regulated on GC B cells following PD-1 expression by Tfh cells.

Poly(I:C) mediates a strong Th1 response in normal conditions, but in our settings with high antigen dose, we found that cDC depleted mice were defective in their Th1 differentiation and failed to express IFN-γ and T-bet. Further demonstrating defective Th1 polarization, 15 weeks after immunization, we saw poor IgG2c production with low antigen affinity, even though the total IgG levels and number of plasma cells during memory response was comparable in mice primed with or without cDC. Conversely, IgG1 antibodies was significantly up-regulated and showed high affinity. Th2 associated genes are proposed to be indirectly suppressed by T-bet according to a recent study, and consequently, a lack of T-bet would lead to up-regulation Th2 genes [254]. In contrast, we have not found evidence of an up-regulated Th2 phenotype, as expression of GATA3 could not be detected in our system (data not shown). The observed IgG1 isotype switch was likely induced by Tfh-mediated IL-4, given that

activated Tfh cells produce IL-4 in a Th2-independent manner, predominantly by SLAM and Ly108-mediated SAP-signaling [181,255].

Injection with anti-CD40 antibody not only abrogates the formation of GCs but also perturbates ongoing GC reactions, indicating an essential role for GC Tfh cells in clonal selection and not only in the formation of GCs [256]. In addition, it demonstrates that limited availability of FDC-bound antigen is not the sole factor of affinity selection. This scenario quite resembles our system where we ablate GCs with DTx-injections. It would be interesting to compare these two systems more carefully to see if they reach the same level of Tfh-differentiation.

Regardless of which system used to remove a certain cell type in vivo there is always the chance that some cells are not depleted and that they, if they are extremely efficient can still perform the function. DTx-mediated depletion of cDCs in the spleen is extremely efficient, ablating more than 95-98% of cDCs. Is it therefore possible that the remaining cDCs can be responsible for the activation of $CD4^+$ T cells after DTx treatment? We find this unlikely, as the development of Th1 phenotype was totally abrogated. In addition, when a lower dose of antigen was used, no activation of $CD4^+$ T cells was observed following immunization of DTx treated mice. If the few cDCs that are remaining have the capacity to perform the same function that the entire cDC population usually does then some activation of $CD4^+$ T cells would be expected to be observed. Hence, a more probable explanation is that some other cell type has T cell priming abilities that is unveiled in the absence of cDCs.

Our observation that cDCs are not required for Tfh differentiation was made when the antigen was in high abundance and most likely also available for an extended period of time. This may increase the chance that B cells that otherwise have poor macropinocytotic capacity may take up that antigen. Intravenous administration with a high dose of hen egg lysozyme (HEL) has been shown to increase unspecific uptake by B cells and mediate peptide presentation on MHC-II, whereas no loading on MCH-II could be detected after injection with lower doses [257]. During cDC-depletion and when antigen is not limiting, B cells could possibly acquire enough antigens to activate Tfh cells. However, the fact that OT-II cell proliferation and intermediate expression of CXCR5 was observed in mice with MHC-$II^{-/-}$ B cells and depleted cDCs suggests that other cell types also could be affected by increased antigen concentrations, e.g. PMNs, inflammatory monocytes or pDCs.

Previous reports from our lab and others have described recruitment of side scatter high (granular) cells that expressed high levels of CD11b and Ly6G during cDC-depletion. Some of these cells also expressed MHC-II. This myeloproliferative phenomenon is not believed to be a direct cause of DTx, as it also occurs in genetically cDC-depleted mice. Instead a combination of CXCL1 and CXCL2 mediated release from the bone marrow and possibly accumulation of FLT3L in the absence of DCs seems to be the cause [223,224]. We used the Ly6C/G depleting antibody RB6-8C5, which ablate Ly6G and Ly6C expressing cells. Administration of this antibody successfully blocked recruitment of additional $CD11b^{hi}$ PMNs to the spleen and diminished their presence in blood during cDC-depletion. We found that OT-II

proliferation, CXCR5-expression and germinal center formation remained unaffected in cDC-depleted mice even after anti-Ly6C/G-treatment, indicating that activation of Tfh cells in absence of DCs was not rescued by newly recruited PMNs.

Another cell to take into account is the sub-capsular sinus (SCS) macrophages that is positioned in the endothelial carpet separating the follicle from the SCS, sampling antigens arriving from the afferent lymph. These cells are F4/80^{-}CD169^{+}, may not be affected by macrophage depletion, and have lower proteolytic activity than regular macrophages, enabling delivery of intact antigen to follicular B cells and FDCs. The lower proteolytic activity could actually imply that these cells are better APCs than regular macrophages. Whether these SCS macrophages possess enough APC functions to induce GC formation in absence of DCs is not yet established [198].

We have previously shown that pDCs are not efficiently depleted in CD11c-DTR mice by DTx-treatment, and we could observe an incomplete depletion of Ly6C^{hi} inflammatory monocytes, both of which are able to express MHC-II. Inflammatory monocytes are believed to have poor priming capacity, and although pDCs have been found to prime naive CD4^{+} T cell in LNs, they failed to do so in the spleen [133]. Our previous study also found low dependency of pDCs to activate splenic CD4^{+} T cells [213]. However, we cannot rule out the possible involvement of these cell types in Tfh development. Also the adjuvant poly(I:C) can modulate the T cell priming capacity of APCs. We have in preliminary experiments observed that PMNs, inflammatory monocytes and pDCs express ICOSL following poly(I:C) immunization. ICOS-ICOSL interaction has been shown to be crucial for Tfh development [258,259]. As these APC populations may have overlapping functions, simultaneous depletion of cDCs, inflammatory monocytes, PMNs, and pDCs in mice with MHC-II-deficient B cells will have to be performed. Removal of just one APC population at a time in addition to cDCs makes it possible that some of the other non-depleted cell types can compensate.

In conclusion, we have demonstrated that cDCs were indispensable for T cell activation after intranasal immunization with Ad-vectors, and that targeting of neighboring cells is sufficient for antigen presentation by dendritic cells. Furthermore, we conclude that activation of CD4^{+}, cytotoxic CD8^{+} T lymphocytes, and generation of serum IgG was superiorly induced by membrane-anchored or secreted antigen compared to intracellulary expressed.

We have also shown that the adjuvant effect of orally administered CT requires direct contact with GM1-expressing cDCs in order to induce adaptive immune responses, whereas GM1-expression on intestinal epithelial cells was redundant.

Finally, we found that Tfh cell development and GC formation could be induced in absence of cDCs, during high antigen dose exposure. In contrast, IgG2c isotype switching and induction of Th1 phenotype was totally dependent on cDCs.

Populärvetenskaplig sammanfattning på svenska (Swedish summary)

Celler ur immunförsvaret finns under huden och slemhinnor där vi ständigt utsätts för skadliga ämnen och mikroorganismer, s.k. patogener. Immunförsvaret består av dels det medfödda immunförsvaret, som reagerar snabbt genom att känna igen karaktäristiska strukturer på mikroorganismer, samt det adaptiva immunförsvaret som kan känna igen en enorm variation av strukturer och generera immunologiska minnesceller som är viktiga vid reinfektioner. För aktivering av det adaptiva immunsystemet krävs interaktion med antigen-presenterande celler. De bästa antigen-presenterande cellerna är dendritcellerna. De är lokaliserade vid slemhinnor där de fångar patogener som de transporterar till lymfnoderna. Patogenerna bryts ner till antigen som presenteras för T-celler ur det adaptiva immunförsvaret. T-celler kan bl.a. döda infekterade celler och hjälpa B-celler att producera antikroppar. Vid vaccinering ger man immunförsvaret ett antigen att känna igen tillsammans med ett adjuvans, som är ett ämne som förstärker effekten och hållbarheten i ett immunförsvar. Denna avhandling behandlar vilken roll dendritceller har under immunsvar inducerat med olika adjuvans.

Vi har undersökt var ett antigen bör uttryckas av en cell för att stimulera ett immunförsvar på bästa sätt. För att studera detta har vi använt adenovirus vaccin vektorer. Dessa virus kan infektera celler och få dem att uttrycka gener som virusvektorn bär på, vilket leder till produktion av ett visst antigen. På detta sätt kunde vi styra lokaliseringen av antigenet till cytoplasman, cellmembranet eller till utsidan av cellen som ett extracellulärt lösligt antigen. Vi upptäckte att det intracellulära antigenet inte klarade av att stimulera bildandet av cytotoxiska $CD8^+$ T-celler som kan döda infekterade celler. Inte heller stimulerades bildandet av IgG-antikroppar i blodet. Vi såg även att dendritceller var nödvändiga för att generera ett immunsvar med hjälp av dessa virusvektorer. I möss där alla dendritceller slagits ut kunde varken T-celler aktiveras eller antikroppar produceras. Däremot visar vi att virusvektorerna inte behöver infektera dendritcellerna direkt, utan de kan bli aktiverade genom att plocka upp antigen från andra infekterade celler.

Koleratoxin (CT) är ett av de mest kraftfulla toxiner som finns och kan orsaka livshotande diarréer. CT har även en stark adjuvanseffekt och genererar ett effektivt immunsvar vid immunisering. Idag känner vi bara till en receptor som CT kan binda till, nämligen GM1, som uttrycks på i stort sett alla celler i kroppen. Exakt hur CT fungerar för att vara ett bra adjuvans är inte känt men man vet att dendritceller är nödvändiga för immunresvaret. Vi har undersökt vilka celler som CT behöver binda till för att fungera som adjuvans, d.v.s. vilka celler som behöver uttrycka GM1 på cellytan. Med hjälp av benmärgstransplantation har vi skapat möss där GM1 saknas på antingen vävnadsceller eller immunceller. Efter immunisering med OVA och CT upptäckte vi att möss där GM1 ej finns på tarmepitelceller klarar att generera ett fullgott antikroppssvar. Däremot, om GM1 saknas på immunceller uteblir i stort sett antikroppssvaret. Vi kunde vidare fastställa att det var specifikt dendritceller som behövde uttrycka GM1 för att kunna aktivera T-celler.

Antikroppar produceras av B-celler som har utvecklats i germinalcentra till plasmaceller. För att B-celler och germinalcentra ska aktiveras behöver de hjälp av follikulära hjälpar T-celler (Tfh celler). Man vet att Tfh-celler i sin tur kan aktiveras av dendritceller men om de är helt ensamma om den funktionen är ännu inte utrett. Vi har sett att i möss utan dendritceller kan inte Tfh-celler aktiveras vid immunisering med låg dos antigen tillsammans med adjuvans. Men, med en hög dos antigen så kunde Tfh-celler aktiveras och germinalcentra bildas även i avsaknad av dendritceller. Detta betyder att även någon annan cell kan utföra aktivering av Tfh-celler. Däremot så kunde inte T-hjälparceller av typ 1 (Th1) aktiveras vilket medförde att IgG2c-antikroppar ej kunde bildas. Detta är en viktig antikropp vid infektion med virus eller bakterier. Dessa resultat visar att någon annan celltyp kan aktivera Tfh-celler och bildandet av germinalcentra vid avsaknad av dendritceller, vid närvaro av en hög koncentration antigen. Däremot är dendritceller helt nödvändiga för aktiveringen av Th1-medierat immunsvar.

Acknowledgements

First of all a big hug and thank you to all my co-workers at the department of Microbiology & Immunology. I enjoy the friendly atmosphere and your company during work and various social events we organize. However, there are a few people who I especially would like to thank:

My supervisor **Ulf**, who with great knowledge, passion for the project and lots of pedagogic skills, has helped me through these years. You are always optimistic and find solutions to the problems. Thank you for believing in me and encouraging me to do things I did not believe I could do.

Bengt, thank you for very good cooperation. I have learned a lot from your expertise in B cells and flow cytometry.

Madde D, I'm grateful to have been collaborating with someone as skilled and methodical as you. Many late nights in the lab and the railway is now quite worn down, but we got our results!

My co-supervisor **Petra,** for all your knowledge in adenovirus and genomics. It was really fun working together with the adeno-project.

Johan, for your assistance with irradiating mice, sorting cells, beer tasting and surfing – stoked! Thank you also for all the challenges in Quizkampen that augmented my procrastination.

Jessica, thanks for trying to establish some order in the lab, it's not easy with me around :) You're constantly at good spirits and like a "lab-sister" to me that I always can talk to (except while pipetting…).

Linda Y, for always being friendly and helpful, assisting me with the FACS, ARIA, mice, antibodies, irradiations, medium, you name it...

Megan, a more organized and methodic person is hard to find. I especially liked our laughing lunches with Jessica. Hope you got your dream wedding!

Yu-Jiann, for helping me with ELISA-assays. I know taking care of those pellets is quite a s**t-job…

Carl-Fredrik, for all the help with my PCR problems.

Jan, for the support as a co-supervisor, your knowledge about cholera toxin and mucosal immunology has been a valuable contribution to my work.

Leif, for nice cooperation and for initiating the adenovirus project.

Nils, for all the interesting discussions and questions, both at journal clubs and pub evenings.

Annelie, my oldest co-worker and a good friend who helped me escape from Volvo. Thanks for getting me to the department! Hoping for many new Kodak-moments to come ;)

Veronica, my trustworthy roomie. It was fun sharing office with you and enormously valuable to have someone to share the dissertation-anxiety with during these cold, dark evenings and week-ends! **Calle**, I anticipate much more watersports (and face-plants) now that we are neighbors.

Maria, It's always festival with you around! Looking forward to the next camping trip in the German mud.

Sofia, for thrillseeking surf and ski-trips! Go green! And I'm especially grateful for that moving-in-party you brought me to ;)

Lotta, for your excellence as game- & movie-night hostess, I like your taste in movies and other more unhealthy things.

The office girls, **Susannah & Josefine** – Thanks for putting up with me and my leaky headphones. It's been quite empty without you guy's lately, hope to get us back together in the office soon.

Linda L & Johan, for all the fun we have together at game evenings, after works and ski trips. I think halländska is a nice language –really! :)

Sara, for sharing pre-dissertation problems and for our fun airport adventures.

Lollo, for your storage of 'lussekatter' that saved me during several dark winter evenings in the lab. Thank you very much!

Margareta and Madeleine, for your knowledge, experience and for making the lab a happy place.

Dubi, for keeping the journal club on track and always being nice and helpful.

Patrik & Stefan – my go-to-guys when I need some good car advice (…or gossip).

Peter & Karin, for fun pub nights, hockey-bockey and surf trips. Time to lay off the Canadian bacon and come back to Sweden!

Carro, for all good times at work, parties and Hemsedal.

Martin, -One of the best actors I know, I hope to see you on PSN in the future.

Madde L, -who saved me from living on the street, thanks for letting me rent your apartment.

The Phd students, for nice movie-nights and after-works (and scientific discussions of course)

Susanne, **Anita & Eva**, for all help with autoclaving, buffer preparation, paperwork, organizing etc. **Tinna**, for sorting my financial problems.

Karin Ahlman, for always being contagiously happy. Your laughs still lingers in the hallways (or maybe we just hear you all the way from Medkem…)

Karin Schön, – I know this is your favorite part so I dedicate it to you! Maybe I'll keep my promise and join your running sessions during spring?

Samuel, for letting me be a part of the saints, it has been great fun!

Karolina & Hans, for your tremendous contribution to the independent film industry.

The EBM crew**, Jenny, Anders, Therés, Katrin, Pernilla** and especially **Pia**, for nice conversations and taking good care of our little furry friends.

Biffen & gymgänget, – You're awesome and motivating! keep up the pump!

Säfflepöjkera, **Glenn, Svenna, Nichlas, Petersén & Marcus**, – Tack för en stabil vänskap, även om vi inte träffas jätteofta nu för tiden känns det alltid som att komma hem när vi ses.

Familjen, **Krille & Acki,** – Mina kära syskon, jag är så glad att jag har er. **Mamma & Pappa** – som alltid finns där för mig oavsett vad jag engagerar mig i. Även om ni inte förstår allt kanske denna boken ger en viss insikt i vad jag har jobbat med under alla år. Tack för all uppmuntran.

Kristin, – Ord kan inte beskriva vad du betyder för mig. Du har varit så otroligt tålmodig och stöttande, speciellt under skrivtiden -min livlina till verkligheten. Jag älskar dig så mycket!

This work was supported with grants from The Swedish Research Council, Swedish Foundation for Strategic Research, through its support of the Mucosal Immunobiology and Vaccine Center; Jeanssson Foundation, Åke Wiberg Foundation, Clas Grochinsky Foundation, Magnus Bergvall Foundation, Goljes Foundation, Willhelm and Martina Lundgren Foundation, and the Royal Arts and Society of Arts and Science in Göteborg.

References

[1] Behbehani AM. The smallpox story: life and death of an old disease. Microbiol. Rev. 1983;47:455–509.

[2] Lombard M, Pastoret PP, Moulin AM. A brief history of vaccines and vaccination. Rev - Off Int Epizoot 2007;26:29–48.

[3] Zepp F. Principles of vaccine design-Lessons from nature. Vaccine 2010;28 Suppl 3:C14–24.

[4] Ulmer JB, Valley U, Rappuoli R. Vaccine manufacturing: challenges and solutions. Nat. Biotechnol. 2006;24:1377–83.

[5] Minor P. Vaccine-derived poliovirus (VDPV): Impact on poliomyelitis eradication. Vaccine 2009;27:2649–52.

[6] Rueckert C, Guzmán CA. Vaccines: from empirical development to rational design. PLoS Pathog 2012;8:e1003001.

[7] Pulendran B, Ahmed R. Immunological mechanisms of vaccination. Nat Immunol 2011;12:509–17.

[8] Donnelly JJ, Wahren B, Liu MA. DNA vaccines: progress and challenges. J Immunol 2005;175:633–9.

[9] Schirmbeck R, Deml L, Melber K, Wolf H, Wagner R, Reimann J. Priming of class I-restricted cytotoxic T lymphocytes by vaccination with recombinant protein antigens. Vaccine 1995;13:857–65.

[10] Harris SJ, Woodrow SA, Gearing AJ, Adams SE, Kingsman AJ, Layton GT. The effects of adjuvants on CTL induction by V3:Ty-virus-like particles (V3-VLPs) in mice. Vaccine 1996;14:971–6.

[11] Centers for Disease Control and Prevention (CDC). FDA licensure of bivalent human papillomavirus vaccine (HPV2, Cervarix) for use in females and updated HPV vaccination recommendations from the Advisory Committee on Immunization Practices (ACIP). MMWR Morb. Mortal. Wkly. Rep. 2010;59:626–9.

[12] Salvador A, Igartua M, Hernández RM, Pedraz JL. An overview on the field of micro- and nanotechnologies for synthetic Peptide-based vaccines. J Drug Deliv 2011;2011:181646.

[13] Hoke CH, Snyder CE. History of the restoration of adenovirus type 4 and type 7 vaccine, live oral (Adenovirus Vaccine) in the context of the Department of Defense acquisition system. Vaccine 2013.

[14] Russell KL, Hawksworth AW, Ryan MAK, Strickler J, Irvine M, Hansen CJ, Gray GC, Gaydos JC. Vaccine-preventable adenoviral respiratory illness in US military recruits, 1999-2004. Vaccine 2006;24:2835–42.

[15] Sharma A, Li X, Bangari DS, Mittal SK. Adenovirus receptors and their implications in gene delivery. Virus Res 2009;143:184–94.

[16] Sorensen MR, Holst PJ, Pircher H, Christensen JP, Thomsen AR. Vaccination with an adenoviral vector encoding the tumor antigen directly linked to invariant chain induces potent CD4(+) T-cell-independent CD8(+) T-cell-mediated tumor control. Eur J Immunol 2009;39:2725–36.

[17] Choi J-W, Lee J-S, Kim SW, Yun C-O. Evolution of oncolytic adenovirus for cancer treatment. Adv Drug Deliv Rev 2012;64:720–9.

[18] Seregin SS, Amalfitano A. Improving Adenovirus Based Gene Transfer: Strategies to Accomplish Immune Evasion. Viruses 2010;2:2013–36.

[19] Santosuosso M, McCormick S, Xing Z. Adenoviral vectors for mucosal vaccination against infectious diseases. Viral Immunol 2005;18:283–91.

[20] Zsengellér Z, Otake K, Hossain SA, Berclaz PY, Trapnell BC. Internalization of

adenovirus by alveolar macrophages initiates early proinflammatory signaling during acute respiratory tract infection. J Virol 2000;74:9655–67.

[21] Worgall S, Wolff G, Falck-Pedersen E, Crystal RG. Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. Hum Gene Ther 1997;8:37–44.

[22] Gallichan WS, Rosenthal KL. Long-lived cytotoxic T lymphocyte memory in mucosal tissues after mucosal but not systemic immunization. J Exp Med 1996;184:1879–90.

[23] Wang J, Thorson L, Stokes RW, Santosuosso M, Huygen K, Zganiacz A, Hitt M, Xing Z. Single mucosal, but not parenteral, immunization with recombinant adenoviral-based vaccine provides potent protection from pulmonary tuberculosis. J Immunol 2004;173:6357–65.

[24] Patel A, Zhang Y, Croyle M, Tran K, Gray M, Strong J, Feldmann H, Wilson JM, Kobinger GP. Mucosal delivery of adenovirus-based vaccine protects against Ebola virus infection in mice. J Infect Dis 2007;196 Suppl 2:S413–20.

[25] Yu J-R, Kim S, Lee J-B, Chang J. Single intranasal immunization with recombinant adenovirus-based vaccine induces protective immunity against respiratory syncytial virus infection. J Virol 2008;82:2350–7.

[26] Zhu Q, Thomson CW, Rosenthal KL, McDermott MR, Collins SM, Gauldie J. Immunization with adenovirus at the large intestinal mucosa as an effective vaccination strategy against sexually transmitted viral infection. Mucosal Immunol 2008;1:78–88.

[27] Xu Q, Pichichero ME, Simpson LL, Elias M, Smith LA, Zeng M. An adenoviral vector-based mucosal vaccine is effective in protection against botulism. Gene Ther 2009;16:367–75.

[28] Pandey A, Singh N, Vemula SV, Couëtil L, Katz JM, Donis R, Sambhara S, Mittal SK. Impact of Preexisting Adenovirus Vector Immunity on Immunogenicity and Protection Conferred with an Adenovirus-Based H5N1 Influenza Vaccine. PLoS ONE 2012;7:e33428.

[29] Serre K, Mohr E, Toellner K-M, Cunningham AF, Granjeaud S, Bird R, Maclennan ICM. Molecular differences between the divergent responses of ovalbumin-specific CD4 T cells to alum-precipitated ovalbumin compared to ovalbumin expressed by Salmonella. Mol. Immunol. 2008;45:3558–66.

[30] Kool M, Soullié T, van Nimwegen M, Willart MAM, Muskens F, Jung S, Hoogsteden HC, Hammad H, Lambrecht BN. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. Journal of Experimental Medicine 2008;205:869–82.

[31] Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. Nat Rev Immunol 2009;9:287–93.

[32] Hunter RL. Overview of vaccine adjuvants: present and future. Vaccine 2002;20 Suppl 3:S7–12.

[33] Lambrecht BN, Kool M, Willart MAM, Hammad H. Mechanism of action of clinically approved adjuvants. Curr Opin Immunol 2009;21:23–9.

[34] Gavin AL, Hoebe K, Duong B, Ota T, Martin C, Beutler B, Nemazee D. Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. Science 2006;314:1936–8.

[35] Kool M, Pétrilli V, De Smedt T, Rolaz A, Hammad H, van Nimwegen M, Bergen IM, Castillo R, Lambrecht BN, Tschopp J. Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. The Journal of Immunology 2008;181:3755–9.

[36] Seubert A, Monaci E, Pizza M, O'Hagan DT, Wack A. The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. J Immunol 2008;180:5402–12.

[37] Mosca F, Tritto E, Muzzi A, Monaci E, Bagnoli F, Iavarone C, O'Hagan D, Rappuoli R, De Gregorio E. Molecular and cellular signatures of human vaccine adjuvants.

Proc Natl Acad Sci U S A 2008;105:10501–6.

[38] O'Hagan DT, Wack A, Podda A. MF59 is a safe and potent vaccine adjuvant for flu vaccines in humans: what did we learn during its development? Clin. Pharmacol. Ther. 2007;82:740–4.

[39] Dupuis M, Murphy TJ, Higgins D, Ugozzoli M, van Nest G, Ott G, McDonald DM. Dendritic cells internalize vaccine adjuvant after intramuscular injection. Cell Immunol 1998;186:18–27.

[40] Alving CR, Peachman KK, Rao M, Reed SG. Adjuvants for human vaccines. Curr Opin Immunol 2012;24:310–5.

[41] De Becker G, Moulin V, Pajak B, Bruck C, Francotte M, Thiriart C, Urbain J, Moser M. The adjuvant monophosphoryl lipid A increases the function of antigen-presenting cells. Int Immunol 2000;12:807–15.

[42] De Smedt T, Pajak B, Muraille E, Lespagnard L, Heinen E, De Baetselier P, Urbain J, Leo O, Moser M. Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. J Exp Med 1996;184:1413–24.

[43] Trumpfheller C, Caskey M, Nchinda G, Longhi MP, Mizenina O, Huang Y, Schlesinger SJ, Colonna M, Steinman RM. The microbial mimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine. Proc Natl Acad Sci U S A 2008;105:2574–9.

[44] Longhi MP, Trumpfheller C, Idoyaga J, Caskey M, Matos I, Kluger C, Salazar AM, Colonna M, Steinman RM. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. Journal of Experimental Medicine 2009;206:1589–602.

[45] Tewari K, Flynn BJ, Boscardin SB, Kastenmueller K, Salazar AM, Anderson CA, Soundarapandian V, Ahumada A, Keler T, Hoffman SL, Nussenzweig MC, Steinman RM, Seder RA. Poly(I:C) is an effective adjuvant for antibody and multi-functional CD4+ T cell responses to Plasmodium falciparum circumsporozoite protein (CSP) and αDEC-CSP in non human primates. Vaccine 2010;28:7256–66.

[46] Meylan E, Tschopp J. Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. Mol. Cell 2006;22:561–9.

[47] Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh C-S, Reis e Sousa C, Matsuura Y, Fujita T, Akira S. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 2006;441:101–5.

[48] Lindenmann J, BURKE DC, ISAACS A. Studies on the production, mode of action and properties of interferon. Br J Exp Pathol 1957;38:551–62.

[49] Schoggins JW, Rice CM. Interferon-stimulated genes and their antiviral effector functions. Current Opinion in Virology 2011;1:519–25.

[50] Brinkmann V, Geiger T, Alkan S, Heusser CH. Interferon alpha increases the frequency of interferon gamma-producing human CD4+ T cells. J Exp Med 1993;178:1655–63.

[51] Wenner CA, Güler ML, Macatonia SE, O'Garra A, Murphy KM. Roles of IFN-gamma and IFN-alpha in IL-12-induced T helper cell-1 development. J Immunol 1996;156:1442–7.

[52] Le Bon A, Etchart N, Rossmann C, Ashton M, Hou S, Gewert D, Borrow P, Tough DF. Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. Nat Immunol 2003;4:1009–15.

[53] Cucak H, Yrlid U, Reizis B, Kalinke U, Johansson-Lindbom B. Type I Interferon Signaling in Dendritic Cells Stimulates the Development of Lymph-Node-Resident T Follicular Helper Cells. Immunity 2009;31:491–501.

[54] Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005;11:S45–53.

[55] Woodrow KA, Bennett KM, Lo DD. Mucosal vaccine design and delivery. Annu. Rev. Biomed. Eng. 2012;14:17–46.

[56] Lycke N. Recent progress in mucosal vaccine development: potential and limitations.

Nat Rev Immunol 2012;12:592–605.

[57] Elson CO, Ealding W. Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. J Immunol 1984;132:2736–41.

[58] Raghavan S, Ostberg AK, Flach C-F, Ekman A, Blomquist M, Czerkinsky C, Holmgren J. Sublingual immunization protects against Helicobacter pylori infection and induces T and B cell responses in the stomach. Infect Immun 2010;78:4251–60.

[59] Hörnquist E, Lycke N. Cholera toxin adjuvant greatly promotes antigen priming of T cells. Eur J Immunol 1993;23:2136–43.

[60] Watanabe I, Hagiwara Y, Kadowaki S-E, Yoshikawa T, Komase K, Aizawa C, Kiyono H, Takeda Y, Mcghee JR, Chiba J, Sata T, Kurata T, Tamura S-I. Characterization of protective immune responses induced by nasal influenza vaccine containing mutant cholera toxin as a safe adjuvant (CT112K). Vaccine 2002;20:3443–55.

[61] Holmgren J, Adamsson J, Anjuère F, Clemens J, Czerkinsky C, Eriksson K, Flach C-F, George-Chandy A, Harandi AM, Lebens M, Lehner T, Lindblad M, Nygren E, Raghavan S, Sanchez J, Stanford M, Sun J-B, Svennerholm A-M, Tengvall S. Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA. Immunol Lett 2005;97:181–8.

[62] Merritt EA, Hol WG. AB5 toxins. Curr Opin Struct Biol 1995;5:165–71.

[63] Holmgren J, Lönnroth I, Svennerholm L. Tissue receptor for cholera exotoxin: postulated structure from studies with GM1 ganglioside and related glycolipids. Infect Immun 1973;8:208–14.

[64] Teneberg S, Hirst TR, Angström J, Karlsson KA. Comparison of the glycolipid-binding specificities of cholera toxin and porcine Escherichia coli heat-labile enterotoxin: identification of a receptor-active non-ganglioside glycolipid for the heat-labile toxin in infant rabbit small intestine. Glycoconj. J. 1994;11:533–40.

[65] Paton JC, Paton AW. Pathogenesis and diagnosis of Shiga toxin-producing Escherichia coli infections. Clin. Microbiol. Rev. 1998;11:450–79.

[66] Paton AW, Beddoe T, Thorpe CM, Whisstock JC, Wilce MCJ, Rossjohn J, Talbot UM, Paton JC. AB5 subtilase cytotoxin inactivates the endoplasmic reticulum chaperone BiP. Nature 2006;443:548–52.

[67] Agren LC, Ekman L, Löwenadler B, Lycke NY. Genetically engineered nontoxic vaccine adjuvant that combines B cell targeting with immunomodulation by cholera toxin A1 subunit. J Immunol 1997;158:3936–46.

[68] Mattsson J, Yrlid U, Stensson A, Schön K, Karlsson MCI, Ravetch JV, Lycke NY. Complement activation and complement receptors on follicular dendritic cells are critical for the function of a targeted adjuvant. The Journal of Immunology 2011;187:3641–52.

[69] Norton EB, Lawson LB, Freytag LC, Clements JD. Characterization of a mutant Escherichia coli heat-labile toxin, LT(R192G/L211A), as a safe and effective oral adjuvant. Clin. Vaccine Immunol. 2011;18:546–51.

[70] Summerton NA, Welch RW, Bondoc L, Yang H-H, Pleune B, Ramachandran N, Harris AM, Bland D, Jackson WJ, Park S, Clements JD, Nabors GS. Toward the development of a stable, freeze-dried formulation of Helicobacter pylori killed whole cell vaccine adjuvanted with a novel mutant of Escherichia coli heat-labile toxin. Vaccine 2010;28:1404–11.

[71] Leach S, Clements JD, Kaim J, Lundgren A. The Adjuvant Double Mutant Escherichia coli Heat Labile Toxin Enhances IL-17A Production in Human T Cells Specific for Bacterial Vaccine Antigens. PLoS ONE 2012;7:e51718.

[72] Lu Y-J, Yadav P, Clements JD, Forte S, Srivastava A, Thompson CM, Seid R, Look J, Alderson M, Tate A, Maisonneuve J-F, Robertson G, Anderson PW, Malley R. Options for inactivation, adjuvant, and route of topical administration of a killed, unencapsulated pneumococcal whole-cell vaccine. Clin. Vaccine Immunol. 2010;17:1005–12.

[73] Kawamura YI, Kawashima R, Shirai Y, Kato R, Hamabata T, Yamamoto M,

Furukawa K, Fujihashi K, Mcghee JR, Hayashi H, Dohi T. Cholera toxin activates dendritic cells through dependence on GM1-ganglioside which is mediated by NF-kappaB translocation. Eur J Immunol 2003;33:3205–12.

[74] Rappuoli R, Pizza M, Douce G, Dougan G. Structure and mucosal adjuvanticity of cholera and Escherichia coli heat-labile enterotoxins. Immunol. Today 1999;20:493–500.

[75] Hazes B, Read RJ. Accumulating evidence suggests that several AB-toxins subvert the endoplasmic reticulum-associated protein degradation pathway to enter target cells. Biochemistry 1997;36:11051–4.

[76] Lencer WI, Hirst TR, Holmes RK. Membrane traffic and the cellular uptake of cholera toxin. Biochim Biophys Acta 1999;1450:177–90.

[77] Vanden Broeck D, Horvath C, De Wolf MJS. Vibrio cholerae: cholera toxin. Int J Biochem Cell Biol 2007;39:1771–5.

[78] Levine MM, Kaper JB, Black RE, Clements ML. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. Microbiol. Rev. 1983;47:510–50.

[79] Lavelle EC, McNeela E, Armstrong ME, Leavy O, Higgins SC, Mills KHG. Cholera toxin promotes the induction of regulatory T cells specific for bystander antigens by modulating dendritic cell activation. J Immunol 2003;171:2384–92.

[80] Olvera-Gomez I, Hamilton SE, Xiao Z, Guimaraes CP, Ploegh HL, Hogquist KA, Wang L, Jameson SC. Cholera toxin activates nonconventional adjuvant pathways that induce protective CD8 T-cell responses after epicutaneous vaccination. Proc Natl Acad Sci U S A 2012;109:2072–7.

[81] Lycke N, Eriksen L, Holmgren J. Protection against cholera toxin after oral immunization is thymus-dependent and associated with intestinal production of neutralizing IgA antitoxin. Scand J Immunol 1987;25:413–9.

[82] Luci C, Hervouet C, Rousseau D, Holmgren J, Czerkinsky C, Anjuère F. Dendritic cell-mediated induction of mucosal cytotoxic responses following intravaginal immunization with the nontoxic B subunit of cholera toxin. J Immunol 2006;176:2749–57.

[83] Lycke N, Eriksen L, Holmgren J. Protection against cholera toxin after oral immunization is thymus-dependent and associated with intestinal production of neutralizing IgA antitoxin. Scand J Immunol 1987;25:413–9.

[84] Lange S, Holmgren J. Protective antitoxic cholera immunity in mice: influence of route and number of immunizations and mode of action of protective antibodies. Acta Pathol Microbiol Scand C 1978;86C:145–52.

[85] Krautler NJ, Kana V, Kranich J, Tian Y, Perera D, Lemm D, Schwarz P, Armulik A, Browning JL, Tallquist M, Buch T, Oliveira-Martins JB, Zhu C, Hermann M, Wagner U, Brink R, Heikenwalder M, Aguzzi A. Follicular dendritic cells emerge from ubiquitous perivascular precursors. Cell 2012;150:194–206.

[86] Gonzalez SF, Lukacs-Kornek V, Kuligowski MP, Pitcher LA, Degn SE, Turley SJ, Carroll MC. Complement-dependent transport of antigen into B cell follicles. The Journal of Immunology 2010;185:2659–64.

[87] Allen CDC, Cyster JG. Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function. Semin. Immunol. 2008;20:14–25.

[88] Junt T, Scandella E, Ludewig B. Form follows function: lymphoid tissue microarchitecture in antimicrobial immune defence. Nat Rev Immunol 2008;8:764–75.

[89] Pape KA, Catron DM, Itano AA, Jenkins MK. The humoral immune response is initiated in lymph nodes by B cells that acquire soluble antigen directly in the follicles. Immunity 2007;26:491–502.

[90] Carrasco YR, Batista FD. B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. Immunity 2007;27:160–71.

[91] Mebius RE, Kraal G. Structure and function of the spleen. Nat Rev Immunol

2005;5:606–16.
[92] Cerutti A, Cols M, Puga I. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. Nat Rev Immunol 2013;13:118–32.
[93] Song H, Cerny J. Functional heterogeneity of marginal zone B cells revealed by their ability to generate both early antibody-forming cells and germinal centers with hypermutation and memory in response to a T-dependent antigen. J Exp Med 2003;198:1923–35.
[94] Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. Immunity 2001;14:617–29.
[95] Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. J Exp Med 1974;139:380–97.
[96] Nussenzweig MC, Steinman RM, Gutchinov B, Cohn ZA. Dendritic cells are accessory cells for the development of anti-trinitrophenyl cytotoxic T lymphocytes. J Exp Med 1980;152:1070–84.
[97] Steinman RM, Inaba K, Turley S, Pierre P, Mellman I. Antigen capture, processing, and presentation by dendritic cells: recent cell biological studies. Hum. Immunol. 1999;60:562–7.
[98] Itano AA, Jenkins MK. Antigen presentation to naive CD4 T cells in the lymph node. Nat Immunol 2003;4:733–9.
[99] Shah JA, Darrah PA, Ambrozak DR, Turon TN, Mendez S, Kirman J, Wu C-Y, Glaichenhaus N, Seder RA. Dendritic cells are responsible for the capacity of CpG oligodeoxynucleotides to act as an adjuvant for protective vaccine immunity against Leishmania major in mice. J Exp Med 2003;198:281–91.
[100] Fujii S-I, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. J Exp Med 2003;198:267–79.
[101] Bates JT, Uematsu S, Akira S, Mizel SB. Direct stimulation of tlr5+/+ CD11c+ cells is necessary for the adjuvant activity of flagellin. J Immunol 2009;182:7539–47.
[102] Geissmann F, Gordon S, Hume DA, Mowat AM, Randolph GJ. Unravelling mononuclear phagocyte heterogeneity. Nat Rev Immunol 2010;10:453–60.
[103] Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. Science 2010;327:656–61.
[104] Waskow C, Liu K, Darrasse-Jeze G, Guermonprez P, Ginhoux F, Merad M, Shengelia T, Yao K, Nussenzweig M. The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. Nat Immunol 2008;9:676–83.
[105] Merad M, Manz MG. Dendritic cell homeostasis. Blood 2009;113:3418–27.
[106] Liu K, Victora GD, Schwickert TA, Guermonprez P, Meredith MM, Yao K, Chu F-F, Randolph GJ, Rudensky AY, Nussenzweig M. In vivo analysis of dendritic cell development and homeostasis. Science 2009;324:392–7.
[107] Bogunovic M, Ginhoux F, Helft J, Shang L, Hashimoto D, Greter M, Liu K, Jakubzick C, Ingersoll MA, Leboeuf M, Stanley ER, Nussenzweig M, Lira SA, Randolph GJ, Merad M. Origin of the lamina propria dendritic cell network. Immunity 2009;31:513–25.
[108] Varol C, Vallon-Eberhard A, Elinav E, Aychek T, Shapira Y, Luche H, Fehling HJ, Hardt W-D, Shakhar G, Jung S. Intestinal Lamina Propria Dendritic Cell Subsets Have Different Origin and Functions. Immunity 2009:1–11.
[109] Satpathy AT, Wu X, Albring JC, Murphy KM. Re(de)fining the dendritic cell lineage. Nat Immunol 2012;13:1145–54.
[110] Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245–52.
[111] Weber M, Hauschild R, Schwarz J, Moussion C, de Vries I, Legler DF, Luther SA, Bollenbach T, Sixt M. Interstitial dendritic cell guidance by haptotactic chemokine

gradients. Science 2013;339:328–32.
[112] Haan den JM, Lehar SM, Bevan MJ. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. J Exp Med 2000;192:1685–96.
[113] Pooley JL, Heath WR, Shortman K. Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells. J Immunol 2001;166:5327–30.
[114] Kamphorst AO, Guermonprez P, Dudziak D, Nussenzweig MC. Route of antigen uptake differentially impacts presentation by dendritic cells and activated monocytes. The Journal of Immunology 2010;185:3426–35.
[115] Pulendran B, Smith JL, Caspary G, Brasel K, Pettit D, Maraskovsky E, Maliszewski CR. Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. Proc Natl Acad Sci USA 1999;96:1036–41.
[116] Soares H, Waechter H, Glaichenhaus N, Mougneau E, Yagita H, Mizenina O, Dudziak D, Nussenzweig MC, Steinman RM. A subset of dendritic cells induces CD4+ T cells to produce IFN-gamma by an IL-12-independent but CD70-dependent mechanism in vivo. J Exp Med 2007;204:1095–106.
[117] Maldonado-López R, De Smedt T, Pajak B, Heirman C, Thielemans K, Leo O, Urbain J, Maliszewski CR, Moser M. Role of CD8alpha+ and CD8alpha- dendritic cells in the induction of primary immune responses in vivo. J Leukoc Biol 1999;66:242–6.
[118] Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, Berg PL, Davidsson T, Powrie F, Johansson-Lindbom B, Agace WW. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. Journal of Experimental Medicine 2008;205:2139–49.
[119] Cerovic V, Houston SA, Scott CL, Aumeunier A, Yrlid U, Mowat AM, Milling SWF. Intestinal CD103(-) dendritic cells migrate in lymph and prime effector T cells. Mucosal Immunol 2013;6:104–13.
[120] Edelson BT, KC W, Juang R, Kohyama M, Benoit LA, Klekotka PA, Moon C, Albring JC, Ise W, Michael DG, Bhattacharya D, Stappenbeck TS, Holtzman MJ, Sung S-SJ, Murphy TL, Hildner K, Murphy KM. Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. Journal of Experimental Medicine 2010;207:823–36.
[121] Schiavoni G, Mattei F, Sestili P, Borghi P, Venditti M, Morse HC, Belardelli F, Gabriele L. ICSBP is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8alpha(+) dendritic cells. J Exp Med 2002;196:1415–25.
[122] Collin M, Bigley V, Haniffa M, Hambleton S. Human dendritic cell deficiency: the missing ID? Nat Rev Immunol 2011;11:575–83.
[123] Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP, Ricciardi-Castagnoli P. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol 2001;2:361–7.
[124] Schulz O, Jaensson E, Persson EK, Liu X, Worbs T, Agace WW, Pabst O. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. Journal of Experimental Medicine 2009;206:3101–14.
[125] Johansson-Lindbom B, Svensson M, Pabst O, Palmqvist C, Marquez G, Förster R, Agace WW. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. J Exp Med 2005;202:1063–73.
[126] Stagg AJ, Kamm MA, Knight SC. Intestinal dendritic cells increase T cell expression of alpha4beta7 integrin. Eur J Immunol 2002;32:1445–54.
[127] Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Rosemblatt M, Andrian Von UH. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. Nature 2003;424:88–93.
[128] Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song S-Y. Retinoic acid

imprints gut-homing specificity on T cells. Immunity 2004;21:527–38.
[129] Hammerschmidt SI, Friedrichsen M, Boelter J, Lyszkiewicz M, Kremmer E, Pabst O, Förster R. Retinoic acid induces homing of protective T and B cells to the gut after subcutaneous immunization in mice. J. Clin. Invest. 2011;121:3051–61.
[130] Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science 2004;303:1662–5.
[131] Huang FP, Platt N, Wykes M, Major JR, Powell TJ, Jenkins CD, MacPherson GG. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. J Exp Med 2000;191:435–44.
[132] Reizis B, Colonna M, Trinchieri G, Barrat F, Gilliet M. Plasmacytoid dendritic cells: one-trick ponies or workhorses of the immune system? Nat Rev Immunol 2011;11:558–65.
[133] Sapoznikov A, Fischer JAA, Zaft T, Krauthgamer R, Dzionek A, Jung S. Organ-dependent in vivo priming of naive CD4+, but not CD8+, T cells by plasmacytoid dendritic cells. J Exp Med 2007;204:1923–33.
[134] Kanitakis J, Morelon E, Petruzzo P, Badet L, Dubernard J-M. Self-renewal capacity of human epidermal Langerhans cells: observations made on a composite tissue allograft. Exp. Dermatol. 2011;20:145–6.
[135] Mowat AM, Bain CC. Mucosal macrophages in intestinal homeostasis and inflammation. J Innate Immun 2011;3:550–64.
[136] Mellman I, Turley SJ, Steinman RM. Antigen processing for amateurs and professionals. Trends Cell Biol. 1998;8:231–7.
[137] Watts C. Capture and processing of exogenous antigens for presentation on MHC molecules. Annu Rev Immunol 1997;15:821–50.
[138] Trombetta ES, Mellman I. Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol 2005;23:975–1028.
[139] Shen L, Sigal LJ, Boes M, Rock KL. Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. Immunity 2004;21:155–65.
[140] Jung S, Unutmaz D, Wong P, Sano G-I, De los Santos K, Sparwasser T, Wu S, Vuthoori S, Ko K, Zavala F, Pamer EG, Littman DR, Lang RA. In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. Immunity 2002;17:211–20.
[141] Rojo JM, Bello R, Portolés P. T-Cell Receptor. Advances in Experimental Medicine and Biology, vol. 640, New York, NY: Springer New York; 2008, pp. 1–11.
[142] Janeway CA, Bottomly K. Signals and signs for lymphocyte responses. Cell 1994;76:275–85.
[143] Chowdhury D, Lieberman J. Death by a thousand cuts: granzyme pathways of programmed cell death. Annu Rev Immunol 2008;26:389–420.
[144] Knickelbein JE, Khanna KM, Yee MB, Baty CJ, Kinchington PR, Hendricks RL. Noncytotoxic lytic granule-mediated CD8+ T cell inhibition of HSV-1 reactivation from neuronal latency. Science 2008;322:268–71.
[145] Bartee E, Mohamed MR, McFadden G. Tumor necrosis factor and interferon: cytokines in harmony. Curr. Opin. Microbiol. 2008;11:378–83.
[146] Buller RM, Holmes KL, Hügin A, Frederickson TN, Morse HC. Induction of cytotoxic T-cell responses in vivo in the absence of CD4 helper cells. Nature 1987;328:77–9.
[147] Janssen EM, Lemmens EE, Wolfe T, Christen U, Herrath von MG, Schoenberger SP. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. Nature 2003;421:852–6.
[148] Bennett SR, Carbone FR, Karamalis F, Miller JF, Heath WR. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. J Exp Med 1997;186:65–70.
[149] Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature 1998;393:478–80.

[150] Jenkins MK, Taylor PS, Norton SD, Urdahl KB. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. J Immunol 1991;147:2461–6.

[151] Schwartz RH. A cell culture model for T lymphocyte clonal anergy. Science 1990;248:1349–56.

[152] Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). Annu Rev Immunol 2010;28:445–89.

[153] Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science 1993;260:547–9.

[154] Szabo SJ, Sullivan BM, Stemmann C, Satoskar AR, Sleckman BP, Glimcher LH. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. Science 2002;295:338–42.

[155] Mullen AC, High FA, Hutchins AS, Lee HW, Villarino AV, Livingston DM, Kung AL, Cereb N, Yao TP, Yang SY, Reiner SL. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. Science 2001;292:1907–10.

[156] Kaplan MH, Sun YL, Hoey T, Grusby MJ. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. Nature 1996;382:174–7.

[157] Maizels RM, Pearce EJ, Artis D, Yazdanbakhsh M, Wynn TA. Regulation of pathogenesis and immunity in helminth infections. Journal of Experimental Medicine 2009;206:2059–66.

[158] Zelante T, Iannitti R, De Luca A, Romani L. IL-22 in antifungal immunity. Eur J Immunol 2011;41:270–5.

[159] Infante-Duarte C, Horton HF, Byrne MC, Kamradt T. Microbial lipopeptides induce the production of IL-17 in Th cells. J Immunol 2000;165:6107–15.

[160] Leibundgut-Landmann S, Gross O, Robinson MJ, Osorio F, Slack EC, Tsoni SV, Schweighoffer E, Tybulewicz V, Brown GD, Ruland J, Reis e Sousa C. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. Nat Immunol 2007;8:630–8.

[161] Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 2006;126:1121–33.

[162] Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, Levy DE, Leonard WJ, Littman DR. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol 2007;8:967–74.

[163] Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med 2005;201:233–40.

[164] Le Gros G, Ben-Sasson SZ, Seder R, Finkelman FD, Paul WE. Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. J Exp Med 1990;172:921–9.

[165] Akiba H, Miyahira Y, Atsuta M, Takeda K, Nohara C, Futagawa T, Matsuda H, Aoki T, Yagita H, Okumura K. Critical contribution of OX40 ligand to T helper cell type 2 differentiation in experimental leishmaniasis. J Exp Med 2000;191:375–80.

[166] Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997;389:737–42.

[167] Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev 2006;212:28–50.

[168] Tangye SG, Tarlinton DM. Memory B cells: effectors of long-lived immune responses. Eur J Immunol 2009;39:2065–75.

[169] Ansel KM, McHeyzer-Williams LJ, Ngo VN, McHeyzer-Williams MG, Cyster JG. In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. J Exp Med 1999;190:1123–34.

[170] Goodnow CC, Vinuesa CG, Randall KL, Mackay F, Brink R. Control systems and decision making for antibody production. Nat Immunol 2010;11:681–8.

[171] Haynes NM, Allen CDC, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. J Immunol 2007;179:5099–108.

[172] Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, Dent AL, Craft J, Crotty S. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science 2009;325:1006–10.

[173] Zaretsky AG, Taylor JJ, King IL, Marshall FA, Mohrs M, Pearce EJ. T follicular helper cells differentiate from Th2 cells in response to helminth antigens. Journal of Experimental Medicine 2009;206:991–9.

[174] Goenka R, Barnett LG, Silver JS, O'Neill PJ, Hunter CA, Cancro MP, Laufer TM. Cutting edge: dendritic cell-restricted antigen presentation initiates the follicular helper T cell program but cannot complete ultimate effector differentiation. The Journal of Immunology 2011;187:1091–5.

[175] Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, Kroczek RA. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature 1999;397:263–6.

[176] Mak TW, Shahinian A, Yoshinaga SK, Wakeham A, Boucher L-M, Pintilie M, Duncan G, Gajewska BU, Gronski M, Eriksson U, Odermatt B, Ho A, Bouchard D, Whorisky JS, Jordana M, Ohashi PS, Pawson T, Bladt F, Tafuri A. Costimulation through the inducible costimulator ligand is essential for both T helper and B cell functions in T cell-dependent B cell responses. Nat Immunol 2003;4:765–72.

[177] Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, Lao C, Crotty S. ICOS Receptor Instructs T Follicular Helper Cell versus Effector Cell Differentiation via Induction of the Transcriptional Repressor Bcl6. Immunity 2011;34:932–46.

[178] Cannons JL, Qi H, Lu KT, Dutta M, Gomez-Rodriguez J, Cheng J, Wakeland EK, Germain RN, Schwartzberg PL. Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. Immunity 2010;32:253–65.

[179] Qi H. From SAP-less T cells to helpless B cells and back: dynamic T-B cell interactions underlie germinal center development and function. Immunol Rev 2012;247:24–35.

[180] Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal centre formation. Nature 2008;455:764–9.

[181] Yusuf I, Kageyama R, Monticelli L, Johnston RJ, DiToro D, Hansen K, Barnett B, Crotty S. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). The Journal of Immunology 2010;185:190–202.

[182] Kawabe T, Naka T, Yoshida K, Tanaka T, Fujiwara H, Suematsu S, Yoshida N, Kishimoto T, Kikutani H. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. Immunity 1994;1:167–78.

[183] Xu J, Foy TM, Laman JD, Elliott EA, Dunn JJ, Waldschmidt TJ, Elsemore J, Noelle RJ, Flavell RA. Mice deficient for the CD40 ligand. Immunity 1994;1:423–31.

[184] Takahashi Y, Ohta H, Takemori T. Fas is required for clonal selection in germinal centers and the subsequent establishment of the memory B cell repertoire. Immunity 2001;14:181–92.

[185] Liu YJ, Mason DY, Johnson GD, Abbot S, Gregory CD, Hardie DL, Gordon J, MacLennan IC. Germinal center cells express bcl-2 protein after activation by signals which prevent their entry into apoptosis. Eur J Immunol 1991;21:1905–10.

[186] Jourdan M, Caraux A, De Vos J, Fiol G, Larroque M, Cognot C, Bret C, Duperray C, Hose D, Klein B. An in vitro model of differentiation of memory B cells into

plasmablasts and plasma cells including detailed phenotypic and molecular characterization. Blood 2009;114:5173–81.

[187] Arpin C, Déchanet J, Van Kooten C, Merville P, Grouard G, Brière F, Banchereau J, Liu YJ. Generation of memory B cells and plasma cells in vitro. Science 1995;268:720–2.

[188] Crotty S. Follicular helper CD4 T cells (TFH). Annu Rev Immunol 2011;29:621–63.

[189] Ozaki K, Spolski R, Ettinger R, Kim H-P, Wang G, Qi C-F, Hwu P, Shaffer DJ, Akilesh S, Roopenian DC, Morse HC, Lipsky PE, Leonard WJ. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. J Immunol 2004;173:5361–71.

[190] Dienz O, Eaton SM, Bond JP, Neveu W, Moquin D, Noubade R, Briso EM, Charland C, Leonard WJ, Ciliberto G, Teuscher C, Haynes L, Rincon M. The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4+ T cells. Journal of Experimental Medicine 2009;206:69–78.

[191] Linterman MA, Beaton L, Yu D, Ramiscal RR, Srivastava M, Hogan JJ, Verma NK, Smyth MJ, Rigby RJ, Vinuesa CG. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. Journal of Experimental Medicine 2010;207:353–63.

[192] Shih T-AY, Roederer M, Nussenzweig MC. Role of antigen receptor affinity in T cell-independent antibody responses in vivo. Nat Immunol 2002;3:399–406.

[193] Dintzis RZ, Middleton MH, Dintzis HM. Studies on the immunogenicity and tolerogenicity of T-independent antigens. J Immunol 1983;131:2196–203.

[194] Maclennan ICM, Toellner K-M, Cunningham AF, Serre K, Sze DM-Y, Zúñiga E, Cook MC, Vinuesa CG. Extrafollicular antibody responses. Immunol Rev 2003;194:8–18.

[195] Lanzavecchia A. Antigen-specific interaction between T and B cells. Nature 1985;314:537–9.

[196] Allen CDC, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. Immunity 2007;27:190–202.

[197] Victora GD, Nussenzweig MC. Germinal centers. Annu Rev Immunol 2012;30:429–57.

[198] Cyster JG. B cell follicles and antigen encounters of the third kind. Nat Immunol 2010;11:989–96.

[199] Shaffer AL, Yu X, He Y, Boldrick J, Chan EP, Staudt LM. BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. Immunity 2000;13:199–212.

[200] Shapiro-Shelef M, Calame K. Regulation of plasma-cell development. Nat Rev Immunol 2005;5:230–42.

[201] Jacob J, Kassir R, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. I. The architecture and dynamics of responding cell populations. J Exp Med 1991;173:1165–75.

[202] Cyster JG, Ansel KM, Reif K, Ekland EH, Hyman PL, Tang HL, Luther SA, Ngo VN. Follicular stromal cells and lymphocyte homing to follicles. Immunol Rev 2000;176:181–93.

[203] Allen CDC, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, Cyster JG. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. Nat Immunol 2004;5:943–52.

[204] Berek C, Berger A, Apel M. Maturation of the immune response in germinal centers. Cell 1991;67:1121–9.

[205] Victora GD, Schwickert TA, Fooksman DR, Kamphorst AO, Meyer-Hermann M, Dustin ML, Nussenzweig MC. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. Cell 2010;143:592–605.

[206] Xu Z, Zan H, Pone EJ, Mai T, Casali P. Immunoglobulin class-switch DNA recombination: induction, targeting and beyond. Nat Rev Immunol 2012;12:517–31.

[207] Robertson JM, Jensen PE, Evavold BD. DO11.10 and OT-II T cells recognize a C-

terminal ovalbumin 323-339 epitope. J Immunol 2000;164:4706–12.

[208] Hogquist KA, Tomlinson AJ, Kieper WC, McGargill MA, Hart MC, Naylor S, Jameson SC. Identification of a naturally occurring ligand for thymic positive selection. Immunity 1997;6:389–99.

[209] K Takamiya AYKFSYMSMOSFMHNTKFMSMKHSKFSA. Mice with disrupted GM2/GD2 synthase gene lack complex gangliosides but exhibit only subtle defects in their nervous system. Proc Natl Acad Sci USA 1996;93:10662–7.

[210] Zammit DJ, Cauley LS, Pham Q-M, Lefrancois L. Dendritic cells maximize the memory CD8 T cell response to infection. Immunity 2005;22:561–70.

[211] Kitamura D, Rajewsky K. Targeted disruption of mu chain membrane exon causes loss of heavy-chain allelic exclusion. Nature 1992;356:154–6.

[212] Madsen L, Labrecque N, Engberg J, Dierich A, Svejgaard A, Benoist C, Mathis D, Fugger L. Mice lacking all conventional MHC class II genes. Proc Natl Acad Sci USA 1999;96:10338–43.

[213] Fahlén-Yrlid L, Gustafsson T, Westlund J, Holmberg A, Strömbeck A, Blomquist M, MacPherson GG, Holmgren J, Yrlid U. CD11c(high)dendritic cells are essential for activation of CD4+ T cells and generation of specific antibodies following mucosal immunization. The Journal of Immunology 2009;183:5032–41.

[214] Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat Med 2009;15:410–6.

[215] Tan AM, Chen H-C, Pochard P, Eisenbarth SC, Herrick CA, Bottomly HK. TLR4 signaling in stromal cells is critical for the initiation of allergic Th2 responses to inhaled antigen. The Journal of Immunology 2010;184:3535–44.

[216] Sanders CJ, Moore DA, Williams IR, Gewirtz AT. Both radioresistant and hemopoietic cells promote innate and adaptive immune responses to flagellin. J Immunol 2008;180:7184–92.

[217] Salazar-Gonzalez RM, Srinivasan A, Griffin A, Muralimohan G, Ertelt JM, Ravindran R, Vella AT, McSorley SJ. Salmonella flagellin induces bystander activation of splenic dendritic cells and hinders bacterial replication in vivo. J Immunol 2007;179:6169–75.

[218] Holmgren J, Månsson JE, Svennerholm L. Tissue receptor for cholera exotoxin: structural requirements of G11 ganglioside in toxin binding and inactivation. Med. Biol. 1974;52:229–33.

[219] Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, Wang Y-H, Watowich SS, Jetten AM, Tian Q, Dong C. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity 2008;29:138–49.

[220] Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, Srivastava M, Linterman M, Zheng L, Simpson N, Ellyard JI, Parish IA, Ma CS, Li Q-J, Parish CR, Mackay CR, Vinuesa CG. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. Immunity 2009;31:457–68.

[221] Hebel K, Griewank K, Inamine A, Chang H-D, Müller-Hilke B, Fillatreau S, Manz RA, Radbruch A, Jung S. Plasma cell differentiation in T-independent type 2 immune responses is independent of CD11c(high) dendritic cells. Eur J Immunol 2006;36:2912–9.

[222] Linterman MA, Rigby RJ, Wong RK, Di Yu, Brink R, Cannons JL, Schwartzberg PL, Cook MC, Walters GD, Vinuesa CG. Follicular helper T cells are required for systemic autoimmunity. Jem.Rupress.org 2009.

[223] Birnberg T, Bar-On L, Sapoznikov A, Caton ML, Cervantes-Barragán L, Makia D, Krauthgamer R, Brenner O, Ludewig B, Brockschnieder D, Riethmacher D, Reizis B, Jung S. Lack of conventional dendritic cells is compatible with normal development and T cell homeostasis, but causes myeloid proliferative syndrome. Immunity 2008;29:986–97.

[224] Tittel AP, Heuser C, Ohliger C, Llanto C, Yona S, Hämmerling GJ, Engel DR, Garbi

N, Kurts C. Functionally relevant neutrophilia in CD11c diphtheria toxin receptor transgenic mice. Nat Meth 2012;9:385–90.

[225] Miloud T, Fiegler N, Suffner J, Hämmerling GJ, Garbi N. Organ-specific cellular requirements for in vivo dendritic cell generation. The Journal of Immunology 2012;188:1125–35.

[226] Bliss SK, Gavrilescu LC, Alcaraz A, Denkers EY. Neutrophil depletion during Toxoplasma gondii infection leads to impaired immunity and lethal systemic pathology. Infect Immun 2001;69:4898–905.

[227] Czuprynski CJ, Brown JF, Maroushek N, Wagner RD, Steinberg H. Administration of anti-granulocyte mAb RB6-8C5 impairs the resistance of mice to Listeria monocytogenes infection. J Immunol 1994;152:1836–46.

[228] Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, Wang Y-H, Dong C. Bcl6 mediates the development of T follicular helper cells. Science 2009;325:1001–5.

[229] Eddahri F, Denanglaire S, Bureau F, Spolski R, Leonard WJ, Leo O, Andris F. Interleukin-6/STAT3 signaling regulates the ability of naive T cells to acquire B-cell help capacities. Blood 2009;113:2426–33.

[230] Lu KT, Kanno Y, Cannons JL, Handon R, Bible P, Elkahloun AG, Anderson SM, Wei L, Sun H, O'Shea JJ, Schwartzberg PL. Functional and epigenetic studies reveal multistep differentiation and plasticity of in vitro-generated and in vivo-derived follicular T helper cells. Immunity 2011;35:622–32.

[231] Probst HC, van den Broek M. Priming of CTLs by lymphocytic choriomeningitis virus depends on dendritic cells. J Immunol 2005;174:3920–4.

[232] Lindsay RWB, Darrah PA, Quinn KM, Wille-Reece U, Mattei LM, Iwasaki A, Kasturi SP, Pulendran B, Gall JGD, Spies AG, Seder RA. CD8+ T cell responses following replication-defective adenovirus serotype 5 immunization are dependent on CD11c+ dendritic cells but show redundancy in their requirement of TLR and nucleotide-binding oligomerization domain-like receptor signaling. J Immunol 2010;185:1513–21.

[233] Shen L, Rock KL. Cellular protein is the source of cross-priming antigen in vivo. Proc Natl Acad Sci USA 2004;101:3035–40.

[234] Delamarre L, Couture R, Mellman I, Trombetta ES. Enhancing immunogenicity by limiting susceptibility to lysosomal proteolysis. J Exp Med 2006;203:2049–55.

[235] Bevan MJ. Helping the CD8(+) T-cell response. Nat Rev Immunol 2004;4:595–602.

[236] Wu Y, Liu Y. Viral induction of co-stimulatory activity on antigen-presenting cells bypasses the need for CD4+ T-cell help in CD8+ T-cell responses. Curr. Biol. 1994;4:499–505.

[237] Damjanovic D, Zhang X, Mu J, Fe Medina M, Xing Z. Organ distribution of transgene expression following intranasal mucosal delivery of recombinant replication-defective adenovirus gene transfer vector. Genet Vaccines Ther 2008;6:5.

[238] Li Z, Palaniyandi S, Zeng R, Tuo W, Roopenian DC, Zhu X. Transfer of IgG in the female genital tract by MHC class I-related neonatal Fc receptor (FcRn) confers protective immunity to vaginal infection. Proc Natl Acad Sci U S A 2011;108:4388–93.

[239] Holt PG, Haining S, Nelson DJ, Sedgwick JD. Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. J Immunol 1994;153:256–61.

[240] Probst HC, Tschannen K, Odermatt B, Schwendener R, Zinkernagel RM, Van Den Broek M. Histological analysis of CD11c-DTR/GFP mice after in vivo depletion of dendritic cells. Clin Exp Immunol 2005;141:398–404.

[241] Alfaro C, Suarez N, Oñate C, Perez-Gracia JL, Martinez-Forero I, Hervas-Stubbs S, Rodriguez I, Perez G, Bolaños E, Palazon A, de Sanmamed MF, Morales-Kastresana A, Gonzalez A, Melero I. Dendritic Cells Take up and Present Antigens from Viable and Apoptotic Polymorphonuclear Leukocytes. PLoS ONE 2011;6:e29300.

[242] Harshyne LA, Zimmer MI, Watkins SC, Barratt-Boyes SM. A role for class A

scavenger receptor in dendritic cell nibbling from live cells. J Immunol 2003;170:2302–9.

[243] Lui G, Manches O, Angel J, Molens J-P, Chaperot L, Plumas J. Plasmacytoid Dendritic Cells Capture and Cross-Present Viral Antigens from Influenza-Virus Exposed Cells. PLoS ONE 2009;4:e7111.

[244] Spörri R, Reis e Sousa C. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. Nat Immunol 2005;6:163–70.

[245] Kraehenbuhl JP, Neutra MR. Epithelial M cells: differentiation and function. Annu. Rev. Cell Dev. Biol. 2000;16:301–32.

[246] Anosova NG, Chabot S, Shreedhar V, Borawski JA, Dickinson BL, Neutra MR. Cholera toxin, E. coli heat-labile toxin, and non-toxic derivatives induce dendritic cell migration into the follicle-associated epithelium of Peyer's patches. Mucosal Immunol 2008;1:59–67.

[247] Neal MD, Leaphart C, Levy R, Prince J, Billiar TR, Watkins S, Li J, Cetin S, Ford H, Schreiber A, Hackam DJ. Enterocyte TLR4 mediates phagocytosis and translocation of bacteria across the intestinal barrier. J Immunol 2006;176:3070–9.

[248] McDole JR, Wheeler LW, McDonald KG, Wang B, Konjufca V, Knoop KA, Newberry RD, Miller MJ. Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. Nature 2012;483:345–9.

[249] Chieppa M, Rescigno M, Huang A, Germain R. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. J Exp Med 2006;203:2841–52.

[250] Teter K, Jobling MG, Holmes RK. Vesicular transport is not required for the cytoplasmic pool of cholera toxin to interact with the stimulatory alpha subunit of the heterotrimeric g protein. Infect Immun 2004;72:6826–35.

[251] Lycke N, Tsuji T, Holmgren J. The adjuvant effect of Vibrio cholerae and Escherichia coli heat-labile enterotoxins is linked to their ADP-ribosyltransferase activity. Eur J Immunol 1992;22:2277–81.

[252] Compan V, Baroja-Mazo A, López-Castejón G, Gomez AI, Martínez CM, Angosto D, Montero MT, Herranz AS, Bazán E, Reimers D, Mulero V, Pelegrín P. Cell volume regulation modulates NLRP3 inflammasome activation. Immunity 2012;37:487–500.

[253] Good-Jacobson KL, Szumilas CG, Chen L, Sharpe AH, Tomayko MM, Shlomchik MJ. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. Nat Immunol 2010;11:535–42.

[254] Zhu J, Jankovic D, Oler AJ, Wei G, Sharma S, Hu G, Guo L, Yagi R, Yamane H, Punkosdy G, Feigenbaum L, Zhao K, Paul WE. The Transcription Factor T-bet Is Induced by Multiple Pathways and Prevents an Endogenous Th2 Cell Program during Th1 Cell Responses. Immunity 2012;37:660–73.

[255] Howie D, Laroux FS, Morra M, Satoskar AR, Rosas LE, Faubion WA, Julien A, Rietdijk S, Coyle AJ, Fraser C, Terhorst C. Cutting edge: the SLAM family receptor Ly108 controls T cell and neutrophil functions. J Immunol 2005;174:5931–5.

[256] Han S, Hathcock K, Zheng B, Kepler TB, Hodes R, Kelsoe G. Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. J Immunol 1995;155:556–67.

[257] Zhong G, Reis e Sousa C, Germain RN. Antigen-unspecific B cells and lymphoid dendritic cells both show extensive surface expression of processed antigen-major histocompatibility complex class II complexes after soluble protein exposure in vivo or in vitro. J Exp Med 1997;186:673–82.

[258] Akiba H, Takeda K, Kojima Y, Usui Y, Harada N, Yamazaki T, Ma J, Tezuka K, Yagita H, Okumura K. The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo. J Immunol 2005;175:2340–8.

[259] Gigoux M, Shang J, Pak Y, Xu M, Choe J, Mak TW, Suh W-K. Inducible costimulator promotes helper T-cell differentiation through phosphoinositide 3-kinase. Proc Natl Acad Sci U S A 2009;106:20371–6.

Papers I-III