Regulation of gut IgA induction by helper T cells

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Gothenburg 2019

Cover illustration by **Ērika Gribonika**

Chicken-ovalbumin empowered by cholera toxin marches through the splashing mucosa leaving footprints of secretory IgA

The analogy with a rooster was chosen to mark 20 years of formal education. It all started on September 1st, 1999 and the first textbook - "Ābece" was decorated by a grand rooster…

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Mammai un tētim,

un krustmātei – eņģelim …

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Gothenburg, Sweden, 2019

ABSTRACT

The gut is the largest lymphoid organ in the body. Due to intense and constant exposure to the outside world, it also functions as the most important portal of entry for many pathogens. T cell-dependent secretory immunoglobulin A (IgA) prevents pathogens from spreading to systemic tissues and, hence, oral immunization represents the most effective route for vaccination against these pathogens. The detailed mechanism of oral vaccination-induced protective IgA immunity is not fully understood. The main aim of this thesis was to investigate the role of the gut CD4 T subsets for the induction of IgA responses. By using Ovalbumin-specific TCR-Tg CD4 T cells in an adoptive transfer system and mucosal immunization with or without cholera toxin (CT) adjuvant I show that IgA induction in the Peyer's patch (PP) is regulated in a distinct two-step process, where T follicular helper cells (TFH) and thymus-derived T regulatory cells (tTreg) orchestrate the IgA induction. Effective B cell help in the germinal center (GC) is maintained by antigen-specific TFH cells, while IgA class-switch recombination (CSR) is promoted by tTregs independently of the immunizing antigen.

It should be emphasized that the default response pathway activated by oral antigen administration is oral tolerance. In this doctoral thesis, I demonstrate that the suppressive pathway is regulated by IL-10. Thus, CD4 T cells upon exposer to cognate antigen in the presence of IL-10 differentiate into peripherally induced Tregs (pTreg). In the absence of IL-10 or after addition of CT adjuvant TFH differentiation is enhanced, resulting in a strong gut IgA response. CT has been reported to be the most potent oral adjuvant. Some reports suggest that CT preferentially exerts the adjuvant function via Th17 cells. The immuno-dominant part of CT is its B subunit, therefore, I used CTB-specific tetramer to monitor if CT induced T cell response is dominated by Th17 cells. Surprisingly, the CTB-specific T cell repertoire was nearly absent of Th17 lineage, however that did not prevent adjuvant's ability to induce a strong gut IgA response. Instead, CT induced CD4 T cells were overrepresented by TFH lineage that did not derive from Th17 cells as shown by using IL-17 fate reporter mice. These observations were confirmed using single-cell RNAseq technology. Gene signature of sorted CTB-specific CD4 T cells showed an almost complete dominance of the TFH phenotype with virtually no Th17 signature. Besides, the adoptive transfer of Th17 deficient CD4 T cells (Rorc$^{-/-}$) into nude host allowed for a robust gut IgA induction after oral immunization with CT. These findings argue strongly against the observations that upon CT immunization gut IgA B cell responses are driven by Th17 cells that exhibit great plasticity towards the TFH lineage. Interestingly, obtained data suggest that TFH cells in the PP do not share clonal relatedness with Th17, Th1 or Treg cells which have been a long-standing controversy in this field. Together, these findings provide a new paradigm for how gut IgA responses are regulated and which two types of CD4 T cell subsets are needed; tTregs for IgA CSR and TFH for GC formation and B cell maturation.

Keywords: Immunoglobulin A, oral immunization, helper T cells, Peyer's patch, ovalbumin, cholera toxin, interleukin 10, transforming growth factor β

SAMMANFATTNING PÅ SVENSKA

Tarmen är kroppens största lymfoida organ. Genom att slemhinnan i tarmen ständigt är utsatt för stora mängder av antigener och dessutom utgör en viktig infektionsväg in i kroppen för många sjukdomsframkallande patogener har detta organ utvecklat ett starkt lokalt immunförsvar mot inkräktande mikroorganismer och oönskade substanser. Sekretoriskt IgA (SIgA) produceras av tarmslemhinnans plasmaceller och denna produktion av antikroppar är i högsta grad beroende av CD4 T celler, som ger de aktiverade B lymfocyterna hjälp till vidare differentiering till plasma celler s.k germinal centrum (GC) i tunntarmens Peyerska plaques (PP). Dessa är stationer för lymfocyter som engageras för att bygga upp det lokala immunsvaret. Det övergripande syftet med föreliggande avhandling var att bättre ta reda på vilka olika regulatoriska mekanismer som styr produktionen av tarmens SIgA och vilka CD4 T celler som krävs för att denna production skall bli framgångsrik i tarmens PP. Vi har använt en musmodell i vilken vi kan tillföra CD4 T celler av olika typer och fråga oss vilka direkta funktioner som dessa celler har i det lokala immunsvaret efter vaccination med äggalbumin. Detta antigen tillföres oral med eller utan ett potent lokalt adjuvans, kolera toxin. Denna molekyl är mycket potent, men trots detta är mekanismen för hur den fungerar efter oral tillförsel begränsad. Etta v mina viktigaste fynd är att 2 celltyper måste samverka för att vi skall få ett IgA immunsvar. Dessa är en cell som står för bytet av immunglobulinklasss från IgM till IgA, detta kallas klass-bytes recombination (KBR) och vi fann att regulatoriska T celler (Tregs) från thymus var ansvariga för KBR.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Gribonika I**, Eliasson DG, Chandode RK, Schön K, Strömberg A, Bemark M, Lycke NY.

   Class-switch recombination to IgA in the Peyer's patches requires natural thymus-derived Tregs and appears to be antigen independent.

   Mucosal Immunol. 2019 Nov; 12 (6): 1268-1279.

   doi: 10.1038/s41385-019-0202-0.

II. **Gribonika I,** Eliasson DG, Schön K, Lycke NY.

   Oral cholera toxin adjuvant blocks pTreg-differentiation which allows for strong gut IgA responses.

   Manuscript

III. **Gribonika I**, Strömberg A, Lebrero-Fernandez C, Moon J, Bemark M, Lycke NY.

   Antigen-specific CD4 T cell responses in PP following oral immunizations with cholera toxin are dominated by Tfh cells and independent of Th17 cell differentiation.

   Manuscript

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CONTENT

ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
ADP	Adenosine diphosphate
Ag	Antigen
AhR	Aryl hydrocarbon receptor
AID	Activation-induced cytidine deaminase
AIRE	Autoimmune regulator
APC	Antigen-presenting cell
APRIL	A proliferation-inducing ligand
BAFF	B cell-activating factor
BATF	Basic Leucine Zipper ATF-Like Transcription Factor
BCL-6	B cell lymphoma 6
BCR	B cell receptor
Blimp-1	B lymphocyte-induced maturation protein-1
cAMP	Cyclic adenosine monophosphate
CCR / L	C-C chemokine receptor/ligand
CD	Cluster of differentiation
CNS	Conserved non-coding sequence
CP	Crypto-patch
Cpm	Counts per minute
CREB	cAMP response element-binding
CSR	Class-switch recombination
CT	Cholera toxin
CTA	Cholera toxin subunit A
CTB	Cholera toxin subunit B
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CX3CR1	C-X3-C Chemokine Receptor 1
CXCR / L	C-X-C chemokine receptor/ligand
DC	Dendritic cell
DN	Double negative stage
DP	Double positive stage
DZ	Dark zone
ER	Endoplasmic reticulum
FAE	Follicular-associated epithelium
FDC	Follicular dendritic cell
Foxp3	Forkhead box P3
GALT	Gut-associated lymphoid tissues
GF	Germ-free
GI	Gastrointestinal
GITR	Glucocorticoid-induced tumor necrosis factor receptor
GM-1	Monosialotetrahexosylganglioside
GVHD	Graft versus host disease
ICOS	Inducible T-cell costimulator
IFA	Interfollicular area

Ig	Immunoglobulin
IL-	Interleukin
IL	Intestinal lavage
ILC	Innate lymphoid cell
ILF	Isolated lymphoid follicle
IRF4	Interferon regulatory factor 4
LP	Lamina propria
LPS	Lipopolysaccharide
LT	Heat-labile enterotoxin
LZ	Light zone
M cell	Microfold cell
mAb	Monoclonal antibody
MFI	Median fluorescence intensity
MHC	Major Histocompatibility complex
MLN	Mesenteric lymph node
NF-kB	Nuclear factor kappa-light chain enhancer of activated B cells
OVA	Chicken ovalbumin
PD-1	Programmed death-1
pIgR	Polymeric immunoglobulin receptor
PKA	Protein kinase A
PP	Peyer's patch
pTreg	Peripherally induced T regulatory cell
RA	Retinoic acid
RAG	Recombination activating gene
Rorg	RAR-related orphan receptor gamma
Runx	Runt-related transcription factor
SAP	SLAM-associated protein
SC	Secretory component
SED	Subepithelial dome
SFB	Segmented filamentous bacteria
SFC	Spot forming cells
SHM	Somatic hypermutation
SIgA	Secretory immunoglobulin A
SP	Spleen
SPF	Some pathogen-free
STAT	Signal transducer and activator of transcription
TCF-1	T cell factor 1
TCR	T cell receptor
TFH	T follicular helper cell
TFR	T follicular regulatory cell
Tg	Transgenic
TGFβ	Transforming growth factor β
Th17	T helper 17 cell
TLR	Toll-like receptor
Treg	T regulatory cell
tTreg	Thymus derived T regulatory cell

We all are born old and have to work hard throughout our lives to die young…

/Vello Salo./

PREFACE

Life, irrespective of geographical location, lifestyle, health or age, is a very demanding and challenging process. To protect us against infections we have developed a highly efficient immune system. This is especially evident in the gut, which is our largest lymphoid organ and constantly exposed to food antigens and the microbiota. The commensal bacteria, outnumbering our cells, are thriving in the luminal mucosa. Good control over this community secures intestinal homeostasis and further impact on our wellness. This is partly done by the production of secretory immunoglobulin A (SIgA) antibodies. The secretion of IgA into the lumen of the intestine is receptor-mediated and consumes a significant amount of energy. Hence, we need nutritious food, clean water, and a healthy environment to maintain a good life. Yet, it can be risky, because food or water intake could bring pathogens, which gain entrance through the mucosal membranes. If properly maintained, the composition of the mucus, SIgA and the epithelial cell lining of the gut intestine form a perfect barrier against intruders. Hence, SIgA serves as a flexible frontline defense-factor.

To perform its effector functions, SIgA holds several characteristics that could be both dependent or independent of the unique structure of the antibody molecule itself. Some of these functions are associated with the variable region of the antigen-binding sites, while others depend on the non-binding parts of the molecule. Therefore, SIgA has a privileged status within mucosal secretions to combat infections. Every day the immune system must distinguish between harmful or beneficial antigens. It is geared up to coexist with commensal communities and food antigens in a mutually beneficial relationship via a process called tolerance. Indeed, humoral immunity is capable of both rejecting as well as allowing antigens to pass the mucosal barrier. SIgA induced in the absence of T cells, provide a fairly rapid, short-lived, antibody response of relatively low specificity that is largely directed against the microbiota. By contrast, SIgA in the presence of T cell help is generated via germinal centers (GC) and form a highly specific immune response that also induces long-term memory, which is the ultimate response to oral vaccination. However, it is not clear how this highly specific process is regulated and where exactly the different events take place. Nevertheless, we know that T cell-dependent responses to oral antigens are initiated in the Peyer's patches (PP), which host the GC reaction, where T follicular helper cells (TFH) provide the necessary environment for B cell expansion and differentiation, including somatic hypermutation (SHM) and class switch

recombination (CSR). Recently, however, such notion has been challenged by observations that CSR could occur outside of the GC in the subepithelial dome (SED) or T cell zone (102; 171). This complicates the regulatory requirements and environments needed for IgA responses in the gut. More research is warranted to better understand how regulatory T cells (Tregs), TFH and B cells interact in the PP to form SIgA.

Many questions have been raised concerning the functions and interconnected relationship of Tregs and TFH cells in the context of PP GC reaction, more specifically, whether the Tregs can acquire TFH fate within PP (1, 2). Others, however, have focused on Th17 cells and reported the critical role for SIgA responses via control of both IgA CSR and TFH cell functions (3). Thus, by proposing T helper cell plasticity both Tregs and Th17 cell subsets have been ascribed the sole source of TFH functions in the PP. For example, Treg cells in the PP have been shown to downregulate their suppressive program in favor of TFH functions (1). Complex collaborative networks between commensal bacteria, SIgA and Treg cells have been observed, therefore Treg conversion into TFH cells seemed a likely mechanism of SIgA formation. By contrast following oral immunization – TFH cells were thought to derive from Th17 cells (3). Thus, it is of critical importance that the concepts for SIgA formation and the elements involved are better studied and a detailed account of the regulatory microenvironment in the PP can be delineated. This will provide us with a better understanding of the precise mechanisms that govern IgA responses in the PPs.

Due to the strong preference for tolerance induction, effective SIgA response to oral antigen requires an adjuvant. Cholera toxin is the most potent oral adjuvant known to this date (4). It possesses strong antigenic and adjuvant functions, effectively driving specific IgA responses locally and systemically to most protein antigens after simple admixing to the oral vaccine. Unfortunately, the molecule has been found highly toxic, which precludes clinical use. However, understanding of its adjuvant effects could facilitate the development of next-generation safe and effective oral vaccine adjuvants. The healthy immune system in the gastrointestinal (GI) tract primary exerts tolerogenic functions, however, oral vaccination must overcome tolerance induction in favor of a strong, protective SIgA response. For this reason, the choice of adjuvant is critical. In this thesis I have addressed the requirements for T-dependent gut IgA induction, in particular, I have focused on TFH and Treg roles to explain how gut IgA responses to soluble proteins are induced in the PP.

INTRODUCTION

Oral vaccination

Vaccination is one of the most important achievements in clinical medicine as it can prevent and even eradicate infectious diseases saving millions of lives each year (5). Nearly all available vaccines are injectable, which primarily stimulates systemic immunity and protection. However, most pathogens enter via mucosal epithelium, therefore there is a need for more effective induction of local immune responses (6). Oral vaccination is the route of administration that best protects against enteric pathogens. It provides both humoral and cellular immune responses at systemic as well as mucosal sites. Besides, oral vaccination can also be cost-effective (7). Indeed, needle-free vaccination eliminates the risk of transmitting contaminating blood-borne pathogens and vaccine administration can be performed by health care workers without specialized medical training. Manufacturing of oral vaccines may be made simpler by reducing the requirement for extensive antigen purification, simplifying the overall production process.

Numerous mucosal immunization experiments have shown strong induction of long-term T and B cell memory with local homing of effector T cells and plasma cells to the mucosa upon a secondary antigenic challenge (8). Hence, the barrier functions are effectively reinforced through the production of cytokines and chemokines as well as antigen-specific SIgA and IgM antibodies. However, very few commercial mucosal vaccines exist today, and nearly all are live-attenuated that are more unstable than killed vaccines. In some cases, these have even reverted to give more virulent infections, which have caused disease (9). Killed vaccines of whole bacteria or subcomponent vaccines are safer alternatives, but their immunogenicity is much reduced and they are less efficiently presented by antigen presenting cells (APC). The only licensed non-living mucosal vaccines available for human use are developed against *Vibrio cholerae* of which Dukoral is known as the first and most studied example. It was developed in the 1970s and consists of killed whole *V. cholerae* bacteria admixed with recombinant cholera toxin B-subunit (CTB) (10). Protection persists even 2–3 years after vaccination, and the resulting protective immune response is dominated by antibacterial (LPS) and antitoxin (CTB) SIgA (7).

There are many hurdles for an oral vaccine to reach the immune inductive sites in the intestine and initiate a response. The harsh gut environment degrades most antigenic epitopes that are present in the gastrointestinal lumen.

Therefore, formulations of biodegradable nanoparticles have been developed and studied for their potential as carriers for oral vaccine antigens (11). However, several other challenges exist in the intestine as enzyme-catalyzed hydrolysis and low pH-impact on the antigenic structures, which affect the stability of an oral vaccine. The inductive sites are limited in numbers, restricted in particle size for efficient uptake and hard to reach due to the mucus layer. Nevertheless, the most critical element is the need for an adjuvant that can promote strong mucosal immunity. Whereas, mucosal tolerance protects against unwanted immune responses to digested antigens it is also the default pathway that needs to be avoided or circumvented to stimulate a strong immune response (12). Thus, the adjuvant helps to overcome the natural tolerance-inducing pathway and greatly promotes the induction of a mucosal response. For this reason, the selection of an appropriate adjuvant becomes critical for the efficiency of the mucosal vaccine. For example, Toll like receptor (TLR) agonists or the bacterial enterotoxins, CT or LT, represent two major categories of mucosal adjuvants, which act on the mucosal dendritic cells (DC). However, whereas both TLRs and the GM1-ganglioside receptors are ubiquitously expressed, these adjuvants can give rise to unwanted side effects, such as diarrheal response of nerve paralysis (13). Because it is not entirely clear what are the adjuvant mechanisms of action, it is much warranted to study the regulatory effects that underlay their immune-enhancing effects when given orally. It is fair to say that oral vaccine design is still in the phase of exploration for safer and more effective vaccines. In this regard, better knowledge about mucosal adjuvants is needed.

Oral tolerance

Oral tolerance is the state of local and systemic immune unresponsiveness that is induced by oral administration of innocuous antigens. It is the default reaction to gut microbiota as well as the food proteins that we ingest every day. Noteworthy, tolerance to gut bacteria in the colon does not seem to attenuate systemic responses, whereas tolerance to food antigens induced via the small intestine appears to affect both local and systemic immunity (14). This is because orally administered antigens can disseminate systemically via blood and lymph (15). Tolerance manifests itself as unresponsiveness and an absence of systemic DTH reactions, T-cell proliferation, cytokine production or systemic and local antibody responses. It is a major hurdle for mucosal vaccination.

Oral tolerance is initiated after antigen uptake by DCs in the lamina propria (LP) within minutes after feeding (16). The antigen dose appears to be crucial because only a single large dose or continuous small doses of antigen will elicit

oral tolerance. The ingested antigens reach LP via paracellular diffusion through pores in the tight junctions connecting epithelial cells or via transcytosis, but the most effective is the active uptake that CX3CR1$^+$ myeloid cells do in the mucosa after stretching out cellular processes into the lumen which allows them to sample intestinal antigens across tight junctions of the epithelial barrier (17). Antigen transport from the LP into the mesenteric lymph node (MLN) by CD103$^+$ DCs via the upregulation of CCR7 is the key event and can only be achieved after antigen-handover from CX3CR1$^+$ myeloid cells to CD103$^+$ DCs (18, 19). In the MLN, CD103$^+$ DCs collaborate with local non-hematopoietic stromal cells to induce priming conditions that promote the generation of activated Foxp3$^+$ Tregs via TGF-β and retinoic acid (RA) (20-23). Various Treg subsets exist and are associated with oral tolerance. Their suppressive functions are mediated via IL-10 and TGF-β secretion and their dominance in the gut immune system is a prerequisite for homeostasis. Thus, long-lasting tolerance is critical for a healthy life. While thymus-derived Tregs (tTregs) possess a high affinity for self-antigens and are instrumental for central tolerance, peripherally induced Tregs (pTregs) respond to luminal antigens. The pTregs are responsible for the suppression of responses against, for example, food antigens, antigens of the microbiota or oral vaccine antigens (24). Importantly, to exert their tolerogenic functions, pTregs need to acquire gut homing receptors and migrate from the MLN to the gut associated lymphoid tissues (GALT) and/or the LP (24). It has been shown that the pTregs undergo expansion upon returning to the gut, which depends on the presence of CX3CR1$^+$ myeloid cells and IL-10. Also, the microbiota contributes to pTreg induction and maintenance (24, 25). Indirectly, Tregs are also critical for gut IgA responses and provide the essential TGFβ1, which is the necessary IgA CSR factor at the inductive sites (2). The local SIgA response contributes to homeostasis as it can bind and interact with the commensal bacterial communities to sustain their presence in the gut intestine (26, 27). Oral tolerance against any given antigen may persist for many years (28) and breaking of oral tolerance appears not to be possible, even with the strongest of oral adjuvants, such as cholera toxin (CT) (29).

The cholera toxin adjuvant

The choice of adjuvant for a mucosal vaccine is as crucial as the antigen composition itself. It can dramatically affect not only the immediate immune response but also the long-term protective effect of a vaccine (30). Adjuvants modulate the quality of the immune response — especially the development of high-affinity B cell clones, long-lived memory B cells, and plasma cells. CT has remarkable adjuvant properties; it is perhaps the most potent mucosal adjuvant to this date and considered the gold standard for an effective mucosal

adjuvant (4). Its use is restricted to experimental models as it is too toxic to be included in a human vaccine, but much can be learned about mucosal vaccine design by studying the performance of the CT adjuvant (31). CT is produced by *Vibrio cholerae*, a gram-negative bacterium that is the causative agent of cholera, a potentially lethal enteric bacterial infection. Infected individuals produce many liters of diarrhoeal fluid, which can contain as much as 10^{11} bacterium per liter. CT causes severe disease and promotes bacterial transmission over the intestinal barrier. The holotoxin induces electrolyte imbalance in the lumen by acting on ion channels. This contributes to the effective dissemination of the *Vibrio cholerae* bacteria to the environment (32). Recently, it was shown that CT causes congestion of capillaries in the terminal ileum that increases the bioavailability of haemoglobulin-derived iron. It also increases concentrations of long-chain fatty acids and lactate metabolites in the lumen. All these factors contribute to enhanced bacterial growth (33, 34).

Structurally, CT is composed of an A-subunit consisting of two elements, A1 and A2, and five B subunits (35). Toxicity is associated with the A1 subunit, while the pentameric B subunits are non-toxic and harbor the binding specificity to the GM1 receptors that are expressed on intestinal epithelial cells (36). Following endocytosis, CTB dissociates from CTA in the endosome and CTA is further delivered to ER. There CTA1 is separated from CTA2. Upon release from ER to the cytosol CTA1 initiates ADP-ribosylation of Gsα and acts through adenylyl cyclase by increasing intracellular levels of cAMP (**Figure 1**). This affects many metabolic and gene transcriptional functions that are regulated by the cAMP-responsive elements or dependent on protein kinase PKA (37). PKA phosphorylates and activates chloride ion channels that in turn increase luminal osmolarity and massive water loss (32). Increased intracellular cAMP also induces lipolysis causing an extensive breakdown of lipids in adipose cells (38).

CT is a strong immunogen and an adjuvant. Its toxic CTA1 subunit hosts the most effective adjuvant function, while its antigenicity primarily relies on the CTB subunit (39). Immune responses to CT are T cell-dependent and MHC class II-restricted. CT also strongly potentiates the immunogenicity of most antigens (4, 40). While CT has been described to affect innate immunity in many different ways its adjuvant activity has been attributed to the enhanced antigen presentation observed in several different types of APCs. In particular, the upregulation of co-stimulatory molecules and chemokine receptors in murine and human DCs have been linked to the adjuvant effects (41, 42). But, the exact mechanism of the adjuvant function is not completely clear. For long CT was considered a strong Th2 inducing adjuvant, but recent investigations have proven this assumption wrong. A mixed Th1/Th2/Th17 type of response

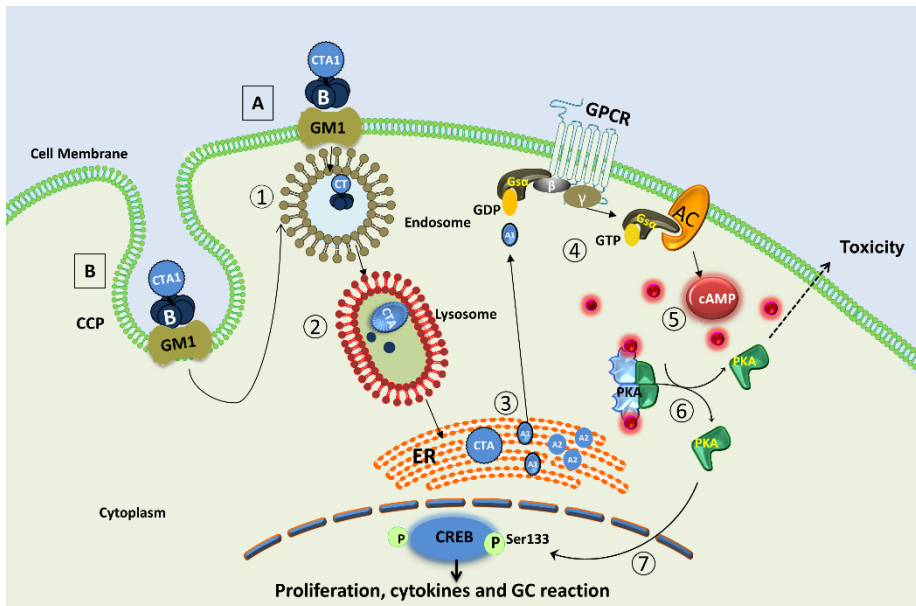is commonly induced (42, 43), but when administered orally, CT initiates a robust mucosal SIgA response (4).

*Figure 1. **Cholera toxin uptake, adjuvanticity, and toxicity.** CT can be taken up via multiple pathways of which receptor-mediated endocytosis (A) and endocytosis via clathrin-coated pits (B) are the most common. CT binds to GM1 and is incorporated into the early endosome (1) that further develops into the endolysosomal complex where CTA and CTB are separated (2). CTA then travels to the ER, where CAT1 and CTA2 are separated (3). CTA1 is released into the cytosol and acts on the GDP to activate Gsa that further acts on AC (4) and produces cAMP (5). cAMP triggers signaling cascade via PKA (6) that promotes toxicity by opening the ion channels, but also initiates the CREB phosphorylation that leads to enhanced immune function (7). Abbreviations: CCP, Clathrin Coated Pits; GPCR, G protein-coupled receptor; ER, endoplasmic reticulum; Gsα, G_{stimulatory} alpha subunit; AC, adenylate cyclase; cAMP, cyclic AMP; PKA, protein kinase A; CREB, cAMP response element-binding protein.*

Unfortunately, CT is precluded for being used in human vaccines due to its toxicity. Apart from the diarrhea-inducing ability when given orally, nasally administered CT is associated with Bell's palsy, facial nerve signaling impairment as a consequence of uptake into the olfactory bulb followed by retrograde transport into the olfactory neurons (44). To circumvent the toxicity, the development of non-toxic recombinant derivatives of CT have been investigated. Although a non-toxic CTB was initially reported to host adjuvant properties it was in the context of purified material from preparations of a holotoxin, while recombinant CTB has little adjuvant effects (45, 46). The best examples of CTA1 adjuvanticity were obtained from studies of *E.coli*

heat-labile toxin LT or mutants with an altered LTA1 that lost their adjuvant functions completely (47, 48). However, other mutations were proven effective at reducing toxicity, while retaining significant adjuvant functions, such as the LTK63 mutation (49). Later, mutations in CTA showed promising adjuvant effects, but the best example of a CTA1-restricted adjuvanticity was achieved in fusion proteins, such as the CTA1-DD adjuvant (50). However, the CTA1-DD does not function after oral administration and, so, its use is restricted to intranasal vaccine formulations.

Secretory IgA

Even though being the most abundant isotype and demanding an enormous amount of energy for its production, IgA remains at large an enigma. While functional redundancy between IgA and IgM antibodies may prevail at mucosal sites a selective IgA deficiency, which is very common, is surprisingly seldom associated with clinical symptoms and few are reported seriously immunocompromised. Occasional studies have suggested an increased risk of the upper respiratory tract or oral infections in IgA deficient patients (51). In IgA deficiency, increased serum and intestinal IgM and IgG levels are observed to compensate for the lacking IgA. In particular, IgM is considered to be effective as it can be transported across the epithelial cell using the same system as IgA via the pIgR (52). Possibly, more serious consequences would arise in populations lacking modern hygiene facilities, but this speculation needs further investigations.

Structure

IgA is the most abundant isotype in the whole body and can be secreted as much as 60mg IgA/kg of body weight each day. Most of the IgA is located at the mucosal membranes of the GI tract (53, 54). In the lumen of the adult rectum, IgA reaches a concentration of 800 μg/mL. While IgA and IgM antibodies are actively transported across the epithelial barrier transport of IgG into mucosal secretions occurs predominantly via a different pathway, namely the FcRn, which is known to function in adults as well as in newborns (55-57). Besides, IgG can passively diffuse via paracellular routes into the lumen, but little IgG is found compared to IgA antibodies in the gut lumen.

IgA is present in all mammals and birds. This isotype is the most heterogeneous of all immunoglobulin isotypes as it occurs in a variety of molecular forms as well as subclasses and allotypes. Patterns of heterogeneity vary significantly

between species of mammals and birds. Mammals, except for rabbits and certain primates, have a single *Ca* gene that encodes for IgA. Rabbits have 13 genes, whereas humans have 2 that encode for IgA1 and IgA2 (58, 59). The IgA1 subtype is a relatively recent evolutionary trait. The most distinct feature is its elongated hinge region that distinctively differs between IgA1 and IgA2 and between IgA from other mammals. All IgA molecules are heavily glycosylated and made up of pairings of two identical heavy chains and two identical light chains. In humans, monomeric IgA is found in the serum in a 9:1 ratio between IgA1 and IgA2. Monomeric serum IgA is produced by plasma cells in the bone marrow, marginal zone B cells or B1 cells. In mice serum IgA is in polymeric form, mainly forming dimers. Dimers are stabilized through disulfide linkages and a 15-kDa J chain (60, 61). Dimeric IgA binds to the antigen through the Fab region. Only a small fraction of the total IgA in the body is found in serum.

Mucosal IgA is dimeric and further stabilized by an 80 kDa glycosylated secretory component (SC) from the pIgR receptor of the epithelial cell (62). One function of SC is to protect SIgA from being degraded by proteolytic enzymes in the gut intestine. Most intestinal SIgA is of the IgA2 type and produced locally by gut LP plasma cells in dimeric form (63). It is important to remember that SIgA operates in an environment that is very different from that of serum or non-mucosal tissues and needs to be well protected against degradation. More specifically, SIgA is well adapted to protect mucosal surfaces against pathogens that invade the body through the mucosal membranes.

Effector functions of SIgA

In IgA deficiency, absorption of food antigens and the formation of circulating immune complexes is increased (64). This leads to food hypersensitivity and increased risk of atopic allergies and autoimmunity (65). SIgA like no other immunoglobulin is designed to constantly survey the microenvironment and secure that intruding pathogens or toxins are eliminated at the same time as food antigens are tolerated. For this reason, the immune system at the GI tract is in a constant balance between induction and suppression of antigen-specific SIgA. Apart from that, antigen recognition by SIgA may involve also a positive selection of antigen, as applies to the microbiota where specific as well as polyreactive IgA antibodies positively select beneficial bacterial species for the gut homeostasis (26, 27, 66). Thus, the existence of two functionally distinct types of SIgA appears to exist. Less specific and polyreactive, "natural" antibodies can play a role in the maintenance of a healthy intestinal microbiota, perhaps produced by B1 cells, while B2 cells produce high-affinity

specific IgA antibodies, which also could select for certain bacterial species from the microbiota or used to eliminate pathogens (67-69). Indeed, SIgA can provide a better uptake and sampling by M cells of an antigen through a mechanism that is known to depend on the receptor dectin-1 (70).

The stability of SIgA depends on the SC, which is covalently bound to the Fcα part and effectively masks potential proteolytic cleavage sites of the antibody (71). This is why SIgA is particularly effective in the enzymatically hostile gut environment. Most biological functions of SIgA depend on the Fc-SC region (72). This region is hydrophilic and negatively charged due to the abundance of N-linked oligosaccharides, containing terminal sialic acid residues in both Fcα and SC and hydrophilic amino acids in the Fc-part (73). Glycan rich SC of SIgA acts as a microbial scavenger and contributes to innate defense by binding to bacterial lectin-like adhesins (74). Such interactions lead to inhibition of bacterial adherence to intestinal surfaces and the elimination of bacteria (75). Also, the negative charge and hydrophilic nature of SIgA may trap microbes with a hydrophilic shell to prevent their attachment to the mucosal membrane. Interestingly, because SIgA can bind to M cells and epithelial cells via CD71, it can compete for the anchoring sites with bacteria thereby further limiting access of pathogens to these sites and subsequent bacterial transcytosis. Concomitantly, such adhesion can selectively capture and deliver bacteria to the PP for the induction of a specific SIgA response to that particular bacterial species (76, 77). Thus, in many regards, a prime function of SIgA is to keep the microbiota at bay using both Fab-dependent adaptive and glycan-mediated innate immune interactions, but some bacteria can exploit lectin-mediated interactions with SIgA for their survival in the gut lumen (78). *E. coli* biofilm formation on fixed epithelial cell monolayers is facilitated by SIgA, suggesting a possible mechanism for intestinal colonization (79). Indeed, recently it was shown that intestinal IgA is required for *B. fragilis* stable colonization of the gut through the exclusion of exogenous competitors (26). Similarly, IgA was shown to promote symbiosis between bacterial species facilitating a complex and healthy microbiota (27).

The SIgA is particularly powerful in the agglutination of viruses and bacteria via antibody cross-linking effectively inhibiting bacterial colonization, and resulting in immune exclusion (80). This mechanism of SIgA relies on the ability to recognize multiple antigenic epitopes, be it on viruses, bacteria or soluble proteins, like toxins. The cross-linking by SIgA of these various antigens in the intestinal lumen can significantly delay or abolish the chance of microbes to infect or toxins to influence host homeostasis. Once aggregated, these antigens become entrapped in mucus and cleared by peristalsis (81). Besides immune exclusion, SIgA binding to bacterial surfaces via epitopes on,

for example, LPS can directly suppress bacterial virulence and limit the potential to infect the host (82, 83). Enterotoxin neutralization is very effective with particularly SIgA, while gut IgM antibodies are ineffective (84). From an evolutionary point of view, SIgA was developed to more effectively neutralize luminal antigens, such as toxins because multivalent binding to these antigens are superior to monomeric binding (85). Viruses can be effectively eliminated both extracellularly and intracellularly. Interestingly, IgA antibodies can inhibit viral replication upon pIgR mediated transport if the same epithelial cell is infected with the virus (86). During vesicular transport virus-specific IgA may come across the viral envelope glycoproteins that emerge from the rough ER. Such an encounter can completely suppress viral replication (86-88). Moreover, specific SIgA can co-localize with *Shigella* derived LPS in the apical recycling endosomes of the epithelial cell and in this way inhibit nuclear translocation of NF-kB and prevent an LPS-triggered inflammatory response (89).

IgA is predominantly non-inflammatory to its nature, mainly regulating commensal communities in the intestinal lumen (67, 90) Thus, SIgA cannot activate complement and, hence, does not drive inflammatory pathways, but rather exerts complement-independent opsonisation of bacteria or viruses to eliminate infection (91, 92). Monomeric IgA can participate in an ADCC reaction, which is mediated by FcαRI (CD89) that is moderately expressed on human neutrophils, monocytes, macrophages, and eosinophils, but it is unlikely that ADCC reactions can be initiated in the gut.

Induction of gut IgA responses

The majority of mucosal IgA plasma cells are derived from B-cell activation in the GALT and Peyer's patches (PP), in particular. The GALT comprises several structures of which the PPs are the most important secondary lymphoid tissue (93). The PPs are strategically located near the mucosal membrane, they lack afferent lymphatics and are covered by a specialized follicular associated epithelium (FAE) that hosts M cells (94). These are specialized cells that transport luminal antigens into the underlying lymphoid follicle through transcytosis (95, 126). Also, goblet cells have the potential to transport antigen via retrograde transport from the lumen (96), however, to what extent goblet cells play a role for oral vaccine stimulation of immune responses has not been evaluated. Noteworthy, M cells are critical for gut IgA responses and in mice lacking M cells severely reduced SIgA responses were recorded (97, 95). Similarly to PPs in the small intestine, the colon host organized lymphoid tissue called colon patches (CP) that may take part in IgA induction. Of note, the cecum has been reported to play a central role for IgA production in the

colon, but the precise function is not clear (98). Also, hundreds of single follicles along the entire small intestine termed isolated lymphoid follicles (ILFs) are potential sites. Outside of the GALT, the MLN is associated with IgA responses, but its relative contribution to the overall total gut IgA B cell pool is poorly known. Even though the MLN is not in direct contact with the gut lumen, these lymph nodes receive afferent lymphatics from the gut carrying activated T and B lymphocytes which could migrate from the PP (94). Spatially separated lymph nodes of the MLN drain distinct parts of the intestine, and they imprint homing receptors on activated T and B cells according to the segment of the intestine that they drain (99). However, MLN is more known as the site for tolerance induction (18). Earlier studies in both mice and humans have indicated that IgA CSR could be induced in the non-organized LP, but more recent work shows little support to this notion rather promoting earlier observations of IgA CSR the PP (100-102).

In PPs directly beneath the FAE is the subepithelial dome (SED) region, where lymphocytes are directly exposed to luminal antigens sampled by the M cells. Active research is ongoing to understand the actual function of the SED region. It appears that activated APCs from SED can migrate to the intrafollicular areas to prime T cells and promote TFH differentiation (103). Somatic hypermutation (SHM) of the BCR drives affinity maturation which regulates the infiltration of antigen-specific B cells from SED into the GCs (104). It appears that some B cells in the GC can down-modulate BCL6 and upregulate CCR6, which allows them to migrate out of the GC to the SED. This way antigen-specific B cells can shuttle between the GC and the SED region as it is the site where CCR6 ligand CCL20 is produced. The activated B cells in the SED have been found to take up antigen from M cells and are thought to carry antigen from the M cell to the GC (105). SED may also serve as the site for IgA CSR, as this is where DCs express integrin αvβ8, which activates latent TGFβ (102).

B cell responses develop in the GC, where expansion, affinity maturation and clonal selection of the activated B cells takes place. Newly formed plasmablasts then migrate through the lymph via the thoracic duct into the blood after which they can home back to the LP in the intestinal villi. The homing process is controlled by expression of specific homing receptors, where PP induced plasmablasts express α4β7, CCR9, and CCR10, restricting their migration to the small and large intestine (106-109, 113). In the gut, long-lived plasma cells survive for many months. It is thought that this could be influenced by the concomitant presence of Tregs and Th17 cells at the effector site in the LP (2, 110). Also, epithelial cells, DCs, and eosinophils, in particular, provide critical survival factors such as CXCL12, IL-1β, IL-6,

BAFF, and APRIL that promote plasma cell survival (111). A fraction of plasma cells also migrates to the bone marrow contributing to the deposition of long-lived plasma cells that are responsible for serum antibody levels (112). However, the longevity of protection after vaccination relies not only on long-lived plasma cells but also on the formation of memory B cells. Only memory cells carrying gut homing receptors, such as α4β7, are effective at contributing to the overall production of SIgA (113). Interestingly, with increasing age the IgA repertoire accumulates highly expanded memory B-cell clones, carrying less SHM than the long-lived plasma cells. Following the complete elimination of gut LP plasma cells, it has been found that memory B cells can renew the plasma cell pool in the gut with the same clonal specificities as seen before (114). Induction of gut memory B cells requires the presence of a strong mucosal adjuvant but once induced, memory B cells are maintained in low frequencies in peripheral lymph nodes, spleen and the PPs for very long, perhaps lifelong, periods. When specific plasma cells have almost disappeared from the gut LP after antigen priming renewal of the response requires a second exposure to the antigen. The oral priming immunization stimulates both memory B cells and long-lived plasma cells, which, interestingly, appear to be clonally unrelated (115). One speculation brought forward is that memory B cells leave the GC reaction at an earlier time point than the long-lived plasma cells (116). An antigen-challenge after a longer period will boost memory B cells in PPs leading to the generation of an oligoclonal IgA plasma cell response in the gut LP (115). This is achieved by an effective selection and maturation process of memory B cells in secondary GC upon antigen reactivation (115, 117). Plasma cells are seeded to many different locations, including the gut LP and the bone marrow, while memory B cells reside in the follicles of secondary lymphoid tissues. Gut memory B cells can be identified by surface expression of CD80, CD73 and PDL-2 and IgA, while in other sites mostly appear to be IgM positive (115). Moreover, gut memory B cells express the transcription factor RORα, while at the systemic sites they tend to express T-bet (118). Very few gut memory B cells are found in mice that lack GC (115). This would argue that most of the memory B cells are specific to T-dependent antigens. However, it has been proposed that memory B cells specific for microbiota-derived T-independent antigens, can use GCs and interact with TFH via CD40-CD40L dependent fashion to acquire a memory phenotype identical to that of memory B cells specific for T-dependent antigens (119).

While the major site for T-dependent gut IgA responses is the GC in PPs, ILFs constitute an alternative site for the induction of mucosal IgA responses. Gut LP IgA plasma cells in mice and humans have undergone extensive SHM and have developed from activated B cells in the PP GCs. By contrast, ILF induced

IgA is distinct and represents a more undiversified IgA repertoire (101). Yet, mice that lack GC in the PP (CD40$^{-/-}$ mice) have almost normal numbers of IgA plasma cells in their gut LP (120, 101). In RORγt$^{-/-}$ mice upon reconstitution with ILC3 cells, ILFs and CPs, but not PPs are induced. This promotes a significant production of gut IgA (120). IgA CSR requires the expression of the AID enzyme that can also be observed in ILFs arguing for a possible site of IgA CSR (120). However, IgA CSR in the ILF may not require Tfh cells as extrafollicular IgA differentiation is GC-independent. In the presence of TGFβ1 B cells can undergo IgA CSR if αvβ8-expressing CD11c$^+$ DCs are located close by as seen in the SED of PPs (120; 102). Several factors derived from macrophages, DCs or local stromal cells may participate and facilitate preferential IgA CSR in ILFs. For example, upon activation with bacteria, APCs in ILF express abundant TNFα, which induces matrix metalloproteases that can activate TGF-β1. In addition to TNF-α and TGF-β1, gut APCs and local stromal cells secrete additional factors, like BAFF and APRIL, which have been found to influence IgA CSR and expansion of IgA committed B cells. Their production can be triggered by TLR ligands to enhance B cell-intrinsic CSR to IgA, independent of T cell help (121).

The germinal center reaction

The prime function of the GC in B cell responses is to provide T cell help, which rests on the TFH cells that express BCL-6 and reside within the GC boundaries (**Figure 2**). Without TFH cells, GC is not formed (122, 123). The GC reaction is known to be the site for SHM and this leads to a selection process of B cells expressing high-affinity BCR and subsequent high-affinity IgA antibodies produced by the plasma cell progeny in the gut LP. However, in the absence of CD4 T cells substantial IgA formation is still observed, which indicates that this production is extrafollicular (67, 124). These responses are short-lived, although the development of extrafollicular memory B cells has also been reported (125). The GC is a result of antigen activation of B and CD4 T cells, but it is still not clear how antigen is transported to entertain the GC reaction. One speculation has identified the SED region to be central in this process because the antigen is being taken up by the M cells and via transcytosis is delivered to DCs and B cells (126, 102, 105). Recently, it was demonstrated that activated antigen-specific B cells can recognize antigen delivered by the M cell without the involvement of DCs (105). This could be a critical pathway for the delivery of antigen to sustain the GC reaction in the PP. Alternatively, the more traditional interaction could occur in the SED region, i.e B cells may bind antigen presented on the surface of APCs (127, 128). Following antigen activation B cells can upregulate the chemokine receptor CCR7, which facilitates the migration via a chemokine gradient

toward the ligands CCL19 and CCL21 expressed in the T cell zone (129). T cells, however, can recognize only peptide-MHC complexes presented by APCs and get further activated via co-stimulation (130, 131). This initiates BCL-6 and CXCR5 upregulation and CCR7 down-modulation, leading to migration of activated T cells toward the T-B border where B and T cells engage via cognate interactions, i.e antigen-specific MHC-restricted interactions, and CD40-CD40L signaling (129, 132). Robust cell proliferation is initiated and those B cells that commit to GC, upregulate the transcription factor BCL-6 and migrate from the T cell zone into the B cell follicles, where they continue to proliferate (133, 134).
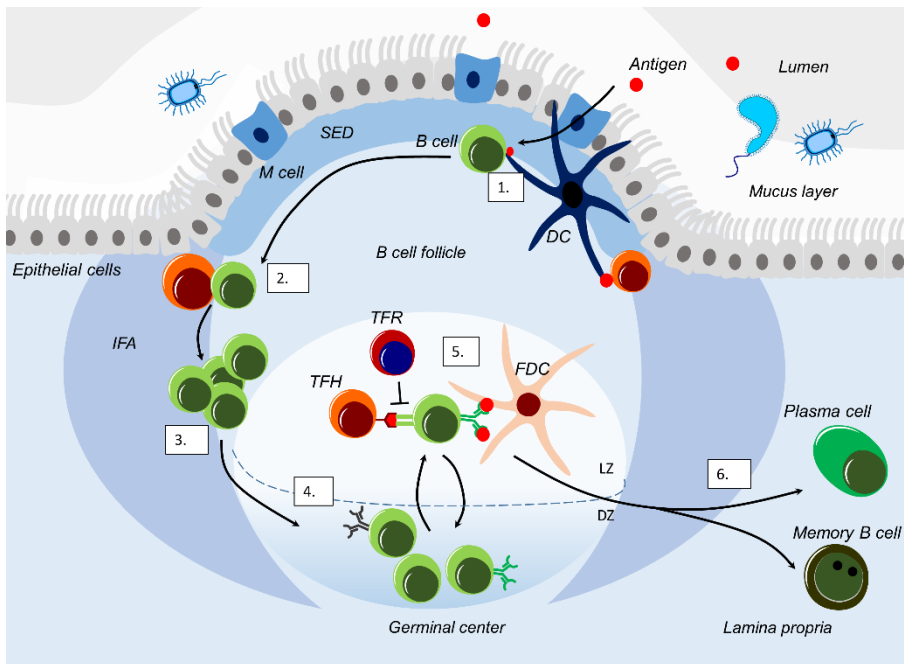
*Figure 2. **Germinal center reaction within PP**. Luminal antigen passes through the M cell and is taken up by DC in the SED region that further activates T cells and B cells (1). Activated B cell migrates to the T-B border to receive further activation signals from the pre-TFH cell (2). This initiates a vigorous B cell proliferation (3) and fate commitment to the GC. Proliferation continues in the DZ of the GC where B cells undergo SHM to enhance the BCR affinity (4). To test their newly formed BCRs and receive the survival signals, B cells migrate to the LZ (5). There B cell affinity selection takes place mediated by FDC, TFR and TFH cells. B cells might reenter the DZ to acquire new mutations for higher affinity. Those B cells that fail to mount an effective BCR undergo apoptosis. Successful GC reaction leads to the generation of the plasma cell and the memory B cell clones (6) that reside in LP. IFA – interfollicular area (T cell zone).*

The GC reaction shapes the humoral immune response to increase the affinity of antibodies over time (135). This increase is the result of AID-driven SHM of the antigen-binding variable regions of the Ig-heavy and light chain encoding genes (136-138). All GC B cells express AID, albeit in different amounts (139). Highly expressing B cells upregulate CXCR4 and continue to proliferate in the DZ where they undergo SHM (140). As a result, antigen receptor affinity is modified creating diversity within B cell pool. Activated B cells downmodulate CXCR4 and AID, but upregulate CD86 and CXCR5 to migrate to the LZ (140). Interestingly, most GC B cells degrade pre-SHM receptors before leaving the DZ, and those B cells that acquire crippling mutations do not reach the LZ. Instead, apoptosis is triggered limiting accumulation of B cell clones in the LZ by the elimination of non-functional BCR (141). The LZ is located close to the source of antigen and occupied with FDCs and TFH cells. It is the prime site for B cells to test their BCR affinity for the FDC presented antigens.

The GC is a highly dynamic structure, where B cells compete for survival signals (133, 140, 142, 143). Due to an enormous range of BCR affinities in the course of the GC reaction, some investigators have proposed that secreted antibodies actively participate in shaping the GC selection process by limiting antigen access. In this way, high-affinity IgA antibodies can substitute for low-affinity IgM or IgG antibodies and thereby establish a selection pressure for more IgA carrying B cells with a high-affinity BCR in the LZ of the GC (144). It is known that FDCs can also provide activated B cells with survival signals, such as BAFF, which would additionally promote high-affinity IgA B cells (145). To what extent the FDCs impact on the TFH population or function is not completely clear. B cells can present antigens obtained from the FDC network to activated CD4 T cells in the T cell zone facilitating TFH fate decisions. TFH cells, thus, can support maturation, differentiation and survival of the activated B cells via ICOS, CD40L, IL-4, and IL-21 production in the LZ of the GC (146, 147). Some activated B cells will re-enter the DZ for further affinity maturation and in this way generate even higher affinity, as seen in long-lived plasma cells. To prevent the generation of autoantibodies, T follicular regulatory cells (TFR) can dampen excessive GC responses (148, 149) by acting on B cells and TFH cells directly (150). TFR cells in the PP also may facilitate B cell maturation via IL-10 secretion (151). Those B cells that fail to receive survival signals in the LZ undergo apoptosis (152). Importantly, the GC output of activated B cells appears to be regulated by TFH-derived IL-21, which supports the production of plasmablasts and by APRIL that is derived from fibroblastic reticular cells located at GC-T zone interface (153). Weather B cell differentiates into plasma cell or memory B cell may depend on its BCR affinity, but also CSR and Tfh factors have been shown to be

instrumental to the B cell fate (154-157). Hence, multiple stages of the GC reaction are involved in the B cell commitment to long-lived plasma cell or memory B cell formation.

Uniquely to the PP, as opposed to other peripheral lymph nodes or spleen, the GCs are constantly present due to the microbiota (158). Because of this fact it has been hypothesized that newly activated B cells in the GALT can enter already existing GC, and in this way help synchronizing the IgA responses to select only high-affinity clonal repertoires (159, 160). A single GC can host many diverse B cell clones but the same B-cell clone most likely appears in the GCs of multiple PPs in the same mouse (160, 161). Another very unique feature to PP GCs is that they are dominated by a single isotype – IgA, however, to some extent CSR to IgG subclasses also occurs in the PP (162).

Class-switch recombination

In the bone marrow, B cells develop and acquire diversity – by recombining V(D)J gene segments into unique BCRs (163). These processes are regulated by RAG1–RAG2 endonuclease complexes, which generates an enormous diversity of antigen specificities among the newly formed naïve IgM and IgD B cells (164). The naïve B cells, upon leaving the bone marrow, migrate, among many different secondary lymphoid tissues, to the PP. In the GALT the main IgA CSR factor, TGF-β1, is produced in abundance (165, 166). Many cell types in the GALT are potential sources of this cytokine. In the PP GC, upon TLR activation, TGF-β is secreted by FDCs (145), also activated B cells (167), Foxp3$^+$ Tregs that are abundant in the T cell zone (168) and mucosal DCs (102, 169) produce TGF-β1. Although CSR has been viewed as a GC process, it is likely that IgA CSR also occurs at a pre-GC stage at the SED region or in T cell zone (101, 102, 170, 171). TGFβ is the major IgA CSR factor for both T cell-dependent and independent IgA responses (120). Noteworthy, in the absence of TGFβR only very few IgA-expressing B cells can be detected (166). Thus, it has been proposed that lymphoid as well as stromal cells can function as the major source of TGFβ1 in its inactive form. The latent form of TGFβ can be activated in several ways, one of which is the expression of the integrin $\alpha v\beta 8$ on DCs or Tregs, in particular (169). Besides the proliferation of the activated B cells, expression of AID, which can be induced by TLR stimulation must take place for successful CSR. Importantly, mucosal IgA CSR and IgA B cell development are influenced by several cofactors such as RA, vasoactive intestinal peptide, BAFF, and APRIL (121, 172, 173). Some of these factors can partly affect transcription of germline alpha transcripts via Iα, thereby enhancing TGF-β-induced transcription.

During the IgA CSR process DNA is deleted from the antibody heavy chain constant (C_μ) region to allow the juxtaposition of the IgA C region to the antigen-binding variable (V) region, which carries the gene sequences responsible for affinity selection. (174). C region determines isotype class and function. Each C region is accompanied by a switch (S) region. It is located 5' to each C gene of the heavy (H) chain and varies among different classes, but share short common, evolutionary conserved sequences (175, 176). Each S region is preceded by a short intronic (I) exon and a promoter that initiates germline C_H gene transcription when the B cell is exposed to activating stimuli. Various T cell cytokines have strong and opposing effects on the B differentiation and CSR processes, with IFNγ promoting IgG2a and IL-4 promoting IgG1 or IgA responses (177). In IgA CSR, TGFβ activates a signaling cascade that culminates with activation of a promoter upstream of the Cα genes (178-179). The activated TGFβ1 binds to the TGFβ receptor (TGFβR) on the B cell membrane and activates SMAD 2 and 3. These will then associate with SMAD4 and Runx3 that together bind to a tandem repeat element in the promoter forming α germline transcripts (180, 181). Signaling via CD40 in the presence of TFH induces AID expression in the B cell via the NF-kB pathway (182). Also, T cell-secreted IL-21 can promote AID expression (183). The switch process is controlled by AID-mediated deamination of cytosine residues in the C_μ S region (139), generating double-stranded DNA breaks. After looping-out and deletion of the intervening DNA segment, non-homologous end-joining pathways replace the C_μ region with C_α by joining distal S regions. CSR commonly occurs via direct C_μ to C_α replacement, but in some cases generation of high-affinity IgA antibodies may be sequential and occur via C_γ (IgG) CSR (184, 185).

CD4 T cells and helper functions

To ensure the gut homeostasis various T helper lineages are employed. Tolerance to the gut microbiota and dietary antigens is induced by distinct Treg subsets, high-affinity antibody production is mediated by germinal center T follicular cells, but Th17 cells seem to impose dual effector functions by supporting both tissue homeostasis and pathology (**Figure 3**). All of them have been ascribed as potent IgA inducers through the lineage plasticity and TFH fate commitment. In this section, I discuss the complex inductive pathways, phenotype, and functions of each effector subset.
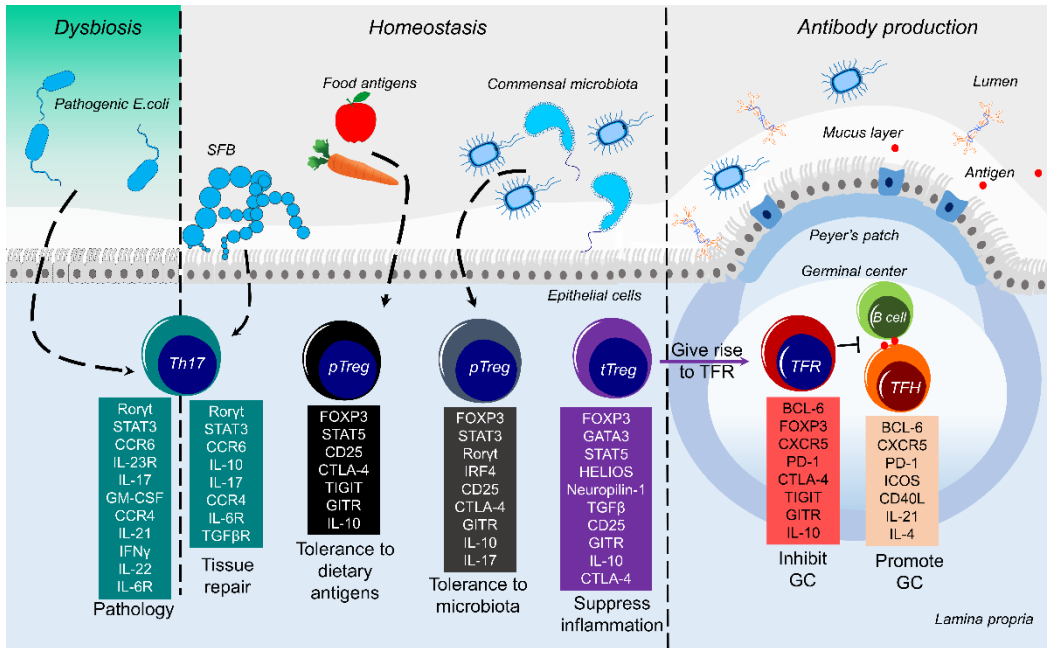
*Figure 3. **CD4 T cell subsets in the small intestine lamina propria.** T-dependent IgA induction is mediated by TFH cells in the germinal center via B cell affinity selection, maturation, and CSR. This process is kept under control by TFR cells that inhibit excessive GC reaction. TFR cells are derived from Thymus derived tTregs that reside in lamina propria. Peripherally induced pTreg lineage originates from commensal microbiota and food antigen stimulation. In turn, it controls intestinal homeostasis by immune suppression to these antigens. Th17 cells can be induced by commensal microbiota, mainly SFB, but also pathogenic invaders imprinting a dual role in the small intestine - under steady-state conditions they contribute to the homeostasis, however, upon pathogen invasion become destructive.*

Thymic selection

The ability of peripheral CD4 and CD8 T cells to respond to a wide array of foreign antigens while avoiding reactivity to self-antigens is largely determined by a selection process in the thymus. It starts with the migration of T cell precursors from the bone marrow. After the entry into the thymus hemopoietic precursor cells locate to the cortico-medullary junction where commitment to the T cell lineage is made (186, 187). Thus, it is the unique microenvironment in the thymus, that actively promotes the development of a diverse T cell repertoire, which is selected for recognizing foreign antigens while not responding to self-antigens.

The T precursor cells have the potential to differentiate into T cell that carries the antigen-receptor (TCR) of the αβ or γδ subtype. Notch signaling and

enhanced expression of CD117 drives theses precursors into a CD4/CD8 double-negative (DN) stage of T cell development (187, 188). The DN cells migrate through the cortex and begin TCR-β, TCR-γ, and TCR-δ gene segment rearrangements under the influence of RAG1 and RAG2 enzymes (189). A gatekeeper for further αβ T cell development is β selection, controlled by the pre-TCR, composed of the newly synthesized TCRβ chain associated with the invariant pre-TCR α chain and the CD3 complex. Only those cells that express in-frame TCRβ chains are allowed to escape apoptosis and undergo cell proliferation (190). In the subcapsular region, β-selection leads to the upregulation of CD4 and CD8 to enter a double-positive (DP) stage of T cell development with TCR-α gene rearrangements driven by RAG1 and RAG2 (189).

Once DP T cells have successfully rearranged their TCR α-chain to produce an αβ-TCR heterodimer they undergo positive and negative selection and migrate to the medulla. Cortical thymic epithelial cells express unique proteasome equivalent, the thymoproteosome, and L-cathepsin that together generate low potency peptides for the optimal positive selection of CD8 and CD4 T cells respectively. These peptides presented on MHC-I and MHC-II selectively bind to T cells with a matching αβTCR and in this way CD4 and CD8 T cell maturation and commitment is achieved. Whereas a weak TCR–pMHC interaction cannot prevent death by neglect an intermediate signal results in positive selection and promote the development of mature CD4 and CD8 T cells (191, 192). Negative selection is facilitated by thymic DCs that, although enriched in the medulla can bring self-antigens to the cortex to initiate thymocyte deletion of self-reactive T cells. In contrary to cortical thymic epithelial cells, those thymocytes that strongly respond to self-antigens are deleted, while low responders undergo positive selection and proceed to the medulla, which is enriched for thymocytes with a single specificity, but requiring an additional round of negative selection to secure central tolerance (193). Indeed, AIRE-expressing medullary thymic epithelial cells are loaded with tissue-restricted proteins, which are presented to single-positive thymocytes either directly or via thymic DCs that may also harbor imported blood-borne antigens. Besides, costimulatory ligands CD80 and CD86 are highly expressed on these APCs. A strong TCR signal leads to apoptosis in T cells. (192, 194). Once passed this stage, thymocytes receiving strong signals through TCR are not the subject of negative selection, but instead undergo agonist selection. While positive and negative selection mainly depends on thymocyte motility and TCR signaling, agonist selection is influenced by the timing of phagocytosis and cytokine environment. However, the precise mechanism is currently unknown (195). Nevertheless, agonist selection gives rise to Treg development.

Thymic Treg development requires strong TCR signaling, which leads to an upregulated high-affinity α-chain of the IL-2 receptor (CD25), and IL-2-induced signaling via activation of STAT5, which leads to expression of Foxp3 (196, 197). Thymic DCs are a potent source of IL-2 and appear to be the most effective Treg-inducers. This is a highly competitive process because IL-2 supplies are limited (198). Those Treg progenitors that fail to compete for IL-2 may become conventional autoreactive T cells, which are kept under control of bona fide Tregs in the periphery. Similarly to class II-restricted negative selection of thymocytes that occur in a specific time-frame, Treg commitment takes place only in immature single-positive CD4 T cell stage (199). However, mature single positive CD4 T cells can turn into Tregs as they respond to antigen-activation in the periphery, forming pTregs.

Dual TCR expression

If we consider that the fate of the individual B cells is imprinted in the BCR similar reasoning can be applied to TCR signaling and individual T cell effector functions (200, 201). As single TCR expressing cell can recognize only one peptide, but cells that allow rearrangement of the α-chain and pair that with the β-chain can potentially express two different surface TCRs. It is estimated that under natural conditions as many as 30% of all αβ T cells can express dual TCRs with specificities to two different peptides (202, 203). During thymic selection, TCR β-chain allelic exclusion is stringent, sequential and a multifaceted process preventing simultaneous rearrangement of both alleles. Hence, thymocytes expressing two functionally rearranged TCR β-chains are rare (204-206). By contrast, TCR α-chain rearrangements can occur simultaneously on both alleles resulting in dual TCR expression (207, 208). While TCR β-chain rearrangements are initiated in the DN stage of thymic selection and stops with a formation of successful in-frame TCR-β chain, TCR-α rearrangement starts at the DP stage and continues until the thymocyte has rearranged both αβTCR chains or the cell dies from neglect.

Even though the absence of allelic exclusion of the TCRα rearrangements results in dual TCRs expression at the gene transcriptional level, only about one-third of these T cells express 2 functional TCRs. It could be that TCR α-chains compete for pairing with TCR β- (or CD3) chains and that the more favorable pairing of α and β chains dictates whether the complex should appear on the surface or not (209). Alternatively, only one TCR α-chain might transduce signals when engaged, which could promote its surface expression and prevent its internalization (210). In mature T cells, the TCR α and β chains are recycled to the cell surface, and therefore, if the allelic exclusion is maintained, the selected TCR continues to receive low-level stimulation. These

T cells have a disproportionate distribution of TCRα chains on the surface, while cells that carry dual TCRs have similar levels of both the TCRs. Such T cells are functionally specific for two different epitopes (211). TCRs recycled from the cell surface can serve as an intracellular store of functional TCR that can be rapidly directed to the immune synapse after ligand engagement of surface TCR (212). It is, however, unclear to what extent the TCRs are interconnected and inhibition of signaling from one TCR may potentially result in the down-modulation of both TCRs (213). Alternatively, the two TCRs can function independently of each other and without cross-regulation (214).

Interestingly, dual TCR expression seems to be more common in Tregs than in other subsets. On the other hand, dual TCR expression on CD4 T cells has been found to inhibit Treg development, which indirectly could promote an autoimmune phenotype (215). These cells could potentially increase the risk for the development of an autoimmune condition as they allow self-reactive T cells to escape thymic deletion through selection on their non-self-reactive TCR. TCR-Tg mouse models have shown preferential expansion of CD4 T cells in the gut of unstimulated mice, suggesting that these cells are capable of reacting to microbial antigens (211). Recently SFB-induced Th17 cells with dual TCR were shown to severely augment lung autoimmunity (216). Besides autoimmunity, dual TCR T cells could also be alloreactive (217-219). Their role in GVHD is so fundamental, that genetic elimination of secondary TCRs could reduce in vitro alloreactive responses to MHC-mismatched cells by over 40%. Similarly, when stimulated with alloantigens, dual TCR CD4 T cells preferentially expand as compared to non-specific stimulation via anti-CD3 and anti-CD28 mAbs. It has been estimated that dual TCR T cells may comprise as many as 75% of all peripheral T cells in the mouse with severe acute GVHD (218, 220). Of note, the co-expression of dual TCRs by a single T cell is the result of the normal allelic inclusion process. Virtually all humans have dual TCR T cells and it has been suggested that under normal conditions they are beneficial and may extend the immune repertoire to foreign antigens (221).

Tregs

Homeostasis is perhaps the most important function to maintain by the immune system. It is achieved by the vast distribution of Tregs in various tissues. Tregs have a stable expression of the master gene regulator Foxp3, an X chromosome–encoded member of the forkhead transcription factor family, and the IL-2 receptor α-chain CD25. These cells emerge from the thymus with an imprinted mission to suppress B and T cell activation and function (222-224). The absence of Tregs leads to autoimmunity at an early stage of life (225).

Foxp3 is a lineage defining transcription factor and its continuous expression is essential not only for the Treg phenotype but also for the suppressive function (226). Interestingly, ablation of a conditional allele of Foxp3 in cells other than Tregs, such as granulocytes, APCs or epithelial cells does not have any functional consequences, hence, Foxp3 in Tregs is essential for the control of autoimmunity (227). Importantly, enforced expression of Foxp3 in naive $CD25^-CD4^+$ T cells will result in the acquisition of a Treg phenotype and function, with the expression of CD25, CTLA-4, and GITR (223, 224). Using genetic cell-fate mapping it has been found that Tregs are very stable and the maintenance of Foxp3 expression is heritable over the lifespan under physiological conditions (228). However, in humans, transient upregulation of Foxp3 can be observed also on activated conventional T cells (229). While Foxp3 expression is needed for the suppressive activity and lineage stability, CD25 expression is essential for self-maintenance of Tregs. Thus, isolated Tregs are unable to proliferate in response to TCR stimulation, while concomitant IL-2 signaling is necessary for Treg proliferation (230). Due to robust expression of CD25 on all activated T cells, IL-2 signaling via Tregs can limit IL-2 availability for conventional T cell maintenance and serves as a mechanism of suppression (231).

While thymus-derived Tregs (tTregs) recognize self-antigens, CD4 T cells in the periphery after responding to foreign antigens can develop into Tregs, termed peripheral Tregs (pTregs). The function of pTregs is to limit responses to food antigens and commensal microbiota. Phenotypically, they can be distinguished by the lack of expression of Neuropilin-1 and Helios that are upregulated on tTregs (232, 233). Whereas differentiation of tTregs depends on high-avidity interactions with self-peptide-MHC class II complexes and IL-2 receptor signaling, pTregs develop from naïve T-cells that respond to foreign antigen under tolerogenic conditions, i.e. strong TCR signaling, suboptimal co-stimulation, and high concentrations of TGFβ and RA (234, 235). The gut is the main site of pTreg differentiation due to the abundance of microbial and dietary antigens (236). $CX3CR1^+$ macrophages capture antigens by sending protrusions into the gut lumen and deliver antigens to classical DCs (237). Upon the upregulation of CCR7, these DCs can migrate from the intestinal LP to the draining MLN where the generation of antigen-specific $Foxp3^+$ Tregs takes place (18, 24, 238). Classical DCs can produce high levels of RA that together with TGF-β promotes activated $CD4^+$ T cells to acquire Foxp3 expression (24). RA also promotes the induction of gut-homing molecules on pTreg cells such as integrin α4β7 and chemokine receptor CCR9 and facilitates their migration back to the gut mucosa. Tregs specific for dietary antigens preferentially home to the small intestine while those specific for microbial antigens end up in the colon.

Commensal bacteria *Bacteroides fragilis* and *Clostridium* species have been shown to promote pTreg differentiation and their accumulation in the colon (25, 239). Interestingly, *B. fragilis* can induce IL-10 secretion from Tregs and DCs mediated by a single bacterial component – namely polysaccharide A (239). *Clostridium* bacteria also stimulate IL-10 and TGFβ secretion from epithelial cells (25). The underlying mechanism is dependent on short-chain fatty acids, particularly butyrates, during the starch fermentation process (240). Butyrates can bind to the intronic Foxp3 cis-regulatory element, CNS1, and facilitate the increased expression and enhanced stability of Foxp3 (240, 241). In the early phase of differentiation pTregs are less stable, due to decreased levels of MicroRNA-10a (242) – a potent suppressor of the pro-inflammatory program - and a lack of stable expression of Foxp3, which is acquired over time and requires demethylation of the Foxp3 cis-regulatory element, CNS2 (241). Importantly and contrary to tTregs, pTreg-depletion does not lead to multi-organ autoimmunity or uncontrolled pro-inflammatory responses. However, mice deficient in pTregs develop pronounced Th2-type pathologies at mucosal sites which can lead to allergic inflammation, asthma or altered gut microbial communities (243, 244). Majority of colonic pTregs that are induced by *Clostridium* species, strongly express RORγt, a molecule that normally antagonizes Foxp3-induction. Such cells have enhanced suppressive capacity during T-cell-mediated gut inflammation (244, 245). Although the vast majority of Tregs in the GALT react to dietary antigens or the microbiota, a fraction of GATA3$^+$ tTregs can also be found. They seem to respond to intestinal tissue damage and occupy a unique, MHC II-independent niche in the intestinal LP (246). Therefore, it appears that the microbiota is not only important for the induction of pTregs but also the maintenance of gut-resident tTregs.

Treg-mediated immune suppression is central for clearance of inflammatory responses, but also has an impact on numerous autoimmune disorders, cancer, and allergy. Apart from IL-2 consumption, the suppressive effect of Tregs is orchestrated by a highly expressed ectoenzymes CD39 and CD73, which convert extracellular ATP to adenosine and directly inhibit effector T cell proliferation and APC functions. Tregs also secrete other immunomodulatory molecules, such as IL-10, TGF-β, and granzymes, that control unwanted immune reactions (247). Moreover, high expression of CTLA-4 can suppress CD80 and CD86 co-stimulation and reduce the priming efficiency of DCs (248). Besides suppressive activity, Tregs in the gut have been associated with IgA B cell responses. They were suggested to drive IgA CSR and TFH functions in the PP (1, 2, 66). Besides, tTregs can give rise to TFRs that dampen excessive GC response and contribute to the maintenance of balanced microbiota (66). With their multiple effector mechanisms and involvement in

many different immunological conditions, Tregs are central for gut immune homeostasis and appear to be influential in gut IgA response.

Th17 cells

Th17 cells are best known for driving autoimmunity and various inflammatory disorders including the immunopathology of inflammatory bowel disease (IBD) (249, 250). On the other hand neutralization of IL-17 has been found to exacerbate disease symptoms in patients with Crohn's disease, suggesting a possible protective role of Th17 cells (251). Indeed, IL-17-producing Th17 cells that line the gut mucosa do not induce inflammation but are necessary to maintain the intestinal barrier functions and reduce effector T cell-driven intestinal inflammation (252). Emerging evidence has pointed to the beneficial effects of Th17 cells in protection against fungal, bacterial and virus infections. Recently their impact on the microbiota has also been documented (253). Th17 cells can regulate pIgR-mediated transport of IgA in a response to IL-17, therefore orchestrating mucosal immune responses and promoting clearance of pathogens (110). They are also involved in maintaining an effective barrier function as injured epithelial cells promote the accumulation of Th17 cells by secreting large amounts of IL-6 (254). Hence, the Th17 subset is central for our understanding of how homeostasis and protection can be maintained in the gut mucosa.

The seemingly opposing nature of Th17 cells could be explained based on two subtypes, one with a pro-inflammatory effect and the other with a tissue homeostatic function (255). Th17 cells develop in the presence of TGF-β1 and IL-6, they co-produce IL-17 and IL-10 and do not drive tissue inflammation, but upon exposure to IL-23, a "switch" occurs, which converts the non-pathogenic cells to become pathogenic (256). By dampening tolerogenic IL-10 production, IL-23 rich environment promote IL-22 and GM-CSF secretion by Th17 cells which completely changes the gene transcriptional program in favor of pro-inflammatory phenotype (256). Whereas homeostatic subtype does not drive tissue inflammation, pathogenic Th17 cells acquire plasticity and practice aerobic glycolysis in addition to oxidative phosphorylation typical for inflammatory effector cells (255). The microbiota plays a critical role in the generation of homeostatic Th17 cells in the gut LP and the most potent inducer is SFB (257). In humans, *Bifidobacterium* species, *Citrobacter rodentium*, and even *Escherichia coli* are reported to promote Th17 differentiation (258, 259). Fungi such as *Candida albicans* and *bacteria Staphylococcus aureus* are pathogenic to humans and mice but are both effective at promoting pathogenic Th17 responses, albeit with different Th17 cytokine profiles. While fungal antigen-loaded APCs have extensive IL-12,

IL-23, IL-6, and prostaglandin E2 production to initiate pathogenic IFNγ and IL-17 producing Th17 cells, *S.aureus* promote more of a "homeostatic" IL-10 secreting cells (260, 261). This demonstrates how evolutionary pressures have fine-tuned different effector cell functions for clearing pathogens in the context of tissue inflammation and utilized the same cells for the induction of tissue inflammation or protection.

The induction pathway of Th17 cells in the gut resembles and overlaps with that of pTreg, Th22 and Th1 cell induction. This introduces plasticity and heterogeneity within a lineage that further may contribute to pathology or a more flexible immune response. While, RORγt and STAT3 are essential transcription factors required for Th17 lineage differentiation, RORα, AhR, IRF4, BATF, and Runx1 are also required to regulate optimal development (262-267). IRF4 and BATF are thought to act downstream of the TCR signaling and initiate a Th17 gene transcriptional program (267). IL-6 is a critical differentiation factor for the generation of Th17 cells (268). Binding of IL-6 to its co-receptors IL-6R and gp130 results in activation of STAT3, which induces IL-17 via activation of RORγt. IL-6 also induces the production of IL-21, which in turn, promotes the expression of IL-23R. This way IL-21 does indeed promote Th17 pathogenicity in autoimmune diseases by enhancing the effect of IL-23, which promotes Th1 competence of the Th17 cells (249, 268). A strong repressor of the Th17 differentiation program is RA that stimulates Treg development. RA dominance in the intestinal environment strongly supports STAT5-dependent FOXP3-activation, which can be overcome to some extent by IL-6 (269). In other tissues where the presence of RA is decreased, the IL-6 and STAT3 signaling pathways are an effective repressor of Foxp3. Another very essential cytokine for Th17 commitment is IL-1. IL-1β alone induces IL-17 and RORγt in activated naïve human CD4 T cells and overrides RA mediated Th17 suppression (270, 271). Furthermore, IL-1β is critical in dampening a dominant STAT5-signaling that supports a tolerogenic environment in the gut. Treg and Th17 differentiation, however, is interconnected via TGF-β (272). It appears, that homeostatic Th17 cells, in particular, benefit from TGF-β signaling, by suppressing or restricting high T-bet induction that is associated with the pathogenic phenotype (273). However, it is premature to ascribe the actual contribution of TGFβ to a more homeostatic-prone Th17 development, as TGFβ is expressed by many different cell types and is present in serum.

The strong association between Th17 cells and autoimmune disorders can be ascribed to the importance of the IL-17 family cytokines, IL-17A and IL-17F, as well as the production of IL-22 and GM-CSF, which lead to neutrophil recruitment to the tissue (274). A similar effect on myeloid cells has Th1 and

Th2 activity that recruits macrophages and eosinophils respectively (275, 276). However, autoimmune disorders are more common in females than in males, therefore Th17 cells might be more prone to sex hormone influences. An increased prevalence of Th17 cells has been found in female patients with severe asthma (277). Also, an excess of Th17 cells during pregnancy has been associated with a higher risk for the child to develop autism-like symptoms (278, 279).

CD4 T cells in the germinal center

Two major kinds of CD4 T cells are located inside the GCs: The T follicular helper (TFH) cells (280, 281), and the T follicular regulatory (TFR) cells (282, 283). Similar to GC B cells, the generation of TFH and TFR cells depends on the induction of BCL-6 and CXCR5 that promotes their positioning in B cell follicle and GC (284-286). The TFH function is to help B cells to expand and differentiate in the GC and this involves the expression of CD40L, ICOS, PD-1 and OX40 and production of IL-21 and IL-4 (147). TFR cells, on the other hand, phenotypically resemble both TFH and extrafollicular Tregs and, therefore in addition to BCL-6 and CXCR5, express FOXP3, GITR, CTLA-4, and TIGIT (282, 286). TFRs do not provide co-stimulation nor signaling via CD40L or OX40 but produce mainly IL-10. Thus, the dominant paradigm today is that TFRs moderate TFH and GC B cell responses and help to promote high-affinity B cell differentiation in the GCs (150). Uncontrolled TFH activity can lead to autoimmunity, but TFRs act to prevent it. PD-1 is highly expressed on TFH and TFR cells and works as a negative regulator of an excessive GC response. TFR cells may inhibit TFH recruitment via PD-1 by limiting CXCR3 (287). Besides, Tregs and TFRs express high levels of CTLA-4 that controls TFH cell responses and GC development via down-modulation of CD80 and CD86 expression on APCs (288). Hence, the loss of TFR cells is associated with a higher risk for autoimmunity (148, 149). Recent findings, however, suggest that TFR derived IL-10 promotes GC growth and enhances B cell entry into the DZ for high-affinity BCR acquisition (151).

TFR differentiation form tTregs is largely unexplored, however, it involves TCF-1. Indeed, activated Tregs express TCF-1 and LEF-1 that are essential for maintaining an optimal Treg pool and promoting competitive survival by supporting the development of TFRs. Loss of TCF-1 and LEF-1 completely aborts TFR generation and leads to autoimmunity caused by unrestrained TFH and GC B cell responses (289). Also, c-Maf appears to be critical for TFR development. It is induced via IL-6 and TGFβ–dependent manner and controls not only TFR differentiation but also other effector Treg subsets emerging from tTregs (290). However, the hallmark of TFR cells is their ability to

express master regulators of Treg and TFH subsets. As of yet, it is not clear what cytokine signature induces and maintains the co-expression of BCL-6 and FOXP3, but IL-21 suppresses Treg and TFR differentiation in favor of Th17 and TFH cells (291). Also, IL-6 induces destabilization of Treg lineage, which is counteracted by Blimp-1 (292). Uniquely, TFRs can co-express the transcription factors BCL-6 and Blimp-1, even though they take part in a negative feedback loop (282, 286). TFR differentiation seems to be a multistage process ultimately leading to CD25$^-$ TFRs. In comparison to CD25$^+$ pre-GC TFR and effector Tregs, CD25$^-$ TFR cells partially down-regulate IL-2-dependent canonical Treg features, but retain suppressive functions, while simultaneously up-regulating genes associated with GC-TFH cells (293). While TFRs develop in a polyclonal and Ag-independent manner from tTregs the TFH cells are Ag-specific CD4 T cells that proliferate after Ag stimulation, (282, 286, 294). So far, a single study has challenged this notion by proposing that TFRs can be generated from conventional CD4 T cells (295).

In human lymph nodes, it was shown that the majority of TFR cells reside at the border between the T cell zone and B cell follicle and express low levels of PD-1. Only very few are found in GCs and express higher levels of CD38, CTLA-4, PD-1 and GARP, a TGFβ associated molecule, but both populations seem to suppress antibody production in vitro (296). Interestingly, unlike TFH, the TFR gene transcription profile is very different between peripheral lymph nodes and the PPs. It appears that PP resident TFR cells are largely unresponsive to IL-2 and express high levels of *IL10*, *IL4* and to a lesser extent also the *IL21* gene. Secreted IL-4 and IL-21 appear to control transcription factors BATF1 and c-maf (297). This information suggests that the classical tTreg-TFR developmental axes do not fully apply to the unique environment of the PP and could be complemented with TFR generation from conventional T cells (295). Indeed, it has been demonstrated that TFRs influence the intestinal microbiota, suggesting that they originate most likely from pTregs (66).

Plasticity is a cardinal feature of CD4 T cell lineages that often results in TFH differentiation (1, 3). On the contrary, TFH can fulfill the pre-stage function before further commitment to a certain effector lineage (299). Initiation of a TFH program starts with the cognate activation and co-stimulation via CD80, CD86 and ICOSL expressed on the DC. ICOSL binds to ICOS on pre-TFH cells, a molecule, that however not restricted to TFH has an important role in lineage development and maintenance. Signals through ICOS-ICOSL are also critical for TFH mediated B cell survival and activation, antibody class switching and GC formation. Upon initial activation in cytokine environment of IL-1β, IL-6, IL-12, IL-21, IL-23, and TGF-β, T cells mount BCL-6

expression program that further promotes transcription of PD-1, CXCR4, CXCR5, and SAP. Cytokine gradient is very important because the early activation events are shared by multiple T cell lineages. Besides, it differs between species, because TGF-β is a negative regulator in mice but a positive regulator in human TFH cells. Once BCL-6 is upregulated that initiates cell migration to GC by allowing for high expression of CXCR5 and reduced CCR7 (280, 300). CXCR5 guides cells to CXCL13 rich FDC areas in B cell follicle, while a reduced CCR7 expression allows cells to leave the paracortical T cell zone.

BCL-6 is an enhancer of expression of several migration-related genes and a lineage-defining transcription factor that regulates early differentiation of TFH cells in an IL-21- and IL-6-independent manner (285, 301). The antagonist to BCL-6 is Blimp-1, which has an inhibitory effect on BCL-6 expression and, thus, suppresses TFH differentiation (302). Because Blimp-1 has a critical role in the differentiation of other T cell lineages one can speculate that TFH develops independently of these effector subsets. Another BCL-6 inhibitor is STAT5. Signaling via IL-2-CD25 activates STAT5 and inhibits BCL-6 and CXCR5 via Blimp-1 induction, but lack of IL-2R leads to BCL-6 expression (303). BCL-6, however, abrogates STAT5 phosphorylation (304). Also, Roquin negatively regulates TFH development. It represses ICOS and other TFH related gene expressions. The combined loss of Roquin-1 and 2 results in spontaneous TFH and GC development (305). TFH development closely resembles that of Th17 cells. Indeed, IL-21 and IL-6/STAT3 were first described to be essential for Th17 cell differentiation. Because STAT3 can form a complex with the Ikaros zinc finger transcription factor Aiolos, which regulates BCL-6 expression, STAT3-deficiency is detrimental to TFH development (306, 307). In the early stages of TFH development IL-6, but also TGFβ in humans is important for driving STAT3 and STAT4 activation of the TFH differentiation program (308). Another transcription factor c-Maf is highly expressed in both mature TFH and Th17 cells. It increases the production of IL-21 and the expression of CXCR5. In cooperation with BCL-6, it upregulates ICOS, PD-1, and CXCR4, but the loss of c-Maf leads to the inhibition of TFH cell differentiation (309, 310). Also BATF, which regulates BCL-6 and c-Maf expression and IRF4 that strongly promotes IL-4 production are typically associated with Th17 cells (311-313). The final fate decision of either TFH or Th17 differentiation is controlled by TCF-1. It positively affects BCL-6 gene expression but prevents IL-17 and Blimp-1 induction (314, 315). Besides, it upregulates IL-6R indicating that TCF-1 might act upstream of STAT3 and STAT5 (316). Importantly, TFH development does not stop with the lineage commitment phase as it continues inside the GC and is guided by BCL-6. First, TFH cell localizes close to mutating B cells, secrete IL-21 and

act to select high-affinity B cell clones, but in due course the TFH cells switch from IL-21 to IL-4 production and express more CD40L to promote B cell maturation towards antibody secretion (plasma cell stage) (147). Hence, in a given GC the TFH cells can represent different stages of their functional repertoire.

CD4 T cell plasticity

For long it was assumed that lineage commitment is a stable phenomenon. However, several studies have challenged this notion and demonstrated that CD4$^+$ T cell subset fates are not final, but rather flexible, and allow for several functional outcomes. Such plasticity appears to be particularly prominent in the GALT. Although Th17, Treg, TFR and TFH cells are present in the GALT, the most critical T cell in B cell responses is TFH. Whether this cell can provide the switch factors for IgA CSR is poorly understood. Both Tregs and Th17 cells have been implicated in IgA responses and both have been claimed to undergo extensive adaption to acquire TFH function (1, 3). Earlier studies have suggested that IgA induction relies on two distinct types of T cells – "helper cell", that expands the B cell population and a "switch cell", that does not perform the helper functions, but ensures IgA CSR (317). It is unclear, whether TFH cells can also perform the "switch cell" functions.

Treg transformation into TFH cells has collected much support over the years and IgA-Treg- axes were suggested by several investigators (2, 66). Induced pTregs are the result of naïve helper T cell exposure to metabolites of commensal bacteria and therefore essential for intestinal homeostasis (25, 239). Exclusively to PP, Tregs were shown to down modulate their suppressive program and acquire the TFH phenotype for enhanced IgA production (1). This observation conflicted with that of Rudensky and colleagues, who demonstrated that under physiologic as well as inflammatory conditions Tregs are stable (228). Treg-plasticity could be attributed only to a small population of pTregs that lack CNS2 demethylation for stable Foxp3 expression (241). Also, a low level of microRNA miR-10a has been shown to promote plasticity. Robust expression of miR-10a in tTregs effectively suppresses TFH conversion by dampening BCL-6 (242).

In the context of IgA induction, it is important to know whether the feature of IgA CSR resides in the Tregs that convert to TFH phenotype or whether there is a separate CD4 T cell subset that controls this response. "Switch cell" must either provide TGFβ or its activators, such as integrin αvβ8. So far, no investigator has demonstrated that the two functions; an initiator of IgA CSR and the helper functions of TFH are hosted by the same cell, be it Tregs

converting into TFH functions or some other constellation of CD4 T cells in the PP. In this context, it is interesting to note that also Th17 cells have been described as highly plastic cells in the PP (3). Hirota et al. reported that Th17 cells, but not Tregs, upon transfer to a TCRα$^{-/-}$ host converted to a TFH phenotype and supported gut IgA responses (3). Upon adoptive transfer of Rorc$^{-/-}$ CD4 T cells, oral immunization with CT failed to stimulate IgA induction, suggesting that Th17 cells are critical for the gut IgA response (3). However, it should be noted that microbiota-specific Th17 cells have been found to contribute to intestinal homeostasis by regulating intestinal pIgR expression and promoting IgA transport to the lumen (110). These contradictory results could potentially be due to different model systems or the composition of the microbiota in the animal facilities. Importantly, Treg –Th17 axes have also been described. The decision of an antigen-stimulated cell to differentiate into Th17 or Treg cells depends on the cytokine-regulated balance between RORγt and FOXP3 (318, 319). However, such balancing may also promote the increased frequency of highly suppressive pTregs that coexpress FOXP3 and RORγt (245).

It is unclear whether plasticity and a highly dynamic environment can explain how IgA responses are regulated. Nevertheless, a better understanding of the cellular interplay in the PP awaits to be further investigated. In this doctoral thesis, I have tried to dissect the Treg, TFH and Th17 lineage contributions to IgA induction following oral immunization.

I honestly believe it is better to know nothing than to know what ain't so.

/Josh Billings, 1874./

AIM

The overall aim of this thesis was to investigate the requirement of specific CD4 T cell subsets for the induction of gut LP IgA responses following oral immunization.

Specific aims:

1. To examine which CD4 T cell subsets are involved in IgA class-switch recombination and IgA plasma cell responses in the gut (Papers I and III)

2. To study the natural suppressive Treg element known to govern oral tolerance and to investigate which mechanisms of action the CT adjuvant uses to overcome or break this suppressive state (Paper II)

3. To identify CTB-specific CD4 T cell subsets in the PP following oral immunization and to compare them with the overall (non-CTB specific) population using single-cell RNAseq analysis of sorted CD4 T cells from PP (Paper III)

Questions and Hypothesis

To induce a successful gut IgA response following oral immunization it is essential to stimulate strong antigen-specific TFH cells in the PP. However, conflicting findings have been reported concerning the precursor cells for the TFH population. Do TFH cells originate from highly plastic Th17 or Treg subsets in PP? Can TFH cells provide necessary functions to drive IgA CSR? Given that the gut IgA responses in IL-17$^{-/-}$ mice are comparable to WT, my doctoral research was based on the hypothesis that Th17 cells are not necessary for gut LP IgA induction, but Tregs, due to reported lineage stability, do not develop into TFH cells. The present study is an attempt to dissect the regulatory CD4 T cell requirements in the PP for gut IgA immunity and challenge current dogmas.

KEY METHODOLOGIES

This section gives a general overview of the methods and techniques that were used in this doctoral thesis work. A more detailed description of the experimental approach can be found in the *Materials and Methods* section of each paper.

Mice

Research in immunobiology is powerless in the absence of in vivo model systems. Due to ethical concerns, permits are granted through a tightly regulated process, but studies must be performed with the greatest respect for the animals used and the people involved. In this doctoral study, I have used several mouse strains as models to investigate the regulation of gut immunity. There are many advantages in using murine models, in particular, the rapid reproductive cycle allows for the development of genetically defined and/or crippled models with, if not identical, highly similar immune system to that of humans. However, unlike humans, these mouse strains are genetically inbred for the desired gene mutations or expression of specific transgenes. Therefore, unlike humans, they are poor models of genetic diversity, which is also their strength as we can address complex questions in a genetically more restricted animal than we normally do in a human test situation. Throughout my doctoral research I have used mainly 3 different murine models on either C57BL/6 (H-2^b) or Balb/c (H-2^d) backgrounds – WT mice, albeit inbred mice, represent some sort of naturally occurring biological diversity of the immune system; OVA TCR-Tg mice DO11.10 (H-2^d) and OT-II (H-2^b) carry an MHC-II restricted TCR for the Ovalbumin (OVA) peptide p323-339; and athymic nude (nu/nu) mice that completely lack mature T cells. OVA TCR-Tg mice were mainly used as T cell donors for intravenous injections of CD4 T cells into nude or WT mice. CD4 T cells from DO11.10 mice can be easily traced with KJ1-26 mAb that specifically binds to transgenic TCR Vα13Vβ8.1. OT-II mice, however, are crossed onto a congenic mouse strain expressing the allelic variant of CD45.1. WT mice express CD45.2, therefore transferred cells can be found by using anti-CD45.1 mAb. Additionally, in paper I, B1-8^{hi}-GFP (H-2^b) mice carrying B cells with an Ig lambda-chain of a very high affinity for the NP hapten were utilized. Also, germ-free mice (H-2^b) completely lacking microbiota were used. These mice were kindly provided by Fredrik Bäckhed's group. In paper II, IL-10KO (H-2^b) mice that have aborted IL-10 production and BATF3$^{-/-}$ (H-2^b) mice that are deficient in cDC1 cells (CD103$^+$CD8aa$^+$ dendritic cells) were employed in separate experiments. IL-10KO mice were generously offered by Gunnar Hansson. Finally, in paper III, an IL-17 fate

reporter mouse was used, IL17^{cre}eYFPxRosa26 (H-2^b), a kind gift from Birgitta Stockinger, London, UK. This mouse permanently marks IL-17 expressing cells with YFP protein. Besides, Rorc$^{-/-}$ (H-2^b) mice that completely lack GALT, ILC3 and Th17 cells were used.

Adoptive transfer and immunization

Adoptive transfer of traceable B cells or T cells into a recipient host mouse is a powerful tool to study individual cells in an immune response. Throughout this doctoral thesis work, nude mice and occasionally also WT mice have been used as recipients of the transferred cells. Enriched donor cells ranging from 0.5 to 2 million per i.v. injection via tail vein were used. After 24h mice were orally immunized with 10 mg OVA alone or together with 10 µg CT adjuvant in 3% NaCHO$_3$. This way donor cells have a time window between the transfer and the first immunization to find their niche. Effective B cell responses required initial immunizations within 24 h post-transfer and p.o immunizations had to be given at least twice. However, most oral immunization protocols hold three doses given with ten days apart. Six days after the last immunization mice were sacrificed and IgA responses were analyzed. The adoptive transfer model followed by oral immunizations is used in all 3 papers.

Antibody detection

ELISA is a robust assay for measuring secreted antibodies in various biological fluids or cell culture supernatants. Effective oral immunizations lead to massive B cell responses in GALT, which are detectable not only in the gut LP but also at systemic sites, such as spleen and serum. For this reason, intestinal lavage and serum samples were commonly harvested to study antibody production by ELISA. Briefly, plates were coated with the immunizing antigen one day before the assay was performed. The next day, plates were blocked using 0.1% BSA to avoid unspecific binding and samples were added. All specific antibodies within a sample specific for the coated antigen, irrespective of isotype, bind to the antigen-coated plate. After 2-3 hours plates were thoroughly washed and developing antibodies of specific isotype were added. This way all the excess "non-specific antibodies" are washed away but developing antibodies can detect specific plate-bound antibodies of a given isotype. ELISA results were given in the log10 titer. For a total isotype measurement within a biological sample, mostly culture supernatants, internal standards were used. This way, results are more specific and given relative to an internal standard concentration, usually in pg/ml.

ELISPOT is a highly quantitative assay which uses antigen-coated plates and freshly isolated mononuclear/antibody-secreting cells from the LP or SP. A fixed number of isolated cells were added into the coated wells and after 3h incubation at 37°C, 5% CO_2 individual coated antigen-specific antibody-secreting cells were detected as spots. Simple recalculation allows expressing obtained results as spot forming cells (SFC)/10^7 mononuclear cells. However, a good cell isolation technique is central to an accurate result, which applies, in particular, to gut LP preparations. Isolation procedure employs repeated rounds of enzymatic tissue digestions and mechanical disruptions, to achieve a sufficiently high quality of isolated cells, therefore protocol optimization is particularly important to allow for reproducible results.

Flow cytometry

Cell phenotypic characterization is central to immunological research and also to the work performed for this thesis. Cell surface molecules, intracellular cytokines, and intranuclear transcription factors can all be defined by flow cytometry. This technique uses fluorochrome-labeled monoclonal antibodies reactive to specific cell proteins that can function as markers for cell identity or function. Using flow cytometry labeled cells pass through a laser beam that at a certain wavelength excites the fluorochrome that in turn emits light at a higher wavelength. Flow cytometry utilizes systems with multiple lasers, but each laser can excite several fluorochromes with distinct emission spectra. The emitted light is separated by various wavelength-specific mirrors and filters and recorded by detectors. Cell size and shape gives an additional layer of information defined by light scatter. This allows to identify distinct leukocyte populations within a sample based on size and granularity. Such technology permits the simultaneous analysis of multiple target molecules on a single cell. Throughout my doctoral research flow cytometry has been the most used technique. Adoptively transferred CD4 T cells were characterized into TFH, TFR, pTreg, tTreg and Th17 cell lineages based on cell surface markers PD-1, CXCR5, CD25, GITR, CTLA-4, Neuropilin-1 and intranuclear transcription factors BCL-6, FOXP3, Rorγt, and HELIOS. Also in flow cytometry, the quality of the isolated cell preparation is critical for an optimal analysis; dying cells are more prone to auto-fluorescence and have abnormal protein composition, therefore, only live cells at the time of surface staining should be analyzed. I used the DNA binding dye 7AAD to exclude dead cells. However, in the case of intracellular/intranuclear staining membrane dyes were used. They are less bright and require a longer staining protocol, but they allow for cell fixation and subsequent permeabilization to gain access to the intranuclear compartment without interfering with its staining. Depending on the source of cells, single-cell preparations may differ in the percentage of dead cells per

sample, e.g., spleen and lymph nodes usually host 80-90 % of live cells, while preparations from intestinal tissues are more difficult to process and, hence, cell preparation is considered good when the viability is above 50 %. DNA released from the dead cells is sticky and clumps living cells together leading to single-cell droplets containing duplicates and triplicates of cells. Such cells are excluded before analysis as they otherwise can give false-positive results. This is done by a simple forward and side scatter gate FSC-H/FSC-W. Finally, most focus must be given to the composition of panels for multiple parameter detection. For a proper panel design and effective analysis, a dump channel (negative gate) must be used in combination with an inclusive positive gate. In the case of T cell phenotypic analysis, I constantly used MHC-II staining in combination with CD3e or TCRβ to specifically identify T cells (MHC-II$^-$CD3e$^+$TCRβ$^+$) as shown in **figure 4**.
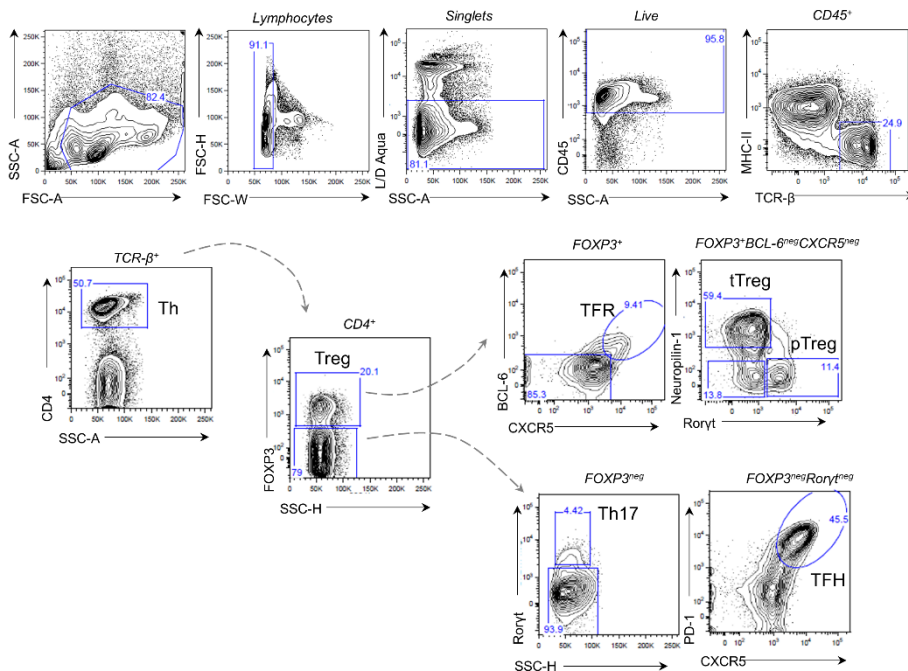
*Figure 4. **Gating strategy of CD4 T cells frequently used throughout the doctoral thesis.** The single-cell suspension was stained with the membrane dye live/dead Aqua, followed by surface antibodies reactive to CD45, MHC-II, TCR-β, CD4, CXCR5, Neuropili-1, and PD-1. Cells were fixed and permeabilized to allow for the intranuclear staining with antibodies reactive to transcription factors FOXP3, Rorγt, and BCL-6. Gating strategy starts with the broad inclusive lymphocyte gate that further is narrowed to Singlets>Live cells>CD45$^+$ cells> T cells (TCR-β$^+$)>CD4 T cells. Based on the surface and intranuclear factors, CD4 T cells are separated into Treg, TFR, tTreg, pTreg, Th17 and TFH lineages.*

In addition to cell phenotyping, flow cytometry can be used for cell sorting. By applying a high voltage on acquired cell droplets it is possible to sort individual cells of a gated population into tubes for further analysis or experiments. This technology was used in papers I and III, in which antigen-specific CD4 T cell subsets were studied via adoptive transfer systems, cell cultures, and single-cell RNA sequencing.

Single-cell RNA Sequencing

Today, novel techniques enable the evaluation of immune responses at the single-cell level. This has revolutionized the assessment of complex immune cell interactions. Cell function may now be defined not only at the protein level, but more accurately at the gene transcriptional level by RNA sequencing. With barcoding technology, it is possible to detect unique gene transcripts at the single-cell level in several thousand cells at the same time. Such an approach allows for the analysis of complex networks of effector CD4 T cells and the cellular heterogeneity in the response. Single-cell RNA sequencing was extensively used in paper III. The combination of antigen-specific cell sorting using MHC-II restricted CTB tetramer and 10X technology was employed to simultaneously characterize the CTB-specific CD4 T cells in PP and MLN. Sorted CTB-specific CD4 T cells were highly enriched with very little contamination of non-CTB CD4 T cells (<1%). By using Chromium 10X Single Cell platform sorted cells went through the reverse transcription reaction to form a cDNA and each single-cell transcriptome was tagged with barcodes for gene library preparation. The resulting barcoded library was then sent for RNA sequencing and finally analyzed by using the Seurat analysis platform – an R package designed for quality control, analysis, and exploration of single-cell RNAseq data. The acquired data set allowed for a detailed analysis of CD4 T cell phenotypes and subset functions.

RESULTS

A model for unadjuvanted oral immunizations stimulating optimal gut IgA responses

The initial response to most protein antigens given orally is tolerance induction (18). Hence, successful oral immunizations must circumvent this suppressive response to generate a distinct SIgA response, therefore, mucosal immunizations require strong mucosal adjuvants. Unexpectedly, in our laboratory, we could show that OVA TCR-Tg mice on both Balb/c and C57BL/6 backgrounds have a strong responsiveness to oral immunization with OVA antigen alone (paper I – fig. 1). The response was comparable with that of adjuvanted with CT. It was CD4 T cell-dependent because an adoptive transfer experiment into nude mice could replicate the magnitude of gut IgA induction independently of adjuvant (paper I – fig 1). OVA TCR-Tg mice have been known for their high frequency of dual TCR expressing CD4 T cells i.e. besides transgenic Vα13Vβ8.1 TCR DO11.10 mice tend to express also an endogenous WT TCR (211, **Figure 5A**). DO11.10 mice on a scid background, however, do not possess dual TCR CD4 T cells as they cannot rearrange the α-chain of the TCR (**Figure 5A**). Adoptive transfer of TCR-Tg CD4 T cells on a scid background did not allow for a response to OVA alone and required a CT-adjuvant (**Figure 5B**, paper I – fig. 3). This led to speculation that the dual TCR CD4 T cells are responsible for the TFH function to an immunizing antigen driven by commensal microbiota as a natural adjuvant. Indeed, an adoptive transfer of the TCR-Tg CD4 T cells without immunization exhibited strong T cell proliferation and presence in the individual PPs (paper I – fig. 3). By contrast, CD4 T cells on a scid background failed to expand and were not found in the peripheral lymphoid tissues unless CT-adjuvant was added to the oral immunization. It appeared that endogenous TCR activation via recognition of microbial antigens was the probable mechanism for the activation and gut homing of these cells. It was not possible, however, to prove this assumption completely as TCR-Tg CD4 T cells could not be transferred into germ-free WT or nude mice. However, evidence from antibiotic-treated OVA TCR-Tg mice supported this notion as oral immunizations led to a reduced TFH level suggesting that microbial recognition promotes OVA-specific SIgA responses in general. Similar observations were made in naïve germ-free mice, suggesting that the microbiota functions as a natural adjuvant to ensure sufficient presence of TFH cells in PP GC (Paper I – fig. 3).
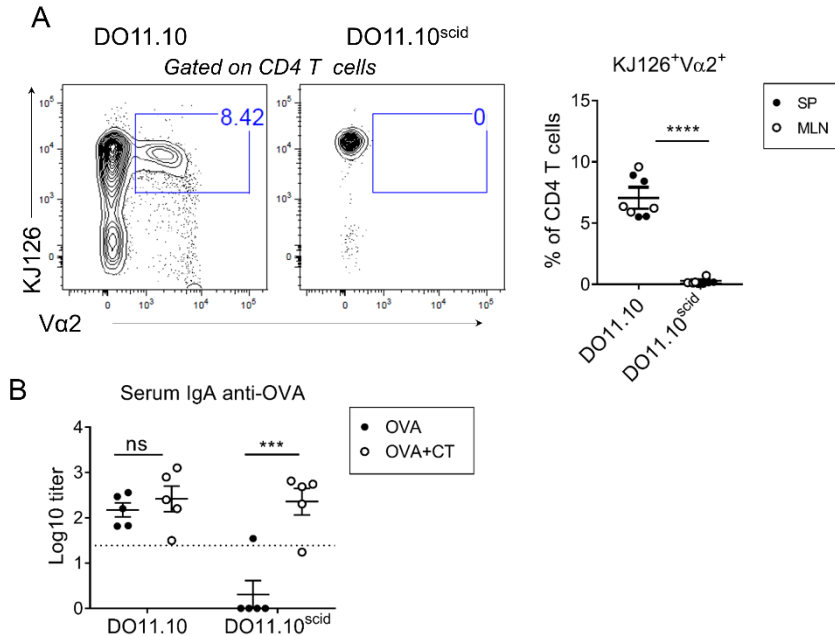
*Figure 5. **Dual TCR CD4 T cells in the unadjuvanted model system.** (A) FACS representation and frequency of splenic (SP) and mesenteric LN (MLN) dual TCR CD4 T cells in naive DO11.10 or DO11.10^scid mice. (B) Resulting OVA-specific serum IgA responses in nude mice assessed by ELISA assay 26 days after reconstitution with either DO11.10 or DO11.10^scid CD4 T cells and 3 oral immunizations with OVA or OVA+CT. Statistical significance was calculated using two way ANOVA with Tukey's multiple comparison test. * p <0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = not significant.*

tTregs are critical for gut IgA CSR

Tregs were shown to exhibit plasticity in the PP microenvironment and were proposed to be prone to become TFH cells that directly could support gut IgA response (1). To test this notion in the unadjuvanted mice, I performed stringent splenic Treg sorting based on surface expression of the markers GITR and CD25 from naïve OVA TCR-Tg mice (paper I – fig. 2). The isolated cells were adoptively transferred to nude recipient hosts (**Figure 6B-C**). Of note, nude mice are athymic and do not host any CD4 or CD8 T cells and, therefore, donor CD4 T cells are the only source of T cells in these animals. OVA TCR-Tg mice have a generally impaired Treg compartment that, in fact, hosts mainly tTregs (paper I – fig. 2, **Figure 6A**). Interestingly, donor cells homed to PPs even 26 days post transfer but showed little plasticity. Up to 90% of cells found following Treg transfer expressed FOXP3 and did not switch to a TFH phenotype (FOXP3⁻BCL6⁺) (**Figure 6B**). Such adoptive transfer also failed to

induce gut IgA responses but demonstrated lineage stability in the tTregs as shown before (228, **Figure 6C**). Interestingly, tTreg deficient adoptive transfer (CD25⁻GITR⁻) also failed to support gut IgA response, while perfectly well responded with systemic IgG responses. Thus, the SIgA response was tTreg-dependent, because upon analysis around 6% of donor cells had acquired pTreg phenotype that was comparable to the total CD4 T cell population in OVA TCR-Tg mice (**Figure 6A-B**). Because Treg deficient mice could promote specific IgG responses, it appeared that these cells gave rise to TFH cells. Hence, the experiment indicated a loss of IgA CSR factor, possibly, TGFβ (165). Using Treg-B cell co-culture system, I could show that only tTreg culture had a remarkable increase in latent TGFβ surface expression (paper I – fig 4). Tissue micrographs of the PP revealed that tTregs expressing TGFβ are found within the T cell zone closely to the GC, however, a lack of CD4 T cell restricted TGFβ deficiency model prevented me from undertaking the final clarifying experiment: namely to test whether TGFβ derives directly from the tTreg population or whether tTregs express the activating αvβ8 integrin that activates latent TGFβ and in this way enables IgA CSR.
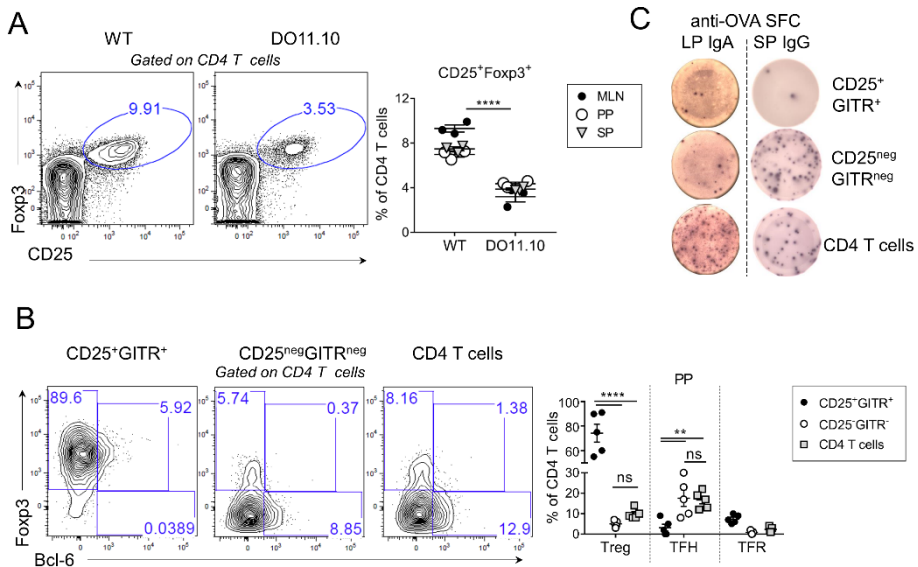
*Figure 6. **Stable Treg phenotype that alone does not drive gut IgA response.** (A) FACS representation and frequency of Treg cells in naïve WT or DO11.10 mice within spleen (SP), mesenteric lymph node (MLN) and Peyer's patch (PP). (B-C) Representative FACS plots and ELISPOT wells of CD4 T cell phenotype and IgA B cell responses in nude mice 26 days after the adoptive transfer of sorted DO11.10 CD4 T cell populations based on GITR and CD25 surface expression. Mice received 3 oral immunizations with OVA. Statistical significance was calculated using two way ANOVA with Tukey's multiple comparison test. * p <0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = not significant.*

tTregs appear to support IgA CSR independently of the immunizing antigen

Tregs emerge from the thymus with a high-affinity TCR for self-antigens. A fraction of these cells also home to the gut and due to a reduced frequency of self-antigens these cells can be maintained in an MHC-II independent way (246). Yet, the role of these cells in the GALT is poorly understood. Based on the results obtained in my work one possible mechanism could be to provide TGFβ for IgA CSR in the PP. Indeed, to test if tTregs are dependent on OVA stimulation to execute their IgA CSR function, I undertook an adoptive co-transfer experiment of Treg depleted OVA TCR-Tg cells with tTregs from OVA TCR-Tg or WT mice (**Figure 7A**). Such combinations resulted in enhanced gut IgA responses especially in WT tTreg transfer, clearly demonstrating that the tTreg function is independent of the immunizing antigen. Microbial stimulation could also play a role, however, unlike TFH cells, the tTreg compartment in germ-free mice is intact and comparable with conventional mice. Similarly, TGFβ expression in tTregs from both mouse strains remained the same (**Figure 7B**). tTregs may require TCR stimulation for TGFβ production, however, no bystander activation was observed in the PP microenvironment. Yet, the activating factors or cellular interactions required for IgA CSR and their specific location in the PP remain elusive.
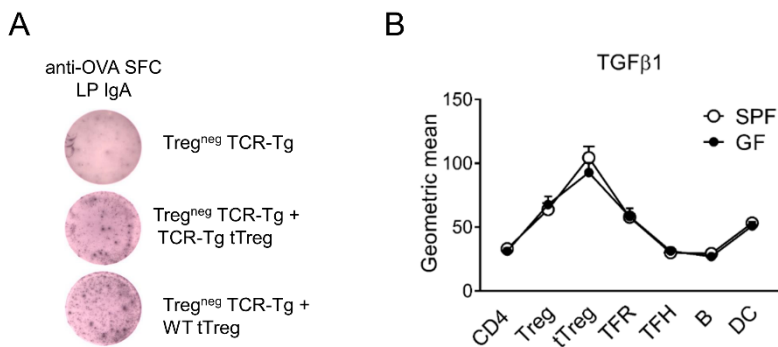
*Figure 7. **tTregs support gut IgA response independently of immunizing and microbial antigens.** (A) Representative ELISPOT wells of B cells responses in LP of nude mice 26 days after adoptive transfer with Treg depleted OVA TCR-Tg CD4 T cells with/without OVA TCR-Tg or WT tTregs followed by 3 oral immunizations with OVA. (B) Surface expression of latent TGFβ1 on CD4 T cells, Tregs, tTregs, TFR, TFH, B cells and dendritic cells in PP of naïve germ-free (GF) or conventional some pathogen-free (SPF) mice. Statistical significance was calculated using two way ANOVA with Tukey's multiple comparison test. * p <0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = not significant.*

Gut homing IL-10 producing pTregs are critical for oral tolerance

To mimic a more natural situation I adoptively transferred WT CD4 T cells together with the OVA TCR-Tg CD4 T cells to nude mice at a 1:1 ratio (paper II). This completely abrogated the OVA-driven oral IgA response observed previously (**Figure 8B**). Hence, the WT CD4 T cells appeared to have imposed strong suppression on the TCR-Tg CD4 T cell population, which now were unable to provide helper functions to stimulate a gut IgA LP response. Albeit
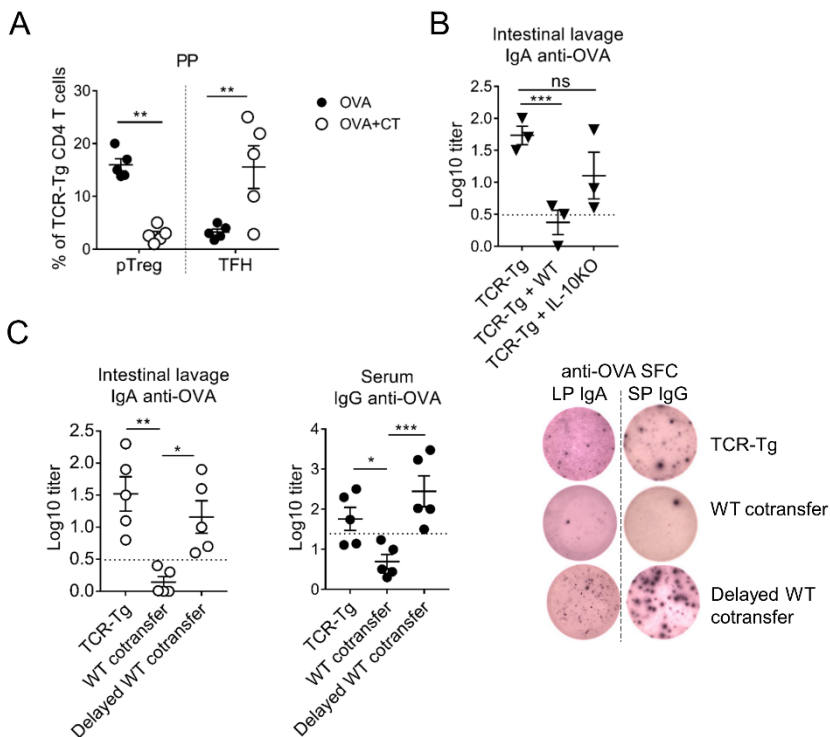

Figure 8. **Gut homing CD4 T cells of WT origin impose tolerogenic phenotype on TCR-Tg cells via IL-10 in priming.** *(A) OVA TCR-Tg CD4 T cell phenotype upon adoptive co-transfer with WT CD4 T cells into nude host 7 days after oral priming with OVA or OVA+CT. (B-C) Adoptive transfer of OVA TCR-Tg CD4 T cells in nude mice with or without the co-transfer of WT or IL-10-deficient CD4 T cells (B) or WT CD4 T cells that were introduced to the host 9 days after oral priming (C). IgA and IgG B cell responses assessed by ELISA and ELISPOT 26 days after oral priming. Mice received 3 oral OVA immunizations (B, C). Statistical significance was calculated using two way ANOVA with Tukey's multiple comparison test.* *$p <0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns = not significant.*

transferred WT CD4 T cells expanded 10 times stronger than TCR-Tg CD4 T cells in the recipient nude mouse host, the proliferation of TCR-Tg CD4 T cells was still significant, leading to the conclusion that the lack of gut LP IgA responses was not a consequence of lack of OVA-specific CD4 T cells, but rather a result of a different regulatory environment in the PP (paper II – fig. 3). In agreement with this notion, I observed that the TCR-Tg CD4 T cell phenotype in the PP was now skewed towards a pTreg lineage (**Figure 8A**). As IL-10 is a typical Treg inducing factor, co-transfer of OVA TCR-Tg CD4 T cells with CD4 T cells from IL-10 deficient mice failed to suppress the gut LP IgA response to OVA alone (**Figure 8B**). This also correlated with the enhanced TFH phenotype of donor TCR-Tg CD4 T cells in the PP. Interestingly, if WT CD4 T cells are given with several days of delay from the TCR-Tg CD4 T cells, the suppressive capacity of these cells on the OVA-specific response is lost (**Figure 8C**). This indicated that the defining role of pTreg-derived IL-10 is at an early stage of naive CD4 T cell priming. Thus, suppression by pTregs for any given antigen is specific and not transmissible as a bystander phenomenon. Hence, it seems that an already active TFH population is difficult to tamper even for pTregs.

CT does not act to break established tolerance, but promotes TFH differentiation

Cholera toxin is the most potent oral adjuvant known to this date (4). However, its well-studied, adjuvant function still needs to be better understood. To test if CT dampens IL-10 production of pTregs, WT mice were orally immunized with CT and 3 days later 2 most proximal PPs isolated (**Figure 9 A**). FACS analysis revealed that CT did not suppress IL-10 but quite the opposite. Then, co-cultures of OVA TCR-Tg CD4 T cells and WT pTreg cells from untreated or CT-treated mice were analyzed, but the latter did not rescue the cell proliferation in vitro relative to that of untreated WT CD4 T cells, suggesting that CT did not block the suppressive nature of WT pTreg cells before culturing (**Figure 9B**). Besides, if CT is added to the oral immunizations of a tolerated antigen, only CT-specific gut IgA was induced, clearly demonstrating that the CT adjuvant does not break oral tolerance (paper II – fig. 4). Finally, to challenge the concept that CT -adjuvant acts via the same pathway as antigen feeding for stimulation of tolerance I undertook an experiment in Batf3$^{-/-}$ mice, which are known to lack cDC1 cells that are effective pTreg inducers (238). Strong gut LP IgA responses were recorded speaking in favor of that CT-adjuvant acts to promote TFH development and does not use the same pathway as used for oral tolerance induction (paper II – fig. 5). In support, co-transfer

of TCR-Tg and WT CD4 T cells into nude mice following CT adjuvanted oral immunization preferentially differentiated Tg cells into the TFH subset. By contrast, PBS or OVA treatment led to a Treg-dominated phenotype (**Figure 8A**). Importantly, CT adjuvant is most needed in the oral priming immunization and therefore is essential for the early fate decision of naïve TCR-Tg CD4 T cells (paper II – fig. 1).
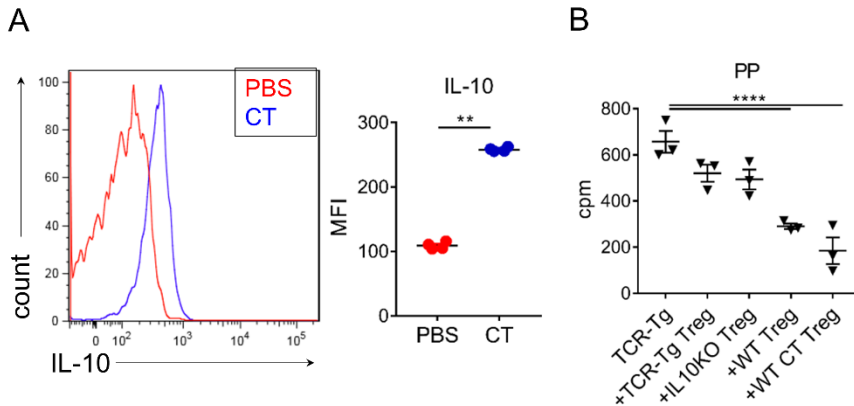

*Figure 9. **Cholera toxin does not act on suppression**. (A) Representation of IL-10 expression by FACS of CD4 T cells in proximal PP 3 days after oral immunization with CT. (B) TCR-Tg CD4 T cells from PP co-cultured with Tregs from TCR-Tg, IL-10KO, WT or WT mice that 3 days earlier received oral CT immunization. Cell proliferation assessed after 96h stimulation with OVA peptide p323. Statistical significance was calculated using two way ANOVA with Tukey's multiple comparison test. * p <0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = not significant.*

CT-induced responses in the PP are dominated by CTB specific TFH cells

Cholera toxin itself is a strong immunogen and it has been reported that CTB is the immunodominant subunit hosting several MHC class-II restricted epitopes (39). To confirm this, ELISA and ELISPOT assays were undertaken to asses if CT- immunized WT mice developed antibodies against CTB or CTA. Indeed, antibody responses were directed mostly against CTB (**Figure 10A**). By using a CTB peptide-MHC-II tetramer I could isolate CD4 T cells from orally CT-immunized mice. Previous reports suggested that gut IgA responses depend on local Th17 cells in the PP as oral CT-immunization failed to stimulate the gut LP IgA response in Th17-deficient mice (3). Surprisingly,
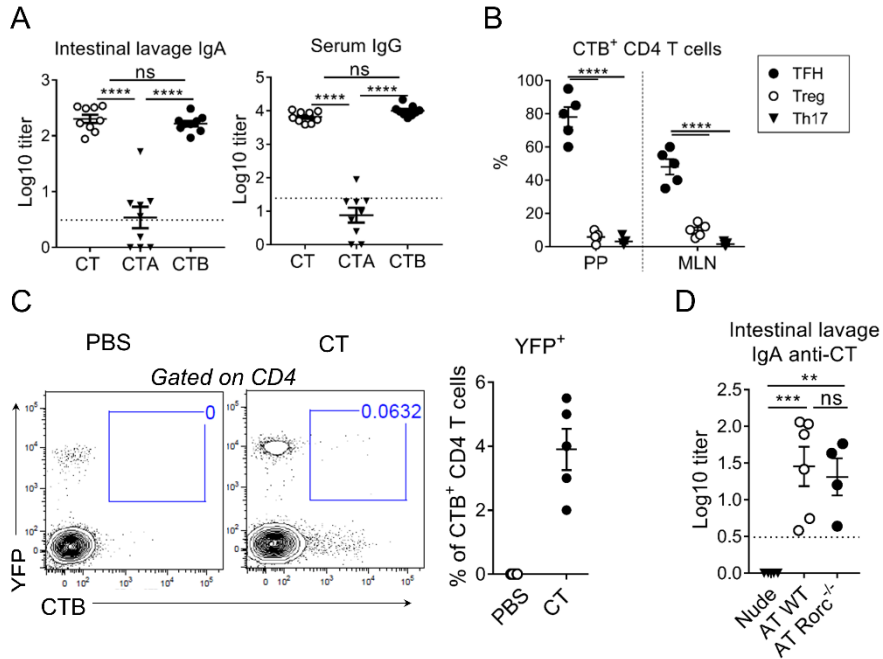
Figure 10. CT induced immune response is mainly CTB specific and dominated by TFH cells but the adjuvant function is independent of Th17 cells. (A) CT induced antibody response in WT mice following 3 oral immunizations. (B) FACS analysis of CTB specific CD4 T cells in WT mice after 3 oral immunizations with CT. (C) FACS analysis of CT induced CTB-specific CD4 T cells in IL-17[cre]eYFPxRosa26 mouse after two oral immunizations. (D) B cell response assessed by ELISA 26 days after the adoptive transfer of WT or Rorc^{-/-} CD4 T cells to nude host. Mice received 3 oral CT immunizations. Statistical significance was calculated using two way ANOVA with Tukey's multiple comparison test. * p <0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = not significant.

CTB-specific CD4 T cells saw little contribution of Th17 cells as determined by FACS analysis (**Figure 10B**). Also, single-cell RNA-sequencing revealed that the early CTB-specific response had very few Th17 cells, which also did not increase in frequency during the course of the response (paper III). Instead, the oral CTB-specific response was dominated by TFH cells that significantly increased by subsequent immunizations from 40% in priming to nearly 90% after 3 oral doses of CT (**Figure 10B**). It was suggested that Th17 cells demonstrate great plasticity towards TFH cells in the PP (3). However, oral CT immunization of the IL-17 fate reporter mice IL-17[cre]eYFPxRosa26 that permanently mark IL-17 producing cells with YFP expression, resulted only in very few double-positive CTB-specific CD4 T cells demonstrating that the CTB-induced TFH cells were not derived from IL-17 secreting/Th17 cells (**Figure 10C**). Technically, only if CTA plate coating ability is poor, it is

possible that ELISA and ELISPOT results of CTB dominating response were exaggerated and significant CTA contribution remained undetected. It has been shown that CT is a potent inducer of Th17 cells via cAMP production (320). Importantly, only the CTA subunit promotes cell toxicity via cAMP and, thus, induces Th17 cells. This could be the reason why analyzed CTB specific CD4 T cells are not Th17 cells.

Th17 cells were shown to be essential for CT induced gut IgA response (3). CT has also been reported to induce mixed-type of the immune response (42, 43). To address the central role of Th17 cells, I performed an adoptive transfer experiment to nude host with enriched CD4 T cells from Rorc$^{-/-}$ mice. IgA response to CT appeared perfectly normal and unperturbed in these mice, indicating that Th17 cells are completely dispensable for oral IgA responses following oral immunizations (**Figure 10D**). As nude mice have no other T cells available the result from this experiment is completely clarifying.

Peyer's patch as the inductive site for oral immunization

Already in the paper I, the B1-8^{hi} mouse model demonstrated that orally induced NP-OVA B cell responses are primarily located to the PP. However, to confirm this also at the CD4 T cell level, unselected T cells from both PP and MLN were analyzed by gene profiles and TCR clonotypes using the 10X platform (paper III). CD4 T cell populations were very diverse in both sites but clustered together in the different functional subsets, and, thus, surprisingly most TCR clonotypes were shared between the two organs. Only one exception was found, a TFH-like population termed "M2" that appeared unique to PPs and absent in the MLN (**Figure 11A**). This population expressed high amounts of ICOS and CD40L (paper III – fig. 3). In agreement with previous reports, Th2 cells were not represented in PP nor MLN – a population that is observed in the gut in response to helminth infections, for example (321). Strikingly, very few cells clustered as proliferating cells and both significant naïve and memory CD4 T cell clusters were observed (paper III – fig. 1). Thus, this may indicate that CD4 T cell proliferation in response to the microbiota is not a dominating element in the regulation of the gut CD4 T cells (paper III). Interestingly, TFH and Th1 cells shared clones between PP and MLN. The CTB-specific CD4 T cell repertoire also exhibited strong sharing between the clusters in PP and MLN, suggesting that the cells originated from PP, and highlighting the interconnected relationship between these two sites (**Figure 11B**). Importantly, clonal sharing between various TFH subsets was extensive

between PP and MLN, but virtually no overlap in TCR clones was seen between TFH and Th17 cells or TFH and Treg cells (**Figure 11C**). This confirmed the previous observations that TFH in PP does not derive from either Th17 or Tregs. TGFβ activating integrins αvβ8 were mainly found on tTregs and TFRs that derive from tTreg population (286), but not on pTregs, which supports the findings in paper I (paper III – fig. 3). On the other hand oral tolerance is mediated by pTregs that produce IL-10. In single-cell data set pTreg cluster was the only to be enriched for IL-10 (paper III – fig. 3). A published report from Sakaguchi and colleagues has shown that murine TFRs have reduced expression of CD25 in germinal centers (293). Also in the RNAseq data set high CD25 expression was restricted to naive tTregs and pTregs but not exhibited by TFRs (paper III – fig. 3). These results with single-cell gene profiling strongly support the observations made in papers I and II.
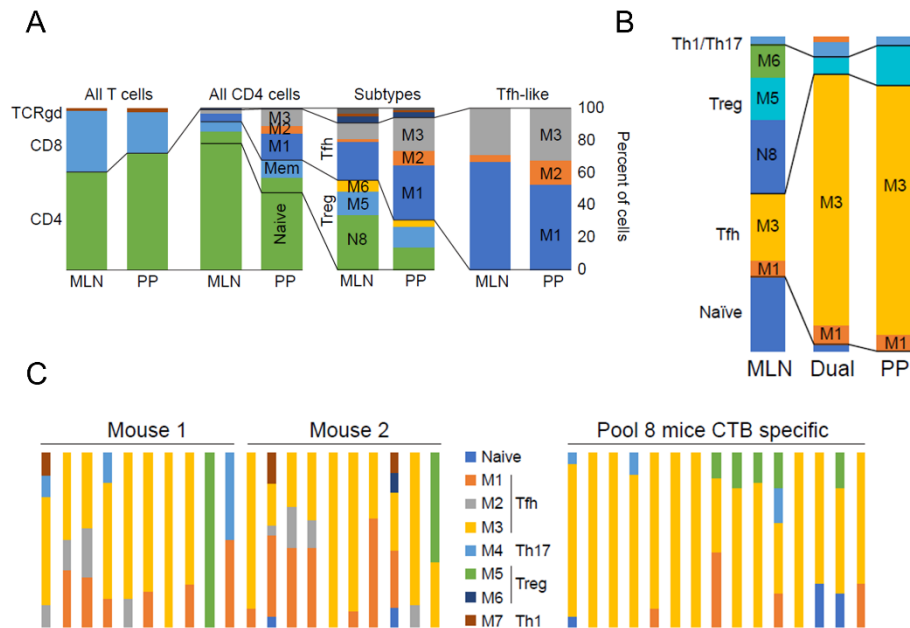
Figure 11. ***T cell repertoire diversity in PP and MLN revealed by Single-Cell RNAseq.*** *(A) Distribution of different cell types in MLN and PP (among total cells). (B) Clone sharing of CTB-specific CD4 T cells between PP and MLN. (C) 10 largest clones in PP in two non-immunised mice to the left, and the sorted pool from PP to the right. Analysis performed using Seurat software.*

DISCUSSION

My doctoral research has addressed several controversies in the field, in particular, whether Th17 or Treg cells are required for optimal gut LP IgA responses and to what extent IgA CSR is mediated by TFH cells or a specialized switch T cell identified more than 3 decades ago (317). It is worth pointing out that CD4 T cell lineage instability could be associated with a fine-tuned immune response and reflect a rapidly changing environment. Similarly, the use of different adjuvants can uniquely polarize the response towards a specific T cell lineage. This could also dramatically influence the representation of a certain subclass of antibodies. In such a situation the TFH cells may fulfill a messenger role by bridging T effector cells to B cell responses. In the gut, two major effector CD4 T cell subsets exist – Th17 cells and Tregs. Both can impact on antibody production and both have been reported to exhibit great plasticity to give rise to TFH in the PP (1, 3). By using an unadjuvanted mouse model I could directly evaluate the impact of TFH and Treg cells on gut IgA response in the complete absence of CT adjuvant. It appeared that TFH cells alone are not sufficient for IgA CSR and a highly stable Treg population is needed. Further experiments showed that antigen-specific TFH induction in PP is independent of Tregs or Th17 cells and that TFH is a prerequisite for an effective IgA B cell response that is also a primary target for CT-adjuvant. However, IgA CSR is completed only by the presence of tTregs that appear to function independently of TFH recognized antigen. The early observation of an IgA switch T cell was made already in the 1980s by Strober and colleagues, who identified the "PP switch T cells" that appeared to govern the pathway involving the DNA recombination events needed to form IgA, but did not involve expanding already IgA differentiated B cells (317). At the time it was not possible to assess T cell lineage identity and, thus, researchers were not concerned about CD4 T cell subsets. Of note, Tregs as a unique CD4 T cell lineage was shown only a decade later and since then have been associated with IgA production (1, 2, 66, 222). However, observations have mainly been made in response to bacterial antigens and, therefore, possibly are being orchestrated by pTregs. Indeed, it has been shown that *B. fragilis* and *Clostridium* species are potent Treg inducers in the gut, but it seems less likely that pTregs induced by the microbiota would play a role in the IgA CSR (25, 239). The acquired data presented in this thesis suggest that pTregs are rather highly suppressive to ingested antigens and would, therefore, favor tolerance induction (paper II), but tTregs are the responsible switch subset for gut IgA responses (322). tTregs emerge from the thymus with a strong affinity to prevent self-reactions. The gut, however, is not a prime site for self-antigen recognition. Perhaps that is the reason why otherwise highly

suppressive subset at systemic sites exerts IgA CSR functions in the gut. TGFβ has many biological functions including a class-switch factor for IgA differentiation (165). To exert a suppressive effect, pTregs must be activated by specific antigen recognition. On the other hand, the activation of tTregs to produce TGFβ was independent of such antigen-specific activation (322). However, what stimulates TGFβ production remains elusive. tTregs might be activated elsewhere before gut homing, however, also bystander activation in this highly crowded environment could play a role. It seems that microbiota can support a tTreg niche in an MHC-II independent way (246), however, TGFβ expression may also be seen in the germ-free mice arguing that tTreg production of TGFβ could be independent of the microbiota.

TGFβ is a complex molecule that exists in various forms. Most commonly, the cytokine is found in a latent form on the cell surface that can also be released in the extracellular matrix. However many different cell types in the gut express TGFβ, tTregs seem to be more privileged. Uniquely, Treg surface-bound latent TGFβ complexes are associated with GARP (323). Activation of the latent form is central for the exploitation of the TGFβ effector functions. Integrins αvβ6 and αvβ8 are the best-known activators of TGFβ, however, they work in concert with pH fluctuations, proteases and other proteins including neuropilin-1 (298, 324). Neuropilin-1 is the molecule that distinctly identifies tTregs from pTregs. Indeed, single-cell RNA sequencing analysis evidently showed that the transcriptional signature of the integrin αvβ8 gene was found in the tTreg rather than the pTreg cluster. The current consensus in the field, however, is not so supportive of Treg- TGFβ-IgA CSR hypothesis. While it is clear that TGFβ is needed for IgA CSR, recent publications have suggested that TGFβ is produced by the B cell itself or by the local DC in the SED region of the PP (102). However, the data obtained in the present work, using conventional methods, indirectly suggests that tTreg derived TGFβ is the key for IgA CSR as the lack of tTregs in the adoptive transfer model failed to support a gut LP IgA response following oral immunization; B cell cultures with tTregs significantly enhanced latent TGFβ expression and stimulated IgA production; tTregs expressing latent TGFβ were found in proximity to GC. Neuropilin-1 and αvβ8 surface expression on tTregs may allow superior IgA CSR ability by TGFβ activation. However, additional, more direct, experiments are still needed. A decade ago the mouse model with a conditional knockout allele for *Tgfb1* was reported (325). Provided it was possible to cross this mouse to a FOXP3-cre model it would create a valuable system for a better experimental approach to finally disclose the Treg derived TGFβ effects on gut IgA CSR. Unfortunately, the TGFβ mouse model was withdrawn due to unforeseen genetic alterations and is currently unavailable. However, other tTreg derived factors may positively influence IgA induction.

The TFR subset is derived from tTregs (282, 286). Therefore, it is plausible, that TFR cells regulate IgA CSR. TFRs have been shown to affect the composition of the microbiota by regulating IgA production (66). The accumulated data set of single-cell RNA sequencing demonstrated that both TFRs and tTregs, but not pTregs, were expressing the αvβ8 integrin gene (paper III). Moreover, a helper function has been reported during acute inflammatory responses where TFRs appear to support B cell maturation in the GC DZ by secreting IL-10 (151). GC B cells formed in the absence of Treg derived IL-10 displayed an altered DZ state and exhibited decreased expression of the Foxo1 gene due to impaired nuclear translocation (151). Very recently TGFβ signaling within PP GC was also shown to be critical for the entry of activated B cells into the DZ (326). A lack of TGFβ signaling promoted the accumulation of these B cells in the LZ which resulted in a reduced antibody affinity maturation, likely due to reduced activation of Foxo1. In that study, however, it appeared, that the source of TGFβ was the FDC. However, in the light of previous publication and the presented work in this thesis, TFRs may also contribute to a TGFβ signaling within the GC.

TFR cells have been very difficult to study until recently. A mouse model system where the *Bcl6* gene is deleted in FOXP3 T cells and, thus, absent from TFR cells, was reported to develop late-onset spontaneous autoimmune diseases, highlighting the role of TFR cells for regulating the GC reaction (148, 149). Another very recent advancement in the TFR field is the development of a "TFR cell-deleter" mouse that selectively eliminates TFR cells, therefore, allowing for temporal studies of the subset (327). Using this model system TFR cells were found to regulate early, but not late, GC responses and control antigen-specific antibody production and memory B cell development. These effects were disrupted after TFR cells were eliminated which also resulted in increased production of self-reactive antibodies (327). Upon systemic immunization, TFR cells did not regulate the size of the GC B cell population but rather contributed to the generation of antigen-specific IgG antibodies at the expense of restraining IgA antibody responses. Interestingly, in the lupus model, lack of TFRs facilitated the production of increased anti-dsDNA IgA antibodies in serum (149). Perhaps this observation argues against TFR-IgA axes, but studies addressing a TFR-deficiency in the GALT still awaits to be done.

The key observation made in the present thesis work was that TFH helper functions are distinct from the tTreg mediated IgA CSR and was possible to unfold because of the discovery of the unadjuvanted mouse model for oral immunizations (322). It is driven by OVA TCR-Tg CD4 T cells that express transgenic and endogenous TCRs simultaneously. The dual TCR appeared

critical to activate injected CD4 T cells to home to the gut, which occurred in the absence of oral immunization, but in response to the microbiota. Indeed, the gut is not a closed environment – the microbiota is affecting many bodily functions, having implications even for protection against virus-induced neurological conditions (328). Pathogenic bacteria can infiltrate the brain and cause neurological disorders, as is proposed to occur in Alzheimer's disease (329). Most convincingly, many systemic immune functions have been found to interdepend on the microbiota under steady-state conditions (330). Splenic memory IgM B cells, originating from mucosal GCs, are constantly replenished and form a barrier against systemic bacterial infections (330). Microbiota also regulates CD4 T cell-dependent increases in serum IgA levels and IgA-secreting plasma cells in the bone marrow, which are thought to protect against sepsis (331). It is not clear how the peripheral reactivity to gut bacteria is organized. It could be that systemic sites are occasionally exposed to gut commensals that breach the intestinal barrier and spread systemically. This could be the way the OVA TCR-Tg CD4 T cells were exposed to the microbiota as observed in paper I (322). The oral ovalbumin feeding model has been widely used for studies of oral tolerance, however, under specific conditions can be used to study unregulated gut IgA responses not requiring adjuvant. Hence, also TFH responses can be studied in this model and found to rely on the presence of the microbiota. It is known that SFB is a potent bacterial inducer of gut IgA responses (332). In turn, IgA has been shown to modulate microbial communities and facilitate optimal colonization (26, 27). Results obtained in a paper I indirectly suggest that the microbiota acts on dual TCR OVA TCR-Tg CD4 T cells as a potent natural adjuvant. This is particularly interesting because dual TCR CD4 T cells are not an artificial phenomenon. It is estimated that up to 30% of all CD4 T cells have the potential to express TCR with two specificities. However, they remain unnoticed and are heavily regulated. It is only in transplant rejection or autoimmunity where dual TCR cells have been found to exert a largely destructive activity. In experimental animal models, dual TCR CD4 T cells accumulate in the intestinal tissues (211). This raises the possibility that very specific stimuli contribute to the accumulation and the function of these cells. Certain microbiota composition may provide the switch from harmless to detrimental. Recently, SFB was shown to trigger lung autoimmunity via the induction of dual TCR Th17 cells in the gut (216).

Albeit TFH cells constitute the most important antigen-specific CD4 T cell population for gut IgA immune response, the role of Tregs is significant, not only for the involvement in IgA CSR. Mucosal immune responses are strictly regulated and one of the key players is the pTreg. The pTreg derived IL-10 production is central to oral tolerance and in response to environmental stimuli

it prevents unwanted inflammatory responses dampening IgA immunity and TFH induction. This was particularly obvious in the WT CD4 T cell co-transfer experiment where otherwise reactive OVA TCR-Tg CD4 T cells were completely suppressed by pTregs (paper II). It might be that WT CD4 T cells have a higher TCR affinity for the microbiota than the TCR-Tg CD4 T cells and, therefore, can more effectively outnumber the latter following injection into nude mice. Such a system imposes TCR-Tg CD4 T cells to acquire pTreg phenotype. The addition of CT adjuvant rescued the co-transfer experiment to effectively stimulate gut IgA responses by supporting the development of TFH cells. This could be due to a combination of DC promoting TFH but failing to entertain a Treg response as normally seen after oral antigen administration. The mechanism of action needs to be investigated in greater detail, but it is clear that cDC2 cells are involved and that these alter their co-stimulatory repertoire to favor TFH development. It has been reported that CT itself promotes the induction of Tregs specific for bystander antigens (333). Interestingly, depending on the CT dose, DCs were shown to differentiate into pTreg or Th17 polarizing subsets (334). My data confirms that CT does not act directly to suppress IL-10 activity, but rather the differentiation of CD4 T cells to Tregs. CT cannot break already induced Treg suppression. It might be that upon CT immunization observed decrease in the pTreg frequency is due to the enormous expansion of the TFH cells.

Several studies have proposed that CT induces strong Th17 immunity in the GALT following oral immunization (3, 320, 335). Also, my work supports this observation. However, Th17 cells appear to be non-specific to the immunization as determined by CTB tetramer and therefore were considered a pharmacological effect. Importantly, CT is composed of CTA and CTB subunit and by using CTB tetramer, CTA induced responses remain undetected. Th17 induction is likely CTA specific. Indeed, cAMP that mounts CT induced toxicity via CTA subunit was shown to promote Th17 induction (320). CTB-specific CD4 T cell response showed very minor priming ability of Th17 cells and instead was predominantly expanded in TFH cells that did not derive from the Th17 population. Both lineages share the IL-6 dependent inductive pathway and it might seem that CT adjuvanticity primarily lays in IL-6 induction (335). However, oral immunization of IL-6$^{-/-}$ mice leads to normal gut IgA stimulation (336). CT is a complex immunogen and has been found to enhance mixed Th1, Th2 and Th17 responses depending on the immunization route (42, 43). Therefore a single cytokine deficiency model might be compensated for the lost adjuvant effect via other pathways. This is what makes CT a powerful adjuvant, yet also complicates the mechanistic studies of its adjuvant function.

Th17 cells were reported to be the cellular subset by which CT enhances gut IgA responses (3). As already discussed, CTB-specific CD4 T cell subsets do not derive from this lineage, however, CTA might possess a profound role in CT induced immunity, that Th17 cells indeed could be responsible for IgA induction. It has already been shown that Th17 cells have a modulating effect on the total IgA transport by the pIgR (110). However, given that IL-17^{-/-} mice mount effective IgA response upon CT immunization, it made me question the central role of Th17 cells in this process (43). I employed the adoptive transfer system to answer this. When Th17 deficient CD4 T cells were adoptively transferred to nude mice, oral immunizations still induced an equally strong gut IgA response when compared to that found in the control group receiving CD4 T cells from WT mice. It might be that the opposing results stem from distinct microbiota composition. Noteworthy, Th17 cells, as well as IgA induction, is stimulated by SFB, which indirectly could mean that observed Th17 effects are the result of reduced SFB community (158, 257, 332).

PP is the major inductive site for gut IgA responses following oral immunization, but CD4 T cell clonal sharing between PP and MLN is rather an active process as revealed by single-cell RNA-sequencing. Also by employing B1-8^{hi} B cells in an adoptive transfer model, subsequent B cell response induced by oral immunization was restricted to PP. Single-cell RNA-sequencing also opposed to popular belief of CD4 T cell plasticity. Clonal T cell sharing between CD4 T cell subsets was negligible. This observation is in contrast to previous reports proposing Treg and Th17 plasticity into TFH functions (1, 3). Extensive clonal sharing was observed only between the TFH cells which most likely represent different functional stages of TFH function within GC (147). Acquired data set of transcription signatures is truly a valuable tool to dissect controversies in the CD4 T cell field, but even so, to confirm already proposed concepts. The data set supported the concept of tTreg stability and TFR relatedness to tTregs because only a few TFR clones had shared the origin with TFH cells (228, 286, 295).

In summary, my doctoral research has dissected the functional complexity and regulatory requirements exerted by CD4 T cell subsets in the PP for gut IgA responses (**Figure 12**). The acquired data demonstrates that 2 distinct PP CD4 T cell populations are needed for IgA B cell responses following oral immunization. In the PP, TFH cells provide help and support the clonal selection of activated B cells in the GC while tTregs promote the necessary IgA CSR. The newly gained results from this thesis work emphasize the stringent regulation of the induction of immune response in the PP. IgA responses to soluble antigens are normally suppressed by pTreg derived IL-10, which maintains antigen-specific tolerance. This regulatory milieu promotes

naïve CD4 T cell differentiation to be dominated by pTreg cells. Thus, CD4 T cell fate decisions are made at an early stage following oral priming immunization. The CT-adjuvant, on the other hand, shifts the response towards the TFH phenotype and promotes strong IgA response. I demonstrated that CT-adjuvant cannot break oral tolerance. CT does not lift already existing suppression but rather changes the differentiation path of activated naïve CD4 T cells towards TFH cells, which is achieved through co-stimulation and production of cytokines. Oral immunization induces CTB specific CD4 T cell repertoire that is overrepresented by TFH lineage. Experimental data show that CT acts independently of Th17 cells to induce gut IgA responses. Finally, CD4 T cells in the PP represent diverse lineage repertoires that are shared with the MLN. Together, these results shed light on the complex regulatory network that exists in the PP to support gut IgA responses following oral immunizations.
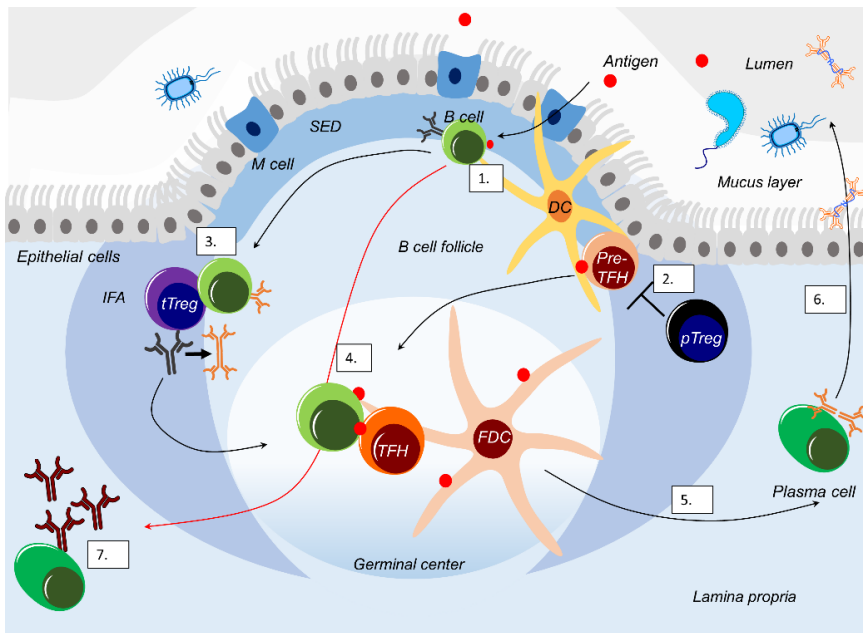
*Figure 12. **Proposed model for T cell regulation of gut IgA induction in PP.** 1. B cell is primed by the DC in the SED region and migrates to the T cell zone. 2. Also, naïve T cell is primed by DC to initiate the TFH program, however under steady-state conditions, it is suppressed by pTreg presumably via IL-10. Those T cells that escape pTreg suppression complete their differentiation in the GC. 3. B cell engages with tTreg in an antigen-independent way to undergo IgA CSR. 4. Within GC B cell undergoes antigen-dependent BRC affinity selection and overall maturation mediated by TFH. 5. Fully educated B cell exits GC and resides in LP as IgA producing plasma cell. 6. Plasma cell secretes IgA that is transported to the lumen. 7. If B cell completes the GC reaction without tTreg engagement, the resulting plasma cell secretes IgG.*

No tree, it is said, can grow to heaven unless its roots reach down to hell.

/Carl Gustav Jung./

REMAINING QUESTIONS AND FUTURE PERSPECTIVE

Most pathogens attack via mucosal surfaces but IgA is the most abundant isotype in mucosal secretions. To mount an effective defense response, not only local IgA but also systemic IgG responses are induced by oral immunization. The SIgA protective role against infectious diseases is well documented and typified by protection against cholera or rotavirus infection. However, not all infectious diseases rely on local IgA production, as found with influenza virus infection (337). Pathogen-specific SIgA antibodies in mucosal secretions might effectively prevent the spread of the infection. For this reason, understanding how gut IgA responses are induced and regulated is critical for successful oral vaccine development. My thesis work dissects these complex regulatory circuits of T-dependent IgA induction and provides critical information on the mucosal adjuvant activity of CT. However many questions remain.

Paper I addresses TFH and tTreg independent roles for IgA B cell responses, but what is the spatiotemporal positioning of these two acting subsets and whether both are fulfilling GC intrinsic functions remain to be investigated. Using confocal microscopy, tTregs were found in various anatomical compartments of the PP, however, the dynamic environment might not be well represented in fixed tissues. Recently, IgA CSR was reported to occur early before GC formation in the lymph node and might also happen in PP, but the location within an anatomic compartment of PP is debated (101, 102, 171). It is important to know if tTreg derived TGFβ is operational for IgA CSR in the GC, SED or perhaps the T cell zone. I have already shown that tTreg deficiency impairs IgA responses, and an experiment that unequivocally resolves how this is done is much needed. Conditional knockout mouse models are essential and would be helpful to test this concept.

In paper II the experimental focus was on understanding the homeostatic regulatory networks in the gut and how these may be overcome by the addition of CT adjuvant. Tolerance induction and CT induced mucosal IgA immunity are induced through separate pathways, but it is still not clear how CT imprints TFH fate in activated CD4 T cells in the PP and more importantly, which APCs are responsible for IgA responses. Detailed phenotypic analysis and transcriptional signatures of DC populations within PP upon CT priming will be useful to explain how the adjuvant works. This could contribute significantly to oral vaccine development and allow for the development of a new generation of oral vaccine adjuvants.

Paper III dissects CT induced CD4 T cell repertoire and its dependency on Th17 cells. By mainly looking at transcriptome data, this paper highlights unique, previously unknown populations. It is a very important data set, however, the results are descriptive and must be verified by using conventional methods to show if gene transcripts are expressed also at the protein level. Such an approach will actively contribute to our understanding of CT adjuvant enhanced gut IgA immune responses.

Finally, it is of fundamental importance to translate laboratory research from murine models to human vaccine development. We all are genetically different and unique; it might be that oral vaccination designs and protocols need to be adjusted for individual populations in different parts of the world. The microbiota plays an important role in the body functions and immune responses are not excluded. Its composition is influenced by our diet, lifestyle, age, environment and health condition. Over a century ago Ilya Metchnikoff proposed that health could be enhanced by manipulating the intestinal microbiome with a certain diet (338, 339). His hypothesis originated from the observations that among the inhabitants of some counties in the Balkan region had an unusually large number of centenarians. This was particularly striking as these people led extremely simple lifestyles under poor and humble circumstances. It was the combination of clean air, daily physical and mental exercise, personal hygiene, peaceful co-existence with nature and moderation in worldly pleasures that stood them apart from the rest of the Europeans. However, being a founder of cellular immunology, Metchnikoff believed that longevity for these people was the result of phagocyte-microbiome interactions imprinted in their diet. Remarkably, his thinking was that the degenerative consequences of aging are the result of otherwise beneficial phagocyte transformation into the healthy tissue distractors, a process that was orchestrated by *metabolites* derived from putrefactive bacteria residing in the colon. To prevent deterioration, a diet, such as soured milk or yogurt containing lactate-producing bacteria was recommended and practiced by Metchnikoff himself (338, 339). Today microbiota studies resemble perhaps the most active field of research. It is clear indeed, that our food intake shapes the commensal communities and therefore also the functioning of our bodies. Such a dynamic system must have a pronounced impact also on vaccine responses. Indeed, particularly in developing countries due to poor sanitation and impaired micronutrient intake specifically for zinc and vitamin A, vaccine responses tend to be poor – a phenomenon known as the "tropical barrier" (7). Today's Europe is home to progressive youth that chose to avoid animal products in their diet. While extensive meat intake was not among Metchnikoff's recommendations and the moderation was celebrated, studies have shown a correlation of vitamin B12 deficiency and diabetes progression

(340). Could such diet restriction also affect vaccine responses? Aging is yet another aspect defining all corners of today's Europe. Vaccine effectiveness is much reduced in the elderly population in part due to defective GC responses (341) and it is not well studied if a certain diet could rescue vaccine responsiveness. Elderly is a high-risk group for seasonal viral infections, therefore younger individuals must protect them via the herd immunity that lately has been difficult to achieve. The anti-vaccine movement that stems from misinformation on a global scale due to social media has shaken the world. Many young parents choose not to vaccinate their infant children this way putting at risk not only their families but also those social groups that cannot get vaccinated. Unlike other medical treatments, vaccines are administered to healthy individuals and recently have been looked upon as a personal choice. This is a very unfortunate development that puts at risk us all. We tend to forget that mass vaccination is the greatest medical achievement of the 20th century, it has saved millions of lives and has even eradicated the disease. It is of critical importance to study basic mechanisms of antibody induction and environmental aspects of overall vaccine responses, but, equally so, it is to educate and remind people of vaccine importance. Successful vaccination is not only dependent on effective vaccine candidates developed in the laboratory, but also on people who are willing to get vaccinated.

This thesis has clarified some of the controversies on gut IgA induction to oral antigen and has provided valuable information on how antigen-specific immune response in PP is induced. I can only hope that the scientific community will benefit from this research and use it as a basis for new exciting questions to be tackled.

ACKNOWLEDGEMENT

To all the **mice** that participated in the study, without your involvement this book would not exist. 90% of all the experiments done were successful and I hope that the sacrifice made was not in vain. Thank you!

Susanna Cardell, thank you for supporting the outreach activities of the department that allowed me to form a MIVAC team for Göteborgsvarvet and Blodomloppet. Tusen tack to those who were not afraid to participate – **Stefan Isaksson**, **Siggeir Brynjolfsson**, **Stefan Nordqvist**, **Gurdeep Chahal**, **Priti Gupta**, **Filip Ahlmanner**, **Louis Szeponik**, **Rebecca Josefsson**, **Rebecca Andersson**, **Sukanya Raghavan**, **Charlotta Hansson**, **Sarah Straßburger**, **Sophie Schussek**, **Jacob Cervin**, **Jenna Anderson**, **Dace Ābele**, **Rakesh Chandode**, **Ērika Gribonika**, **Esteri Viitanen**, **Lisa Larsson Berglund –** it is of fundamental importance to inform the society about the need for vaccines and research in general. Is there a more fun way to do that than by running 5, 10 or 21km? MIVAC runs on science!

To **Marie Curie Alumni association** for providing the funding for yet another Göteborgsvarvet to promote the universal influenza vaccine research done within the UniVacFlu network. 21 km closer to the universal influenza vaccine!

To **Adlerbertska Stiftelserna**, **Knut och Alice Wallenbergs Stiftelse, Sahlgrenska Akademin** and **Marie Skłodowska-Curie Actions (7FP)** for providing the funding for research and travel. This enabled me to do some very exciting experiments and allowed me to communicate science all around the world.

To all **UniVacFlu fellows** and **partners** in all corners of Europe, thank you for the nice scientific and social interactions. You literally opened a world of science for me!

To my collaborators and friends **Yoshita Bhide**, **Ganesh Shelke, Rakesh Chandode**, and **Kyong-Su Park**, thank you for the given chance to try my skills in slightly different research fields. We did some good work together. I got struck by your enthusiasm, scientific curiosity, work ethics, and precision! You all have a great future in science and I can only hope that our collaborations will continue.

To all my roomies at the department, **Tobias Hedberg**, **Pekka Jaako**, **Frank Liang**, **Stefan Nordqvist**, **Samuel Alsén** and **Astrid von Mentzer** for providing a cozy atmosphere and a great forum to discuss the global problems of a Ph.D. student's /postdoc's life. In particular to those two who have been

tolerating me the longest – **Samuel**, with all my respect, you are the smartest Swede I know! It seems that in this country geography of Northern Europe is taught only to chosen ones, yet, you are one them! Even more, the history of the region is also your thing, how many times you took me on surprise by congratulating on 18th November and 4th of May! Astonishing! **Astrid**, your heaters are currently heating up the world! You have been my role model and inspiration for research ever since I started. Energy and dedication are flowing from you! I wish you good luck and strength to combine the family life in Gothenburg and the research in Cambridge!

To **all my friends** in Gothenburg and Latvia, thank you for being a reason to get out of the lab and have a long walk or a delicious dinner or simply a Fika! Surely, this had a significant influence on my mental well-being! ☺

Es gribu pateikt lielu paldies **visiem maniem skolotājiem** Priekuļu pamatskolā, Preiļu Mūzikas un mākslas skolā un Preiļu Valsts ģimnāzijā par iedvesmu, motivāciju un vienmēr jauniem izaicinājumiem, jo nekas jau nerodas bez smaga darba! Jūs veidojāt mani kā tādu Latgales mālu...

Mols vīn, mols, vysapleik — mols,
Molu meicej pūdnīks vacs,
Izmeicejs, kai izgrīž pūdu,
Pūda vydā — Preiļs taids sēd.
Mozs, a kusteigs, leikom kojom,
Kankarainu mēteli,
Lyudz nu pūdnīka, lai īdūd
Bāslaveņai dālderi.
Nav jau pūdnīks kaids to žņauga,
Dūtu trejs, bet kur jūs jimt?
— Cik tos bādas! — attrauc Preiļs,
— Byus tev tiergs jau aizpareit! —
Un, kai saceits — trešā dīnā
Divu ceļu krystceļūs

Tērga polots — plots kai paltraks,
Tērdzinīki — tyukstūšos!
I zvīdz zyrgi, i kvīc kvīkši,
I tynkš pūdim vopējums,
I skaņ nauda, i smej pūdnīks:
— Dūdu Preiļam riškovu! —
Preiļs, tū peļņu saskaitejs,
Blokus tērgam pili ceļ.
A, kur pils, tī sāta leidzos —
Tai tam Preiļam — piļsāta!
Preiļi, Preiļi zīd kai jasmins,
Tāli tveiksmes byrga sveist —
Dažai daiļovai uz kāzom Stāmačūs pat Laimu leigst!

/Antons Kūkojs. Variaceja par Preilim. 1988./

Vysleluokū paldis gribu saceit sovim **vacuokim**. Jyus esit muna leluokuo dzeivis laime un prīks! Jyus īdedzet zynuotkuori jau agrā bierneibā muocūt pasaulis lykumu kuorteibu, stuostūt par fiziku i bioloģeju iz dzeivis, dūdūt laseit gromotys par kosmosu, ģeogrāfeju i viesturi. Jyus atteistejot munu iztēli, losūt puorsokys. Jyus muocejot pacīteibu, drūsmi caur dorbu. Jyus esit muns suokums, muna leluokuo veiksme, bez Jums es naasu nikas! Itei disertāceja ir muna pateiceiba Jums! Paldis, ka Jyus esit!

Ērci, paldis par skaistū vuoka zeimiejumu! Tu esi patīsi talanteiga personeiba ar naticami lelom dorba spiejom, degsmi un apjiemeibu vīnmār uzvarēt! Jau tagad Tu kolnus guoz un veidoj pasauli! Tu esi vyslobuokuo! Es ar Tevi ļūti lepojūs!

Andeņa, muns mozais pedeļ (kai Preiļu baba kuodreiz saceja...), Tevī sliepās vysys pasaulis spāks! Tu, mozeņa byudama, īvuiceji man atbildeibu i patstuoveibu. Tu, taipat kai es i Ērce, esi laimiejus dzeivis loterejā ar vysbreineiguokajim vacuokim pi obim suonim! Esi pruoteiga un izmontoj labi Tev pīšķiertuos duovonys, es ar Tevi ļūti lepojūs!

Visim munim **vacvacuokim**, jyus īvuicejot man mīlēt un cīneit kotru dzeivū byutni. Jyus īruodejot man dorba tykumu, bet arī lutynavot bez gola! Sīna laiks Grybynānūs un guļbu tolka Dambeišūs ir munys duorguokuos bierneibys atmiņys! Jyus bejot muns paraugs i drūšais krosts vysuos dzeives vātruos. Jyus bejot un esit munys mozūtnis spylgtuokuo zeime.

Krystamuot, eņģeli, paldis, ka Tu beji! Tu vīnmār man liki dareit vairuok, vysur īsasaisteitīs, uzadreikstētīs... Tovys degsmis īspaiduota, es devūs pasaulī i, gluži kai Spreideits sasasmālus pasauļa gudreibu, nuokšu muojuos... Tik ļūti suop, ka navari ar mani šudiņ daleitīs prīkā, bet ticu, ka siedi iz saulis stareņa i smaidi man pretim kotru dīnu. Itys Tev.

Reksi, muns lobuokais draugs! Tu atvieri durovys iz pasauli, īvuiceji sapņuot, nūticēt sev i pīpildeit lelys lītys! Tu vīnmār beji leidzuos, ejūt cauri guņim i iudiņim. Paldis, ka esi ar mani!

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