Intestinal IgA Synthesis:
Localization and Requirements for IgA Class Switch Recombination

Peter Bergqvist

GÖTEBORGS UNIVERSITET

Department of Microbiology and Immunology
Institute of Biomedicine at Sahlgrenska Academy
University of Gothenburg
Sweden, 2009

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”Vad det är skönt, att inte veta sitt eget bästa
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Abstract

Production of IgA at mucosal surfaces is one of the most striking features of the mucosal immune system. Despite that IgA was first discovered in the 1950's and secretory IgA described in gut secretions and breast milk in the mid 1960's we still have limited information about the sites and exact requirements for IgA class switch recombination. The aim of this thesis work was to investigate potential locations for induction of T-independent IgA responses using CD40 deficient mice as a model. Furthermore, as germ free mice have very poor IgA levels in the gut lamina propria (LP) we investigated whether this is because of a lack of IgA CSR at the inductive sites or whether the commensal flora is involved in maintaining IgA plasma cells at the effector site in the LP itself. Finally we used new ways of assessing the development of T-dependent IgA responses during oral immunizations using NP-hapten-conjugated cholera toxin as our oral immunogen.

$CD40^{-/-}$ mice have very low levels of serum IgG, are unable to form GC and as a consequence, cannot respond to TD antigens. However, we found that $CD40^{-/-}$ mice hosted near normal levels of IgA plasma cells in the gut LP, indicating that IgA CSR was intact and could occur in the absence of GC-formations and CD40-signalling. The ongoing controversy between researchers claiming evidence for two types of IgA CSR processes in the gut; one TD in the organized gut associated lymphoid system (GALT), and another pathway dependent on the commensal flora and ongoing in the non-organized LP itself, prompted us to investigate these theories in more detail using $CD40^{-/-}$ mice and molecular markers for IgA CSR. We found no evidence for IgA CSR in the gut LP and that IgA CSR was restricted to the GALT and the Peyer's patches (PP), in particular. In support of this notion, we observed clonally related Ig heavy chain variable sequences in widely separated segments of small intestinal biopsies, suggesting a common source rather than a disseminated process in the non-organized gut tissue. In addition, analyzing the $GL7^{int}$ cells for molecular markers of IgA CSR clearly showed that the cells could undergo IgA CSR despite not being derived from histologically detectable GCs. Therefore, we believe that the main pathway for CD40-independent IgA CSR is via the PPs, as in WT mice, and that the IgA CSR precedes the GC-stage where somatic hypermutations are introduced.

Furthermore, studies in germ free mice revealed that GCs were present and IgA CSR was ongoing in the PPs, despite the lack of commensal gut microflora. Therefore, we hypothesize that the effector site, the lamina propria, is deficient in supporting IgA responses.

Finally, we studied TD IgA responses at a molecular level during oral immunizations using NP-CT conjugates as antigen. We found that repeated oral immunization generated affinity matured and clonally selected IgA responses originating from the GALT. Three immunizations generated 15% antigen specific IgA plasma cells in the LP, distributed evenly thoughout the intestine.

In conclusion, we have provided evidence that TI IgA CSR occurs exclusively in the GALT prior to SHM in GCs. IgA CSR activity was never found in the non-organized LP, and peritoneal cavity B-cells do not significantly contribute to LP IgA plasma cells. Additionally, we show that the induction of IgA CSR is intact in GF mice, but subsequent IgA plasma cell development appears to be impaired, resulting in a 90% reduction in gut IgA plasma cells in the small and large intestine. Finally we show that TD IgA responses are efficiently generated in the GALT and that the responses early on undergo mutational selection events that result in high affinity IgA plasma cells seeding the gut LP.

Keywords: IgA, Intestine, Gut Associated Lymphoid Tissue, Class Switch Recombination, CD40, Germ Free
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Original papers

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

I. **Gut IgA Class Switch Recombination in the Absence of CD40 Does Not Occur in the Lamina Propria and Is Independent of Germinal Centers.**
Bergqvist, P., Gardby, E., Stensson, A., Bemark, M. & Lycke, N.Y.
J Immunol **177**, 7772-7783 (2006).

II. **T Cell-independent IgA Class Switch Recombination is Restricted to the GALT and Occurs Prior to Manifest Germinal Center Formation.**
Bergqvist, P., Stensson, A., Lycke, N.Y. & Bemark, M.
Submitted to J Immunol

III. **Germ Free Mice Express High IgA Class Switch Recombination Activity But Develop Few IgA Producing Plasma Cells.**
Bergqvist, P., Stensson, A., Bemark, M., & Lycke, N.Y.
Manuscript

IV. **The T-dependent specific gut anti-NP ((4-hydroxy-3-nitrophenyl)acetyl) IgA response is oligoclonal and is affinity matured in gut associated lymphoid tissue.**
Bergqvist, P., Bemark, M., Stensson, A., Holmberg, A. & Lycke, N.Y.
Manuscript

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Abbreviations

αCT	Alpha circle transcripts
Ag	Antigen
AID	Activation induced cytidine deaminase
APC	Antigen presenting cell
APRIL	A proliferation inducing ligand
BAFF	B-cell activation factor of the tumor necrosis factor family
BCR	B-cell receptor
CD40L	CD40 ligand
CDR	Complement determining region
CSR	Class switch recombination
CT	Cholera toxin
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbet assay
ELISPOT	Enzyme linked immunosorbet spot assay
EC	Epithelial cell
FACS	Fluorescent activated cell sorting
FAE	Follicle associated epithelia
FDC	Follicular dendritic cell
FWR	Framework region
GALT	Gut associated lymphoid tissue
GC	Germinal center
GFP	Green fluorescent protein
ILF	Isolated lymphoid follicle
IgA	Immunoglobulin A
iNOS	Inducible nitric oxide synthase
LP	Lamina propria
LPS	Lipopolysaccharide
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
NP	(4-hydroxy-3-nitrophenyl)acetyl
NHEJ	Non homologous end joining
PRR	Pattern recognition receptor
SED	Sub epithelial dome
SFC	Spot forming cells
SHM	Somatic hypermutation
RA	Retinoic acid
RAG	Recombination activating gene
RNA	Ribonucleic acid
RSS	Recombination signal sequence
RT-PCR	Reverse transcriptase-polymerase chain reaction
PP	Peyer's Patch
TD	T-cell dependent
TNFα	Tumor necrosis factor alpha
TI	T-cell independent
TSLP	Thymic stromal lymphopoietin

Introduction

The immune system is divided into an innate and an adaptive part, where the innate immune system provides the initial response to a foreign antigen, be it food antigens or microbes. This is especially true for the mucosal immune system found in the gastrointestinal tract. The mucosa of the gut serves as a first line of defence against pathogens (1, 2). At the same time it coexists in homeostasis with the gut commensal flora, which is critical for a healthy life. To be able to discriminate between pathogens and dietary antigens, a delicate regulatory network has been developed at mucosal sites. This includes a physical barrier, production of mucus, sensing of luminal antigens by pattern recognition receptors (PRR) and production of secretory IgA (SIgA) (3, 4).The cells responsible for innate immunity include epithelial cells, monocytes, macrophages, neutrophils and dendritic cells (DC) . Whereas the innate immune system is unable to recognize specific antigens and cannot develop memory functions the adaptive immune system is specific for antigenic epitopes through distinct receptors on T and B – lymphocytes. Following clonal expansion of T and B lymphocytes after antigen recognition long term memory is generated. One of the main functions of B-cells is to produce and secrete high affinity antibodies to protect the host from invading pathogens. To be able to respond to a wide variety of pathogens, toxins and other foreign agents, B-cells go through several selection and maturational events when formed in the bone marrow (5). After antigen activation naïve IgM B cells undergo expansion, class switch recombination (CSR) to downstream isotypes and somatic hypermutations (SHM) in the inductive lymphoid tissues before they become antibody secreting plasma cells (6). I will now explain the major events during B-cell development in the bone marrow (BM) as well as in peripheral lymphoid tissues, such as lymph nodes or spleen, following antigen activation with emphasis placed on gut IgA B-cell development.

Antibody structure and function

The key component of humoral immunity is production of antibodies. High affinity antibodies effectively eliminate invading pathogens by several different mechanisms depending on the antibody isotype. The different isotypes activate and recruit components of the immune system which can effectively target and eliminate an infection. In addition, antibodies can prevent pathogens to adhere to the host cells as well as neutralize toxins, which are thought to be the primary function of secretory IgA (SIgA) at mucosal surfaces (7-9). SIgA is produced by plasma cells in the mucosal lamina propria (LP) and secreted into the gut lumen where it can

adhere to bacteria or bacterial toxins, thus inhibiting bacterial adhesion and neutralizing toxins. Thus, bacteria which are trapped by SIgA antibodies are unable to adhere to or penetrate the mucosal barrier and are efficiently eliminated from the host (10). The multitude of antigens the body can encounter is enormous and in order to deal with such complexity B cells with very diverse antigen-binding ability is developed in the bone marrow (5).

Immunoglobulin structure

Although Ig molecules have widely different antigenic binding properties and effector functions they share a common structure which consists of two heavy chains and two light chains (Fig. 1) (11). Both chains are comprised of a constant (C) and a variable (V) region where the C_H regions carries the effector function of the antibody, and the V regions confer the antigen

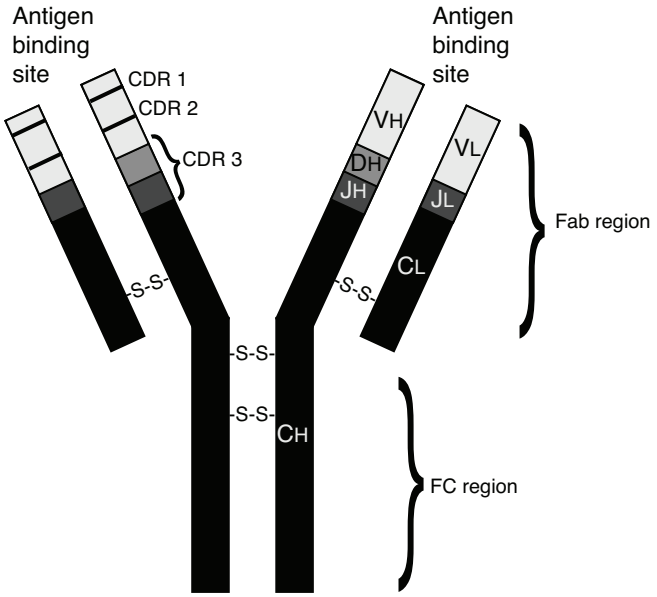

Figure 1
The image shows a schematic drawing of an immunoglobulin. The lower part of the antibody is the C_H chain which determines the activity of the antibody. This part is also named the FC region. The upper part of the antibody consists of both the heavy chain and the light chain and is the part of the antibody which interacts with the antigen and is named the Fab region. The three different hypervariable CDR regions are marked in the figure, which are the only parts of the antibody in direct contact with the antigen. The different chains are held together covalently by disulfide bonds (-S-S-).

specific binding properties of the Ig molecule. The heavy and the light chains are assembled early during B-cell development by combining variable (V) diversity (D) and joining (J) segments to various constant regions by a recombination activated gene (RAG) dependent process (12-14). In addition, the Ig heavy chain locus in mice contains five (eight) different C regions: μ, δ, γ, ε and α, where γ can be subdivided into γ1, γ2a, γ2b (or γ2c) and γ3. (Fig. 2). Naïve B-cells express the IgM heavy chain (15, 16). Following antigen activation this expression is altered by CSR, which generates B cells with down stream isotypes such as IgA in the periphery (17, 18). Depending on where the B-cell is activated and on which cytokines are released in the vicinity of the activated B-cell, different isotypes are favoured. The most important switch factor for

IgA differentiation is TGF-β (19, 20). The light chain consists of two different constant regions, κ or λ, where κ is the dominant light chain in the mouse. In fact 95% of all antibodies in mice carry the κ light chain (21). In contrast to C regions, the V regions of the antibody are highly variable to enable a wide antigen-binding repertoire. This variability is generated through the combination of different V, D and J segments early during B-cell development by an antigen independent process in the bone marrow (22). In addition, following antigen activation somatic hypermutation (SHM) fine tune the binding properties of the antibody to increase the affinity of antigen binding. This is achieved through single base pair mutations of the V-genes and normally requires the establishment of a germinal center (GC) (23).

When an antibody binds antigen, the interaction occurs at three specific sites of the V region called the complement determining regions (CDRs) (24). These regions of the antibody are hypervariable and highly susceptible to SHM to allow a repertoire of antibodies able to bind many different antigens. The framework region (FWR) sequences between the CDRs contain far less mutations compared to the germline sequence than the CDRs (25). These sections are not in direct contact with the antigen, but are merely a framework in the antibody structure. The high variability of the CDR3 region is generated already during VDJ recombination, because the CDR3 includes the VDJ joints, where variability is generated not only by randomly combining different V, D and J segments but also by non-templated genetic alterations (14, 22). CDR1 and CDR2 on the other hand, are located within the V gene and the sequences are determined by the V segment that is used in that particular antibody. In addition to the variability generated during VDJ recombination, the CDR regions are hotspots for SHM during affinity maturation, which results in the high variability and high affinity towards the antigen (25). Together these processes generate antibody repertoires with vastly different antigen binding properties, necessary to protect the host from invading pathogens and toxins.

B-cell development

VDJ Recombination

B-cells develop early in ontogeny from haematopoietic stem cells in the bone marrow and fetal liver (26). During development B-cells have to produce a functional B-cell receptor (BCR) to be able to interact with a distinct antigen. To create such a diverse repertoire of B cells, recognizing an almost infinite number of antigenic determinants (epitopes), the BCR is

generated by VDJ recombination, which requires expression of the recombination activating genes (RAG-1 and RAG-2), as already alluded to (12, 22, 27). In mice there are currently 110 described functional V_H genes, 85 pseudo V_H genes, 23 D_H genes and 4 J_H genes (28, 29). By randomly combining different V_H, D_H and J_H sequences an enormous variation in antigen specificity is achieved. In addition V-J recombination of the light chains adds variability to the produced antibody. However, the diversity is not only generated by combining different VDJ segments, but it is also generated by the recombination process itself. Each coding segment is flanked by a recombination signal sequence element (RSS) which RAG-1 and RAG-2 binds to and generates double stranded breaks (27, 30, 31). These ends of the broken DNA are asymmetrical and create a hairpin loop which has to be resolved before recombination continues. When the hairpins are resolved by Artemis/DNA-PKc complexes, proteins of the ubiquitously expressed non-homologous end joining (NHEJ) pathway, two hanging ends are created (32). These ends can be complemented by P-addition to form blunt ends, which are ligated together (33, 34). In addition, exonuclease activity can cut the non homologous ends and ligate the blunt ends together, and the enzyme terminal deoxynucleotidyl transferase (TdT) can add non-templated (N) nucleotides to the joint (35). Together these series of events ensure that every VDJ rearrangement is unique and highly variable. These recombination processes occur within the CDR3 region of the Ig gene and renders a unique “fingerprint” for each B-cell which can be traced later in the periphery by sequencing the V_H gene and analyzing the CDR3 region (36-38).

B-cell selection

The highly variable assembly of a functional BCR will not always result in a functional Ig gene. Some rearrangements will generate stop codons, or even worse; generate self reactive B-cells. Therefore, constant and careful surveillance of the newly generated Igs is required to ensure that self reactive B-cells are not allowed to circulate the body. Hence, every B-cell that produces a functional BCR is probed for self reactivity before, and shortly after exiting the bone marrow. If a B-cell generates a BCR with high affinity to self antigens at this point of development, the cells are eliminated by apoptosis (39-41). Alternatively, the BCR affinity can be altered by receptor editing to make it less prone to bind self antigens. However, B-cells with a low affinity to self antigens become anergic and these B-cells are unresponsive to their specific antigen, and if they encounter antigen in the periphery, T-cells cannot activate these B-cells (42, 43).

In addition, immature B-cells in the periphery can be selected based on their BCR signalling strength. A tonic signalling in weak self-reactive B-cells is thought to generate marginal zone B-cells in the spleen (44). Thus, B-cells are carefully selected not to interact with self antigens before they are released into the blood and lymph. Follicular B-cells, that encounter antigen, are activated and receive cognate T-cell help to become Ig secreting plasma cells or long lived memory B-cells (45). This control mechanism ensures that the immune system is not directed towards self antigens, but rather reacts to foreign antigens. Should these control mechanisms fail, this could lead to autoimmunity.

B1-B cells

In addition to B-cell development in the bone marrow, a subset of B-cells named B-1 cells develop in the fetal liver (5). B-1 cells have self renewing capacity and occupy the peritoneal and pleural cavities in adult mice but can also be found in the spleen (46, 47). B1 cells can be further subdivided based on their surface expression of CD5 where B-1a B-cells are $CD5^+$ and IgM^{high} and B-1b B-cells are $CD5^-$ and IgM^{high} (46). B1 cells have been shown to contribute to natural serum IgM antibodies and studies have indicated that maybe as much as 50% of the mucosal IgA production originates from B1 B-cells in the peritoneal cavity (48, 49). However, at variance, other studies have failed to confirm this notion and have not found B1 cells to contribute significantly to the overall IgA production in the gut (50). The theory that B1 cells contributes significantly to gut IgA production has been incompletely challenged. Although it is thought that B1 cell responses are predominantly T-cell independent and directed mainly against bacterial antigens abundant in the gut lumen, it is still incompletely understood where the B-1 cells are triggered by antigen and undergo IgA CSR (51, 52).

T-cell dependent and independent antigens

B-cells in the periphery respond to antigen, but without co-stimulation and T-cell help B-cells die by apoptosis. Newly generated B-cells exit the bone marrow and circulate to the blood and lymph because there is a constant production of new B-cells with diverse antigenic specificities. B-cells are able to directly interact with soluble protein antigens through their BCR. B-cells are also capable of processing and presenting peptides on MHC class II molecules on the cell surface (53). $CD4^+$ T cells, which are unable to interact with soluble antigens alone, recognize complexes with MHC class II presented peptides on the membrane of the antigen presenting

cell (APC) with the TCR. This recognition leads to activation of the T cell and upregulation of CD40L and other surface molecules. Since CD40 is the corresponding receptor for CD40L and expressed on the B cell surface the T cell can through cognate interaction stimulate the formation of GCs. In these hotspots for B cell expansion two critical processes are occurring , namely Ig CSR and SHM (54). Therefore, antigen responses that require T cell help for driving B cell expansion and differentiation are termed thymus dependent (TD) responses. One example of a TD antigen is cholera toxin (CT) which is widely used as a mucosal immunogen and adjuvant in experimental systems. The immunogenicity of CT is highly dependent on $CD4^+$ T-cells, and no CT specific antibodies are generated in the absence of appropriate T-cell help (9, 55-57).

Some B-cells can be activated without cognate interaction, not requiring T-cells, and such B cell responses are named thymus independent (TI) responses. Normally, activation of B-cells by TI antigens does not lead to GC formation and hence very low levels of Ig CSR and SHM are observed in TI responses. Depending on the nature of the antigen, and how they activate B-cells, they can be grouped into TI type 1 (TI-1) and TI type 2 (TI-2) antigens. TI 1 antigens are typically polyclonal activators of B-cells (mitogens), such as bacterial lipopolysaccharides (LPS) which activate B-cells regardless of their antigenic specificity. TI-2 antigens are molecules with highly repetitive epitopes, such as bacterial flagellin, which are able to crosslink the BCRs which leads to B-cell activation (58). A major difference between the two types of TI antigens is that TI-2 antigens require a mature and functional BCR and they will not act as polyclonal activators. TI-1 antigens on the other hand, can activate immature B-cells without functional BCRs. In addition, TI-2 antigens require cytokines derived from $CD4^+$ T cells to efficiently stimulate B-cell responses (59).

Germinal center reactions

During TD antibody responses B-cells interact with $CD4^+$ T-cells via cognate interactions at the border of the B-cell follicle. In response to antigen exposure the T-cells upregulate CD40L, that interacts with CD40 on B-cells (60, 61). As aforementioned, these signalling events result in the formation of GC. The GC reaction is responsible for generating high affinity plasma cells and memory B-cells, and it can be divided into a dark zone and a light zone (62). Until recently, the hypothesis was that B-cell blasts named centroblasts, down-regulate their surface Ig expression and undergo heavy proliferation in the GC dark zone. During proliferation

mutations accumulate in the variable regions of the Ig locus in the B-cells by SHM. Following several rounds of proliferation the B-cells acquire surface Ig molecules, which are mutated compared to the germline sequence, and could have an enhanced affinity for the antigen (63). These so called centrocytes, migrate to the light zone of the GC, where they interacted with antigen trapped on follicular dendritic cells (FDC). Based on an increased affinity towards the antigen such B-cells receive survival signals and are enriched (64). However, recent studies using multi photon microscopy have shown that the GC reaction is even more complex than originally thought. In fact, it appears that B-cells within a given GC are highly motile and long lived interactions with FDCs are rare. Evidence that B-cells constantly migrate between the dark and light zone in both directions, as well as documentation of substantial cell proliferation in both zones have been presented (65-68). Thus, the exact sequence of events and mechanisms in the GC remains to be elucidated.

Activation induced cytidine deaminase

The antigen-independent VDJ recombination in the bone marrow generates naive B-cells with can interact with a wide variety of antigens (12, 22). Whereas, Cμ is always expressed on the surface of naïve B-cells high affinity antibodies with different effector functions require that the B-cells alter their genomic composition and in this way increase their affinity for a specific antigen. This is achieved by two antigen-dependent processes; CSR and SHM (17, 18, 23, 66, 69). Both these processes are dependent on the activity of the enzyme activation induced cytidine deaminase (AID) (70-74). Originally AID was described as an RNA editing enzyme based on homology to apolipoprotein B (apoB) mRNA-editing enzyme, catalytic polypeptide 1 (APOBEC-1) (72). However, accumulating data suggest that AID acts directly on single stranded DNA by deaminating deoxycytidine (dC) residues to deoxyuracils (dU), which initiates Ig CSR and SHM (75-78). The deamination occurs preferentially at dC recidues at WRC hotspots where W=A or T and R=purines, which are frequent in the switch regions of the V_H genes (79, 80). In addition, AID only targets dCs on single stranded templates which are formed during transcription, and therefore, transcription is essential to initiate Ig CSR and SHM (78, 81-85).

Class switch recombination

The different Ig isotypes have distinct and unique effector functions and they bind to different Fc receptors. For example mucosal IgA binds to the pIg receptor (pIgR) which enables transport of dimeric IgA to the mucosal lumen (86, 87). Naive B-cells express IgM, but during CSR the V_H gene is rearranged to express different isotypes. The VH locus originally contains all the different C regions lined up together with the respective promoters and switch (S) regions (Fig. 2), but initially only the Iμ promoter is actively transcribing the Cμ gene segments. However, the different promoters are activated by different cytokines and this way can transcribe the distinct S regions, located between the promoter regions and the respective C_H region, a process called germline transcription (18). The Iα promoter is activated in response to TGFβ which initiates germline transcription through the downstream Sα and Cα exons (19, 20). This germline transcription is non-productive and often referred to as a sterile transcript, facilitating

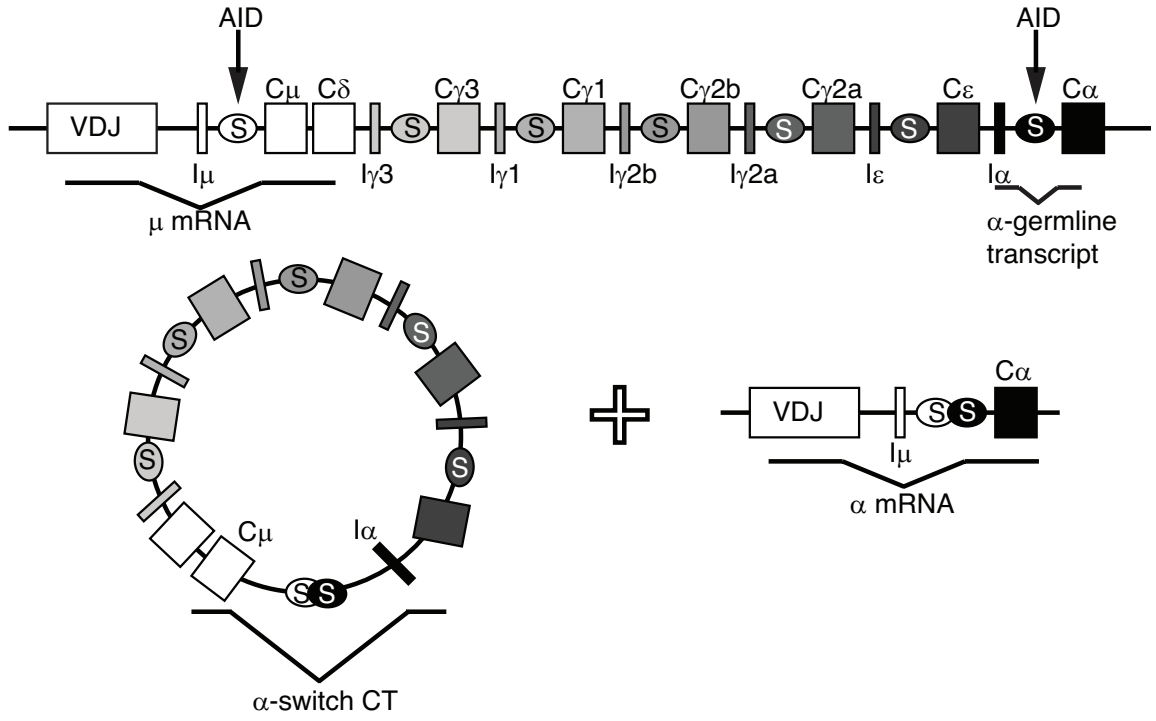

Figure 2

The figure shows the typical events during IgA CSR and the resulting producs. The top shows the Ig heavy chain locus in mouse after completed VDJ recombination. At first the Iμ promoter is active and generates IgM expressing cells. With the correct stimuli (TGFβ for IgA) downstream promoters become active and generate germline transctipts. During CSR double stranded breaks are induced in the switch (S) regions by an AID dependent process, which results in the generation of a switch circle by an intra chromosomal deletion of the excessive DNA. The activated Iα promoter is still active but transcribes the circle now, creating a nonsense α-switch circle transcript. These transcripts can be detected by PCR and serve as excellent markers for recent IgA CSR. To the right is the newly synthesized IgA generating product.

subsequent Ig CSR (18, 88). Therefore, during germline transcription AID is recruited to the hotspots in the actively transcribed switch regions where it deaminates dC residues to dU to initiate CSR. The endogenous base excision repair mechanism repairs the introduced dU in an orderly fashion, including the activity of several important enzymes. First, uracil DNA glycosylase (UNG) removes the dU, creating an abasic site (89, 90). Second, apurinic/apyrimidinic endonuclease (APE) generates double stranded breaks at the abasic sites in the switch regions of the donor isotype and the acceptor isotype (82, 91), and an intrachromosomal deletional event occurs, where the intervening DNA is looped out (92, 93). The donor and the acceptor S regions are joined together by DNA polymerase β (pol β) to form a new chromosomal rearrangement and the looped out DNA is degraded (18) (Fig. 2). The transcription of the newly synthesized DNA is directed through the Iμ promoter, but the Iα promoter is still active a short period of time after the CSR events occurred. Importantly, the transcription directed from the Iα promoter is now directed through the excised DNA circle creating a nonsense product that can be detected by PCR. Because these circular transcripts are retained for a short time after completed CSR, they can serve as excellent markers of ongoing and recently completed Ig CSR (94).

Affinity maturation

Somatic hypermutation (SHM)

The second major genetic alteration in antigen activated B cells is achieved by SHM, which generates high affinity antibodies against a particular antigen. During SHM point-mutations are generated in the Ig V regions, which can increase the antigen binding properties of the Ig molecules. Similar to CSR, this process is triggered by antigen, requires adequate T-cell help and occurs within GCs in the lymph nodes or spleen (69, 95). This process requires AID which initiates SHM the same way as CSR, by deaminating dC residues to dU (23).The mismatch repair mechanisms can either remove the dU, creating an abasic site, or creates a new strand using the dU as a template (89, 90, 96). If the dU is used as a template, the original dC information is lost and the newly replicated strand will contain a dT instead of the complimentary dG as the original dC residue would generate. Thus the net result for this type of replication is a mutation from a GC pair into an AT pair (85, 97). Additionally, the dU can be removed by UNG, creating an abasic site, and if replication occurs across the abasic site, error prone polymerases will insert any base to repair the damaged DNA (75, 96, 98). Thus, this pathway will generate an

unbiased array of mutations but sometimes the inserted bases will also be the same as the initial deaminated dC. Finally mutations at AT base pairs are introduced by dC to dU deamination, but instead of being excised by UNG the mismatch repair pathway enzymes MSH6/MSH2 recruits the exonuclease EXO1 which removes a stretch of bases surrounding the original mismatch (23, 69, 96, 99).

Selection

As previously mentioned AID acts on the Ig genes at specific hotspots where mutations are introduced. Some mutations will be silent whereas others will be replacement mutations which lead to amino acid changes. Depending on the mutation, the affinity for the antigen is altered, where some mutations lead to higher affinity for the antigen and others to lowered affinity (100). To ensure that only cells with mutations that render high affinity antibodies escape the GC it is thought that the newly inserted mutations are screened against trapped antigen on the FDC (101). Centrocytes with different affinity are thought to compete for the antigen trapped of the FDC and centrocytes with high affinity for the antigen will be selected for survival (64). Centrocytes with unaltered affinity, or lower affinity for the antigen, die by apoptosis and are engulfed by macrophages (62). Recent data show that FDCs are able to retain the captured antigen and interact with B-cells >1 week after immunization. This prolonged presentation assures that even rare B-cells are able to interact with antigen captured on the FDC (67). Additionally, it allows B-cells to recirculate the GC and go through several rounds of mutations and selection processes (65, 68, 102, 103). This quality control of ensures that only high affinity B-cells are selected to become plasma cells or long lived memory cells.

The gut mucosal immune system

The gut mucosa and its local immune system serves as a defence barrier against pathogenic bacteria, viruses and parasites that otherwise would gain access to the body. Simultaneously, the mucosal barrier allows uptake of food antigens and has established a symbiotic relationship between the commensal bacterial flora and the host. Of note, the large intestine can carry up to 10^{12} microorganisms per ml of luminal content (104). Only a single layer of epithelial cells protects the host from the luminal content, but critical for the barrier function is also the mucus layer and above all the generation of SIgA antibodies. These are generated in response to the bacterial flora as germ free mice exhibit a dramatically reduced gut IgA level. The SIgA is produced by

plasma cells in the gut lamina propria (LP) (105-108). The production of SIgA is one of the cardinal features of the mucosal immune system. In humans, every day approximately 3g of IgA is produced, which is more than all other isotypes combined (109, 110). The production of IgA is essential in maintaining the gut homeostasis with the commensal flora and in preventing attacks by pathogenic microorganisms or harmful toxins (111). Two pathways for generating IgA antibodiers have been proposed. The first is driven by the commensal flora and is T-cell independent, while the second pathway is responsive to T-dependent antigens and initiatiated in the organized lymphoid tissue of the GALT. Both these pathways are dominated by classical B2 cells but to what extent the two pathways contribute to the overall IgA production in the gut is incompletely known. In addition, B-1 cells derived from the peritoneal cavity have been suggested to contribute to the overall IgA production in the gut against the commensal flora (2, 52, 111-115).

Despite an extensive literature on IgA formation and function we still lack a definite understanding of how and where gut IgA responses are generated (10, 116). As early as 1971, Craig and Cebra showed in rabbits that Peyer's patch (PP) B-cells preferentially underwent IgA CSR and were committed to become IgA producing plasma cells in the intestinal LP (117). Additional evidence that antigen specific IgA responses were generated in the PPs were reported by Husband and Gowans in 1978, who used ligated intestinal loops challenged with cholera toxin to demonstrate that PPs were the inductive sites for antitoxin IgA responses. They found that if the loop hosted a PP, cholera toxin specific IgA cells appeared in the draining thoracic duct, whereas no IgA response was found in loops devoid of PPs (118). Thus, it has been known for a long time that PPs are potent inductive sites for specific IgA responses. However, in recent years attention has been given to alternative sites as inductive sites, especially for IgA responses against the commensal flora. These responses have been proposed to be initiated *in situ* in the non-organized LP itself and possibly also in the peritoneal cavity (48, 49, 115, 119). However, just recently a few studies have questioned whether the LP is a site for IgA CSR against the bacterial flora (36, 38, 120).

Factors influencing IgA CSR

Several factors are involved in the generation of gut IgA antibodies. The first cytokine that was described to promote IgA CSR was TGF-β, which was tested on LPS-stimulated naïve B

cells in vitro (19). The authors showed that TGF-β induced IgA CSR in IgM B cells rather than enhanced production of IgA from already IgA-committed cells. This was also confirmed by subsequent studies, showing that TGF-β induced germline transcription through the α-switch regions by activating the Iα promoter (121-123). Several sources of TGF-β have been described, such as B-cells (124), T-cells (125), antigen presenting cells (126) and stromal cells (127, 128). The relative importance of the different sources of TGF-β for IgA CSR though, has not been clearly elucidated. Additionally, IL-5 and IL-6 act synergistically with TGF-β to augment IgA responses, but are not alone sufficient to induce IgA CSR (129, 130).

In addition, two other IgA switch factors are derived from the ECs or DCs. These are termed a proliferation inducing ligand (APRIL) and B-cell activation factor of the tumor necrosis factor family (BAFF) (131-133). These factors act directly on the activated B cell independently of the presence of T cells and in this way could promote IgA responses to the commensal flora in the complete absence of an organized lymphoid tissue that supports cognate T-B cell interactions. B-cells have several receptors for both these ligands; B-cell maturation antigen (BCMA), transmembrane activator and CAML interactor (TACI), BAFF-receptor (BAFF-R) and APRIL-receptor (APRIL-R) (134-136). In a study by He, et al. it was recently shown that human B-cells can undergo CD40-independent IgA CSR when stimulated with BAFF and APRIL. Because DCs express BAFF and APRIL the authors suggested that this could be an alternative pathway for IgA CSR in the non-organized gut tissue in response to the commensal flora (137). Also, TNFα/iNOS producing DCs have been ascribed a possible role in generating IgA responses. It was recently reported that T-cell dependent IgA responses were lowered in $iNOS^{-/-}$ mice, by impairing the expression of TGF-β receptor II. More importantly, T-cell independent responses were dramatically reduced, probably as a result of low production of BAFF and APRIL, in $iNOS^{-/-}$ mice (138). Interestingly, iNOS production is dependent on bacterial ligands, and GF mice are deficient in iNOS-production, which could explain why these mice have few gut IgA producing plasma cells. Such a defect would affect the inductive site and IgA CSR rather than the effector site in the gut. However, whether BAFF and APRIL exert IgA CSR activity in the organized GALT or are restricted to the non-organized LP is still incompletely investigated (116, 138, 139).

The gut associated lymphoid tissue (GALT)

Peyer's patches

Almost 40 years ago, the PPs were described as the main inductive sites for IgA responses and also the predominant site for IgA precursor cells (117, 118). Together with isolated lymphoid follicles (ILF), cryptopatches and MLN, the PPs constitute an important part of the GALT (140, 141). The PP's are easily detectable macroscopically as distinct nodules spread along the intestine at the antimesenteric border (142, 143). The nodules consist of one or more B-cell follicles containing mostly B2 B-cells surrounded by interfollicular T-cell regions and the sub epithelial dome (SED), which separates the follicles from the follicle associated epithelium (FAE) (144-146). Within the FAE the specialized microfold cells (M-cells) are found, which enable antigen uptake from the intestinal lumen (147, 148). Since PPs lack afferent lymphatics, this is how lymphocytes within the PP are exposed to antigen (149). Antigen, taken up by the M-cells, can be captured by B-cells, DCs and macrophages within the SED, which can present antigen to $CD4^+$ T-cells (150-152). The latter cells support B cell expansion and differentiation within the follicles through cognate interactions, where CD40-CD40L binding plays a critical role. These interactions result in the formation of GC (54). Within GCs the antibody response matures through IgA CSR and SHM. Interestingly, due to the high antigenic load in the intestine the PPs constantly host GCs, which is quite exceptional as all other secondary lymphoid organs, including the spleen, do not normally exhibit GC in unimmunized individuals. These GC may not even be driven by specific antigen-recognition because it was observed in a model where the BCR was replaced by signalling through LMP2A, an Epstein-Barr virus protein, and GCs were formed in response to the intestinal microflora and completely independent of BCR-specificity (153)

Isolated lymphoid follicles

In addition to the macroscopically visible PPs, the isolated lymphoid follicles (ILF) are clusters of B-cells which are not visible to the naked eye, but can readily be detected using low power microscopy (143, 154). The developmental pattern differs between PPs and ILFs as no ILFs are visible at birth, while PPs develop during fetal life (154). Subsequently, germ free mice develop PPs but lack ILFs, which require bacterial colonization to develop (155-157). The ILFs are distributed along the antimesenteric border of the small intestine, and the number of ILFs increases from the proximal to the distal small intestine. Different strains of mice have been

found to host quite different numbers of ILFs and in C57Bl/6 mice as many as 100-300 ILFs can be found (154). Although ILFs are smaller than PPs, the organization is similar to that of the PPs. Importantly, also the ILFs have an FAE with M-cells that can sample luminal antigens.

Two different maturational stages of ILFs have been described; immature (iILFs) and mature ILFs (mILFs) (158). These range from loose clusters of $B220^+$ cells in iILF to organized follicles with FDC that can support the GC formations in mILF (159). The maturation of iILFs into mILFS is dependent on TNFRI signalling, which may be triggered by e.g. luminal bacteria (143, 154, 159-161). However, the total number of ILFs appears to be rather constant and differ only in maturation stage (155). To what extent ILFs contribute to the overall production of IgA plasma cells in the LP of normal mice is unknown, but studies in PP-deficient mice have indicated that this could be quite substantial (120, 127, 159, 161, 162).

Mesenteric lymph nodes

Following antigen exposure, DCs migrate from the mucosa to the draining mesenteric lymph nodes (MLN), where they can activate T-cells (163). The MLN separate the gut intestine from the systemic tissues, and in the MLN DCs also imprint gut homing properties in activated T and B cells (164). Studies in mice lacking PPs have suggested that the MLN can be a complementing site for IgA CSR and the generation of LP-homing plasma -cells (165). Furthermore, the MLN have been found to be indispensable as a barrier for preventing bacteria from reaching systemic tissues. (166). The MLN may also function in establishing oral tolerance against fed protein antigens (167-169). Mucosal DCs loaded with antigen migrate to the MLN where regulatory T cells are induced. These T cells are responsible for dampening unwanted reactions to e.g. food antigens and are critical for the homeostasis in the gut (170, 171).

Lamina propria

The lamina propria (LP) is the diffuse non-organized effector site, where plasma blasts terminally differentiate into IgA secreting plasma cells. The plasma cells residing in the LP produce and secrete dimeric IgA, which consists of two IgA antibodies linked together by a J-chain. This complex is actively transported into the gut lumen after binding to the polymeric Ig receptors (pIgR) produced by the epithelial cells (EC) and located to the basolateral side of the cell (86). Immunohistochemical staining of the small and large intestine in adult mice show that the entire LP is filled with IgA producing plasma cells, explaining the enormous IgA production seen every day.

Homing to the lamina propria

Activated IgA-committed B cells in the GALT, migrate to the MLN and thoracic lymph duct via the blood back to the intestinal mucosa and the LP (172). The signals required for this homing process are imprinted in the B cells already at the inductive site. The system relies on the integrin α4β7 and CCR9 or CCR10 expressed on the membrane of the B-cell (173, 174). The key attractant for the integrin in the gut is mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which is expressed on the endothelial cells in the gut LP (175-177). MAdCAM-1 interacts with α4β7 to attract lymphocytes to the LP (178). Additionally CCL25 (TECK), which is expressed on crypt epithelium in the small intestine, interacts with CCR9 expressed on B-cell blasts from the PP and recruits them to the small intestinal LP (179). In the colon CCL28 (MEC) expression interacts with CCR10, to attract IgA plasmablasts to the large intestine (180, 181). The imprinting of homing receptors on the activated B cells is dependent on DCs and the production of retinoic acid (RA) derived from vitamin A (139, 172, 173).

Lamina propria as a site for IgA CSR

In recent years, the LP has not only been described as an IgA effector site, but also as the inductive site for T-cell independent IgA CSR in both the small and large intestine (115, 119). Evidence in support of this theory has been generated in both human and mice and ascribes critical roles for EC as well as DC and the production of BAFF and APRIL (115, 137, 182). EC can produce thymic stromal lymphopoietin (TSLP) and in this capacity can stimulate DCs to produce APRIL, which has been found to support IgA CSR in naïve IgM B cells *in vitro* (115, 183). TSLP production can be upregulated in response to bacterial products, such as flagellin, and hence the bacterial flora may promote IgA CSR locally in the LP itself. Because LP DCs have been shown to extend their dendrites through the tight junctions between ECs to sample luminal antigens it is conceivable that local DCs in the LP are activated to provide BAFF and APRIL for IgA CSR (184, 185). However, other studies have failed to find evidence for IgA CSR in the non-organized LP itself (36, 38, 120). Thus, there is currently an ongoing debate about whether the intestinal LP can support IgA CSR or not (37, 116). The present thesis work is focused on unravelling the requirements and sites for IgA CSR in the mouse intestine using sensitive molecular markers.

Aims of the thesis

The general aim of this thesis work was to investigate the requirements and sites for IgA CSR in the mouse intestine using sensitive molecular markers.

The specific aims were:

- To investigate the localization of IgA CSR in the CD40$^{-/-}$ mouse model, exploring T cell independent gut IgA responses
- To unravel whether IgA CSR occurs in the non-organized intestinal LP or is restricted to the GALT
- To evaluate at what stage of differentiation activated B cells undergo CSR and SHM in relation to formation of GC in the PP.
- To determine whether the bacterial flora influences IgA CSR at the inductive sites or is critical for developing IgA plasma cell responses in the gut LP effector site.
- To develop and study a hapten-based oral immunization protocol for the molecular analysis of T-cell dependent antigen-specific IgA responses in the gut mucosa, especially taking kinetics of build-up and affinity maturation of the response into account.

Materials and methods

The details of the experimental procedures are described in detail each article. Here, I will try to describe and ratify the choice of methods and techniques that we used for this thesis work.

Mice

All mice used in the study were on a C57BL/6 background, bred and housed under SPF or germ free conditions at the experimental biomedicine (EBM) facility at the University of Gothenburg, Sweden. To better control for the bacterial microflora I choose to breed the mice for all experiments in our own facility. This ensured that the variation in the bacterial flora was minimized between different experiments and between $CD40^{-/-}$ and WT mice, despite that we could not work with littermates. The germ free mice were acquired from two different sources; the Karolinska institute in Stockholm or from our own EBM facility at the University of Gothenburg. The results in manuscript III are the aggregated data from mice originating from both germ free units. The germ free status in both locations was carefully monitored by cultivation assays for both aerobic and anaerobic bacteria at KI and by 16S RNA PCR in Gothenburg. The PCR assay is a more reliable instrument to monitor the germ free status since many bacterial strains cannot be cultivated. In comparison our data from the two breeding units for GF mice were similar strengthening the notion that the mice were truly germ-free.

IgA detection

To identify IgA and IgA producing cells in the gut we used several different methods to get an objective measurement of the intestinal IgA production. Immunohistochemistry (IHC) using fluorescent antibodies was used throughout the studies (paper I-IV). This is an excellent tool to get an overview of the localization and the abundance of IgA in the intestine, and by using antibodies with directly conjugated fluorochromes we were able to stain the sections with different antibodies at the same time (e.g. anti-IgA-FITC and anti-IgM-Texas red). However, information about the absolute amount of IgA cannot be aquired by IHC, and therefore we also used the more quantitative ELISPOT analysis to analyze spot forming cells (SFC) from isolated LP lymphocytes. This method gives an objective measurement of the number of IgA secreting cells within the LP. The ELISPOT assay was complemented by ELISA directly determining the amount of IgA found within the gut lumen, by analyzing gut lavages. Together these methods give us an objective measurement of the total IgA levels within the intestine.

Germinal center identification

The lectin peanut agglutinin (PNA) has traditionally been widely used to identify GCs within spleen and lymph nodes (186). More recently, a monoclonal antibody GL7, which labels activated GC B-cells, was developed (187). We used the GL7 antibody in all our experiments. GL7 staining generates less background staining on sections compared to PNA, and it is also easier to use GL7 in the FACS, due to less background staining. Recently, the epitope for GL7 was identified as a sialylated glycan specifically expressed on activated B-cells in a GC (188). To further visualize GCs we identified proliferating cells within the different tissues by using the cross-reacting anti-human Ki67 antibodies, which specifically identifies proliferating cells (189). Since GC B-cells are heavily proliferating, and since Ig CSR requires proliferation (18, 77, 190), the anti-Ki67 staining detects GCs as well as cells potentially undergoing Ig CSR. Together with GL7 staining GCs can easily be detected using both FACS and IHC.

ILF free lamina propria

When analyzing the LP it is important to consider that the preparation could also host naïve and activated B-cells localized to ILFs. To ensure that the analyzed tissues were ILF free we adopted a method previously described by Stoel et al (191). Briefly, we snap froze 2-3 cm pieces of the small intestine and cryosectioned 7µm thick slices of the tissue. Every fifth section was placed on a slide and stained with anti IgA and anti IgM to visualize ILFs. The intervening sections between ILF-negative cryosections were dissolved in buffer RLT (Qiagen) for subsequent RNA isolation. If either one of the cryosections contained an ILF the adjacent sections were not analyzed but considered potential ILF tissue. Finally, if two consecutive cryosections were found to have ILFs (e.g. section 1 and 6) then the tissue was considered to represent an ILF and analyzed. This method also ensured good quality of the isolated RNA since the tissue was submerged and homogenized in buffer RLT immediately after it was sectioned, which can be a problem during laser capture microscopy.

PCR

In the first paper we used traditional reverse transcriptase PCR (RT-PCR) to identify germline α, AID and CD79a transcripts. To get semiquantitative results and to increase the specificity of the analysis of AID transcripts we performed a Southern blot analysis on the amplified material. To correlate the expression of AID to the number of B-cells present in the sample we used

CD79a as a B-cell specific house keeping gene. CD79 forms a complex with the BCR which is expressed on all mature cells but not on plasma cells and therefore CD79 serves as a reliable house keeping gene for normalizing the number of B-cells analyzed in each sample. In paper II and III we developed quantitative qRT-PCR assays to quantify the expression of germline α and AID transcripts. To minimize pipetting errors, we developed a multiplex assay using hybridization probes in different colors. We designed CD79a primers and probes and labeled the probe with Texas red and also added a black hole quencher 2 to the 3' end of the probe. The probes used in the different assays were locked nucleic acids probes (LNA) from Roche's universal probe library which were labeled with fluorescein (FAM) and a dark quencher dye according to the company's website. The primers were designed using Roche's online services (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000) and the system suggests 2 primers and an appropriate probe to be used. This approach allowed us to get quantifiable data with high specificity. To allow comparisons between different runs, all of the reactions included a standard calibrator from a pool of PPs from several different animals. This batch of cDNA was used throughout the thesis work and given the value 1 for relative quantification. All samples were then normalized against the standard sample in each run which gave consistent quantitative data between different runs.

qPCR arrays

The low density PCR arrays were custom designed and ordered from SABiosceinces. The rationale behind the design was to include many different genes that have previously been suggested to be involved in or influence IgA CSR and B cell differentiation. However, we were limited to 12 genes due to the plate layout and one of the genes had to be a housekeeping gene, in this case, HPRT. The plates were pre-validated with the primer sets already loaded onto the plate, thus we mixed the template with a sybrgreen mastermix before loading the samples in the plate. Subsequent to the PCR reaction, we analyzed the samples using ΔΔCT calculations and compared the relative expression of the different genes in the indicated tissue (Paper III). This analysis provided a rapid tool to identify gene expression profiles in several tissues at the same time.

Detection of switch α-CTs by nested PCR

Switch α-CTs could not be detected by one round of RT-PCR amplification, and instead we had to use nested RT-PCR as originally described by Fagarasan et. al (94). However, we modified the original primer set since we had problems with false positive and negative samples. To increase the sensitivity and the specificity of the assay, we used one step RT-PCR for the first round, which meant that the cDNA synthesis and the first round of the RT-PCR was carried out in the same tube in consecutive steps. The one step reaction mix contained a mixture of superscript RT-polymerase and hot start taq polymerase. Hence, all the components necessary for the cDNA synthesis and the actual PCR were included in the reaction tube and RNA can therefore be used as a template directly in the reaction. This minimized the chance of contaminating the samples as we avoided a separate cDNA preparation step. In addition, the cDNA synthesis was carried out using gene specific primers included in the reaction which added specificity and sensitivity to the reaction. The second round of PCR was carried out using the product from the one step RT-PCR as a template and both PCRs ran for 30 cycles which generated pure qualitative and nonquantifiable results. To analyze the sensitivity of the reaction we cloned the amplified product into the plasmid pBSK, and measured the concentration of the plasmid. By calculating the mass of the plasmid we could calculate the number of plasmids present in the mixture at a certain concentration. We serially diluted the plasmid and ran our nested PCR assay and found that our detection limit was as few as 4 gene transcripts .We also attempted to set up a qRT-PCR for the switch α-CTs but we were unfortunately not able to establish an assay with consistent amplification of the product. This may possibly have been due to very low gene expression levels. Therefore, to get semiquantitative values on expression of switch α-CTs in $CD40^{-/-}$ and WT mice we serially diluted the RNA prior to the nested RT-PCR. This allowed us to make relative estimates of how many transcripts that were expressed in $CD40^{-/-}$ and WT PPs .

NP-specific sequence analysis

In manuscript IV we analyzed the induction of an NP specific response following oral immunization with NP-CT. The purpose of these experiments was to analyze the kinetics and quality of a TD-antigen response after oral immunizations by monitoring NP specific V_H –μ and V_H-α gene expression in various gut tissues. From previous studies it is well known that NP responses are dominated by antibodies encoded by a specific VDJ rearrangement containing the V_H gene 186-2, the D region DFL16.1 and Jh2 or Jh4 (192, 193). Many of these studies used an

upstream primer which binds to the leader intron sequence of the Vh186.2 gene but also to some other genes in the J558 family, together with a J specific downstream primer to clone NP binding antibody heavy chain gene sequences using PCR from genomic DNA (194, 195). However, as these primers do not discriminate between antibody class and are not sufficiently NP-specific to give reliable results , we designed an upstream primer that only detected V186.2 gene and a pseudogene, but not other Vh genes within the J558 family, NP leader up (5'-TCT AGA ATT CGG GAT GGA GCT GTA TCA TGC TC-3'), and class specific downstream primers that are specific for IgM; IgM down2 (5'-TTC CTC GAG AGG GCC AGA GAA GCC ATC C-3') or IgA,;IgA down2 (5'-TTC CTC GAG GGA TGG CAG GAA GGA GGA C-3'. To estimate expression of NP binding sequences, PCR products generated using these primers were then analyzed using southern blotting with a probe (5´- CCG TAG TAA TAT CTT GCA CA-3´) which hybridizes across the VD joint specific for NP binding . The fact that a strong signal correlated well with NP binding was also confirmed when individual amplification products were sequenced.

The same strategy was employed when cloning the sequences for subsequent sequencing. Here the V_H-α and V_H-μ segments were amplified by the proofreading enzyme Phusion which generates blunt end products. The products were blunt end ligated into a TOPO-10 vector (Invitrogen) and screened by blue white selection. White colonies were patched onto a new selective plate and grown over night. The patches were then lifted onto a nitrocellulose membrane. The membranes were hybridized with the same NP specific CDR3 probe as described above and positive colonies were inoculated in liquid LB medium for subsequent plasmid preparations. The plasmids were sent off for sequencing, and the sequences were analyzed. Particular points addressed were, how many of the sequences that were likely to encode antibodies that bind NP, the frequency of somatic hypermutation, the clonal relationships of V_H genes, how many of the clones that had the mutation $W_{33} \rightarrow L_{33}$ (107 G>T) which increases the affinity for NP roughly x10 times (196), and if there were signs of affinity selection based on silent and non-silent mutations in the FW and CDR regions. The coamplified constant regions were used as a control for the number of mutations that were introduced during reverse transcription, PCR amplification and cloning since they should have remained unmutated. The rationale behind this approach was to analyze the early NP specific responses following oral immunization with a T-cell dependent antigen (cholera toxin), which could not be analyzed using non-molecular assays.

Results and comments

TI IgA production is unaltered in CD40$^{-/-}$ mice

T cell-B cell cognate interaction is absent in CD40$^{-/-}$ mice which results in failure to develop GCs. As a consequence, CD40$^{-/-}$ mice cannot produce high affinity class switched antibodies. Therefore, they have elevated serum IgM levels because of the inability to undergo Ig CSR (197, 198). This is also seen in patients with mutations in the CD40 or CD40L gene which causes a hyper IgM syndrome where serum IgG, IgA and IgE levels are decreased, but serum IgM levels are normal or elevated (199). Based on this information we expected CD40$^{-/-}$ mice to have decreased levels of IgA in the gut. Surprisingly, CD40$^{-/-}$ mice produced and secreted close to WT levels of IgA in the small intestine. This production was completely GC independent since no GCs were found by IHC in the GALT or the spleen (Fig 3). The lack of GCs was also confirmed by FACS analysis showing low levels of GL7 positive cells in the PP. Thus, the lack of GCs was not reflected in the ability to generate gut IgA. Of note, these mice did not respond to oral immunizations with TD antigens e.g. KLH and cholera toxin, showing that TD IgA CSR critically depends on GC formation. However, CD40$^{-/-}$ mice exhibit TI IgA responses which are GC and CD40 independent.

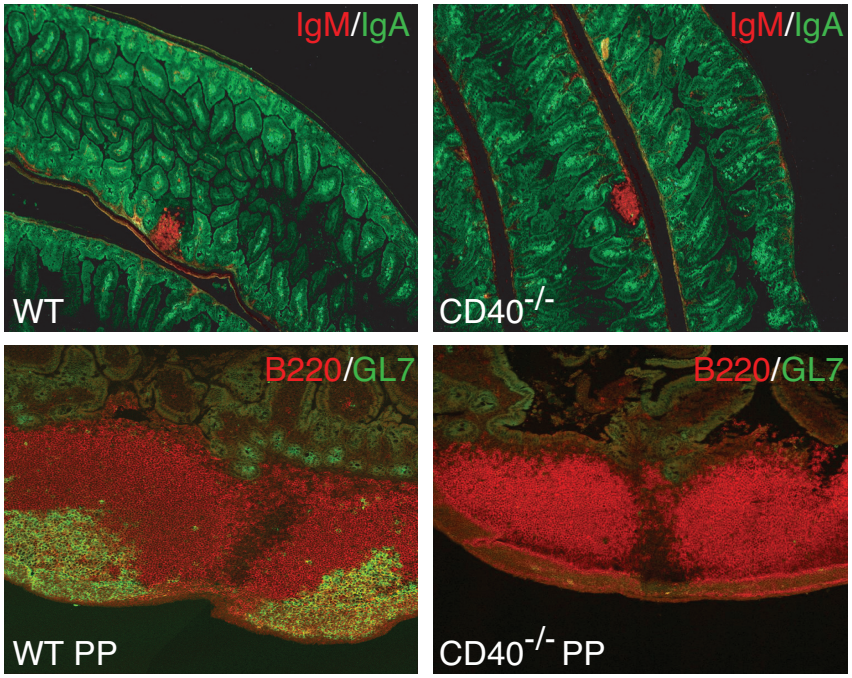

Figure 3
The image shows a comparsion between the intestine and the PP of WT and CD40$^{-/-}$ mice. Top panels shows a representative IgA (green) staining of the small intestine where IgM (red) cells are located in ILFs. The bottom panel shows GC stainings using GL7 (green) and B-cells stained with B220 (red).

Peyer's patches are sites for CD40 independent IgA CSR

In search for an explanation to the normal numbers of IgA plasma cells in CD40$^{-/-}$ mice we searched for a location of TI IgA CSR by analyzing the IgA CSR molecular markers α-germline transcripts, AID transcripts and α-switch CTs (94). RT-PCR analyses showed that the transcripts were expressed in the PPs of CD40$^{-/-}$ as well as WT mice albeit at lower levels in CD40$^{-/-}$ mice. In WT mice almost every PP expressed α-switch CTs and the frequency of α-switch CTs in the PP of CD40$^{-/-}$ mice were lowered by 50% compared to WT mice (Fig. 4). Since the α-switch CT assay was based on a nested PCR the results were purely qualitative and reflected only positive or negative results. Therefore we serially diluted the mRNA prior to the PCR to get semiquantitative data on the concentration of α-switch CTs in WT PP compared to CD40$^{-/-}$ PP. Combining the two analyses, the net result shows that IgA CSR activity in CD40$^{-/-}$ PP is roughly 25% compared to WT activity. That correlated well with the quantitative data on α-germline transcripts and AID mRNA expression which was also lowered to 25% in CD40$^{-/-}$ mice. However, despite the lower activity, PPs were the only site where significant IgA CSR activity was observed and very low levels of IgA CSR was detected in other organized lymphoid structures of the GALT, and not in the non-organized LP of either the small intestine or large intestine.

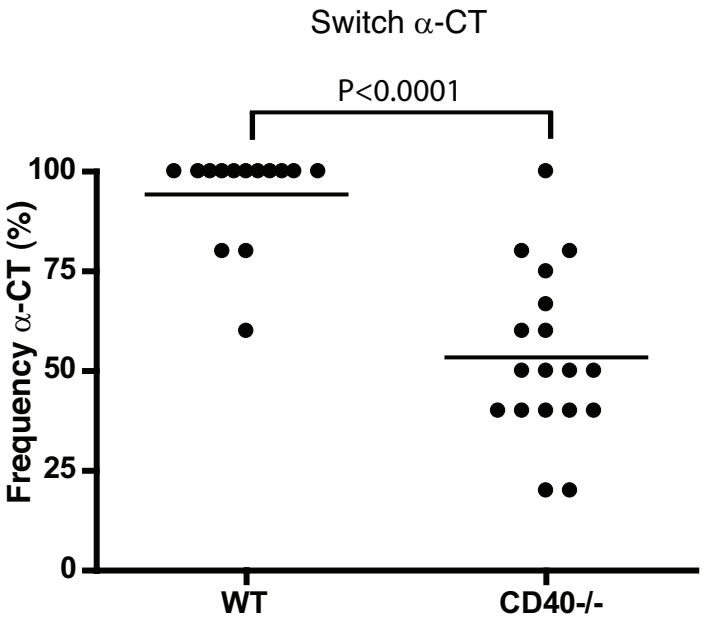

Figure 4
The image shows an analysis switch α-CT in the PP of WT and CD40$^{-/-}$ mice. Every dot represents the frequency of positive PPs in an individual mouse. At least 4 PP are included in each dot in the diagram. The average frequency of switch α-CT is shown by the line

No detectable IgA CSR in the lamina propria

In addition to the PP, we investigated several potential locations which have been suggested to host enriched IgA CSR (119, 162, 165, 200). First, the MLNs displayed very low IgA CSR activity in both WT and $CD40^{-/-}$ mice and are therefore unlikely sources of TI IgA CSR. Second, the LP itself has been suggested to support IgA CSR (119), and therefore ILF free LP was analyzed for evidence of IgA CSR. However, our analysis showed that ILF negative LP never contained molecular markers of ongoing IgA CSR. Also, few IgM^+ B-cells were found in the LP, arguing against the LP as a source of IgA CSR. Instead, IgM^+ B-cells were restricted to the organized GALT. In paper II the colonic LP and colonic ILFs were included in the analysis since a human study had indicated that IgA CSR could occur in the colonic LP (115). In fact, random colonic biopsies (0,5-1 cm) occasionally carried α-switch CTs. However, when ILF negative and ILF positive biopsies was analyzed separately, we found expression of α-switch CTs confined only to the organized colonic follicles and no evidence of IgA CSR was found in the non-organized LP itself. Even though evidence for IgA CSR was found in the colonic follicles, the majority of the follicles (>85%) failed to exhibit molecular markers for IgA CSR.

Peritoneal cavity B-cells do not contribute to gut IgA plasma cells

Apart from the GALT, B1 cells residing in the peritoneal and pleural cavities have been ascribed an important role for gut IgA production (48). Therefore we isolated peritoneal cells and analyzed the presence of molecular markers for IgA CSR. Interestingly, peritoneal cells expressed AID mRNA, but the cells did not express α-germline transcripts or α-switch CTs. Hence, these B-cells cells show an activated phenotype but no IgA CSR appeared to occur within the peritoneal cavity itself. If these cells contributed to the gut IgA production CSR had to have occurred elsewhere. For example, the activated B-1 cells may have migrated to the intestine and completed the IgA CSR, but since no evidence of IgA CSR was found in the LP, this is unlikely. To elucidate if peritoneal cavity B-cells migrated to the gut LP we transferred peritoneal washings from GFP expressing mice into WT and $CD40^{-/-}$ mice. 5 weeks after the transfer 20% of the peritoneal cavity cells were GFP^+. However, very few GFP positive cells were recovered from the GALT and the small intestinal LP. 50% of the plasma cells in the small intestine are replaced after 5 weeks (Pabst, O, personal communication), but very few of these cells appeared to be recruited from the peritoneal cavity. In addition, GF mice which are colonized with a normal bacterial flora rapidly restored IgA levels in the gut (201). If B-1 cells

were recruited to the LP to generate IgA plasma cells we expected that the B-cell composition in the peritoneal cavity would be altered during colonization. However, we did not detect any differences when comparing GF, WT and GF mice being colonized (Fig. 5). Thus, we believe our data argue for that B1 cells, at best, provide a very limited contribution to the overall IgA production in the gut (50).

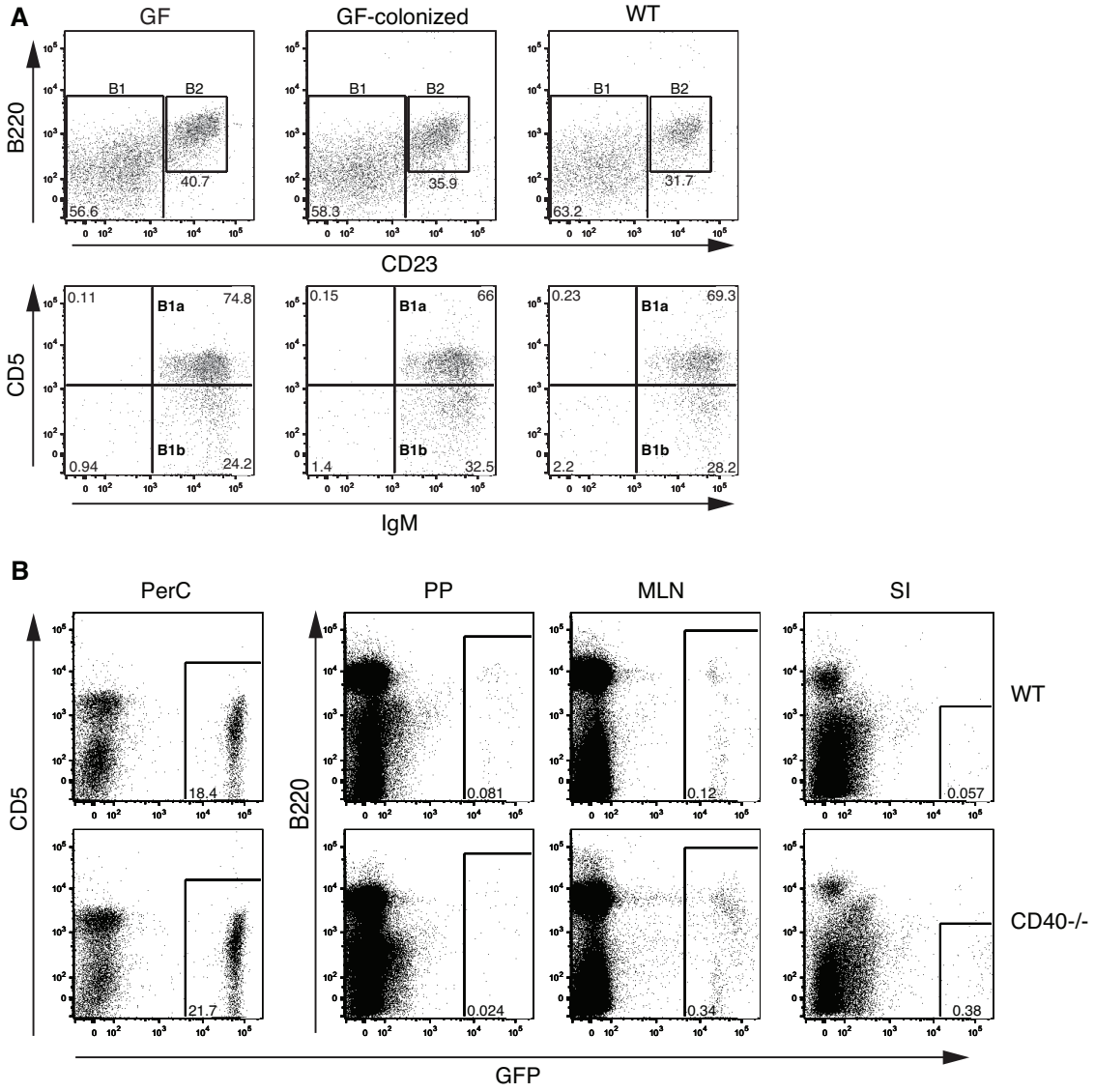

Figure 5

A) An analysis of the composition of B1 and B2 cells in the peritoneal cavity before and after bacterial colonization. The figure shows a FACS analysis of peritoneal cavity cells from the indicated animals. Included in the analysis is only $B220^{+}IgM^{+}$, $B220^{-}$ IgM^{+} and $B220^{+}IgM^{-}$ cells. The B1 cells were further subgated into B1a and B1b populations. The relative frequencies of cells in the different gates are shown. The image shows one representative figure out of animals. **B)** Peritoneal cavity cells from eGFP expressing mice were isolated and transferred i.p. into WT and $CD40^{-/-}$ recipients. 5 Weeks later the cells were isolated and analyzed by flow cytometry to visualize eGFP expressing cells in the indicated tissues. The relative frequencies of eGFP positive cells in each gate are indicated in the respective figures.

ILF frequency and distribution is unaltered in CD40$^{-/-}$ mice

ILFs in the small intestine can be a potential source of IgA CSR (127, 161, 162). Because CD40$^{-/-}$ mice displayed reduced IgA CSR activity in the PP we analyzed the number and distribution of ILFs in the small intestine. Surprisingly, the frequency of ILFs was similar in WT and CD40$^{-/-}$ mice and we detected 200-300 follicles in each mouse strain with increasing numbers in the distal part of the intestine. None of the ILFs showed GC formations or proliferating cells (Ki67^{+} cells) and their relative contribution to the total IgA production in the gut appeared to be low. The comparable numbers of ILFs in CD40$^{-/-}$ and WT mice suggested that the ILFs are unlikely to compensate for the lower IgA CSR activity (25%) in the PPs of CD40$^{-/-}$ mice. Nevertheless, further studies are needed to critically evaluate the importance of ILFs compared to PPs to the overall TI IgA CSR in the intestine.

Plasma cell turnover is similar in WT and CD40$^{-/-}$ mice

A possible explanation to the near normal production of gut IgA in CD40$^{-/-}$ mice could be altered plasma cell turnover in the LP. To address this issue we pulsed mice with BRDU and followed the kinetics of BRDU positive plasma cells in the LP. The changes were similar in WT and CD40$^{-/-}$ indicating that the plasma cell turnover was unaltered CD40$^{-/-}$ mice. Additionally, the level of apoptosis in the LP was also similar between WT and CD40$^{-/-}$ mice further supporting that gut plasma cell turnover were no different in CD40$^{-/-}$ mice compared to WT mice.

GL7 intermediate cells show evidence of IgA CSR

Because of the lack of GCs in CD40$^{-/-}$ PPs, we asked if IgA CSR could have occurred in B-cells prior to the GC stage. When analyzing GL7 expression by FACS, WT and CD40$^{-/-}$ PP both hosted a GL7 intermediate (GL7^{int}) population, which appeared similar in the two strains. This population expressed AID as well as α-switch CTs. Interestingly, the level of AID expression did not reflect the frequency of α-switch CTs, because AID expression levels were highest in the GL7^{high} population, whereas, the α-switch CTs were most frequent in the GL7^{int} populations. Further, when the JH4 intron was analyzed for SHM, the GL7^{low} cells did not show any mutations while in contrast, the GL7^{high} population was heavily mutated. The GL7^{int} population had fewer mutations than the GL7^{high} population, suggesting that this stage preceded the GC stage. Thus, TI IgA CSR occurred in GL7^{int} cells prior to manifest GC (Fig. 6).

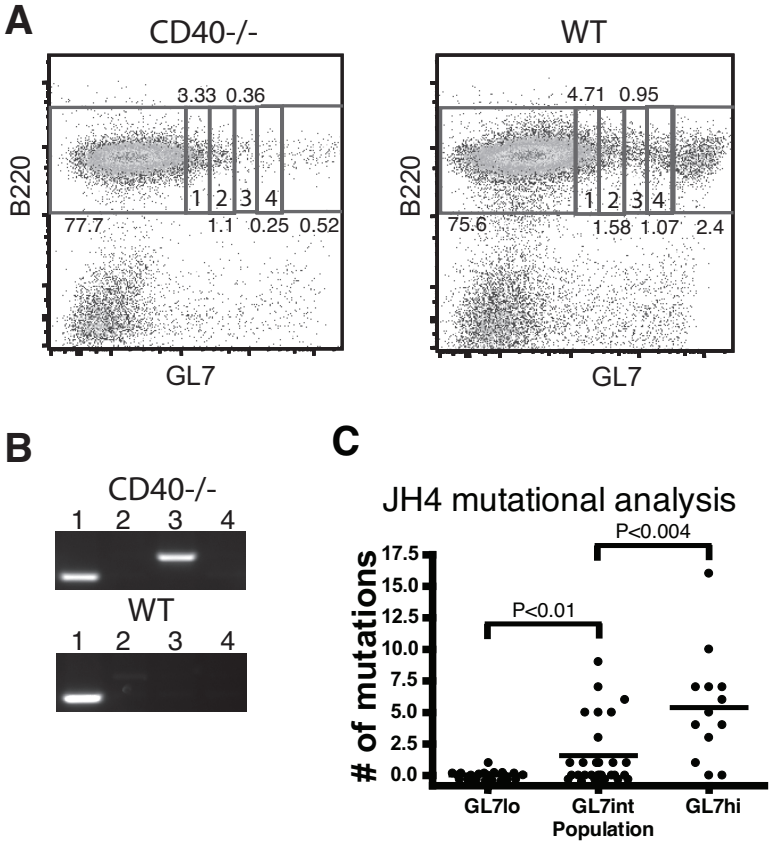

Figure 6
The image shows evidence of IgA CSR in GL7^{int} cells. **A)** A representative FACS image of the frequency of GL7 cells in WT and CD40$^{-/-}$ mice. The numbered gate shows the sorted cells that were analyzed for presence of switch α-CT in B. **B)** one example of presence of switch α-CT in GL7^{int} populations. **C)** Mutational analysis of the JH4 intron in cells isolated from the indicated GL7 populations. Every dot represents one sequence and the number of mutations within each sequence is shown on the Y axis.

Clonally related IgA plasma cells are present at isolated sites in the small intestine

The lack of GC in CD40$^{-/-}$ mice was reflected in the absence of mutations in the LP IgA plasma cells. While WT mice exhibited numerous mutations the V regions from LP IgA plasma cells, the CD40$^{-/-}$ mice showed very few mutations (on average 0,3 mutations/V region) compared to WT (5,2 mutations/V region). Thus, to generate highly mutated IgA responses, GC formation is fundamental. Nevertheless, RNA from spatially different parts of the small intestine showed examples of clonally related sequences, as identified by identical V_H genes and CDR3 regions. While it is highly unlikely that VDJ recombination generates several identical clones that subsequently home to the LP, and independently undergo IgA CSR *in situ* in the LP, this suggests that IgA plasma cells in the gut share a common clonal origin (36-38). Thus, clonal expansion of IgA plasma cell precursors occurs prior seeding the gut with IgA producing plasma cells in organized lymphoid tissues rather than in the diffuse LP itself. Additionally, TI IgA CSR precedes the formation of GCs.

Germ free mice host few IgA plasma cells in the gut, but IgA CSR activity is intact

Germ free (GF) mice host very few IgA plasma cells in the gut (107, 112, 201). However, IgA levels are quickly restored following bacterial colonization. To get insights into early IgA CSR, we used GF mice. The rationale behind the experiments was that we assumed that bacterial colonization would rapidly induce IgA CSR. By monitoring different locations for IgA CSR it would provide us with information as to how IgA is generated in response to bacterial colonization. However, surprisingly the IgA CSR activity in PPs in GF mice was comparable to that in WT mice. The PPs in GF mice exhibited GCs and the frequency of GL7 positive cells was similar to that in WT PPs. In contrast, despite normal GC formations the intestine hosted few IgA^+ cells, suggesting that there was a deficiency in the IgA CSR. However, the PPs expressed α germline trancripts, AID mRNA and α-switch CTs at levels comparable to WT PPs (Fig. 7). Next, we screened the PP, MLN and the small intestinal LP for a number of genes potentially affecting IgA CSR and involved in B-cell activation, survival and homing. In the PP, the only major difference between GF and WT was iNOS, which was downregulated in GF mice. It has previously been reported that $iNOS^{-/-}$ mice have low levels of IgA producing plasma cells in the gut (138), as a consequence of downregulated BAFF and APRIL expression. However, we found unaltered expression of any of these genes in our analysis, indicating that iNOS expression did not directly correlate with lower levels of BAFF and APRIL. Hence, IgA CSR appeared not to have been affected by the germ free status of these mice, arguing that later stages of IgA plasma cell differentiation and survival could be involved.

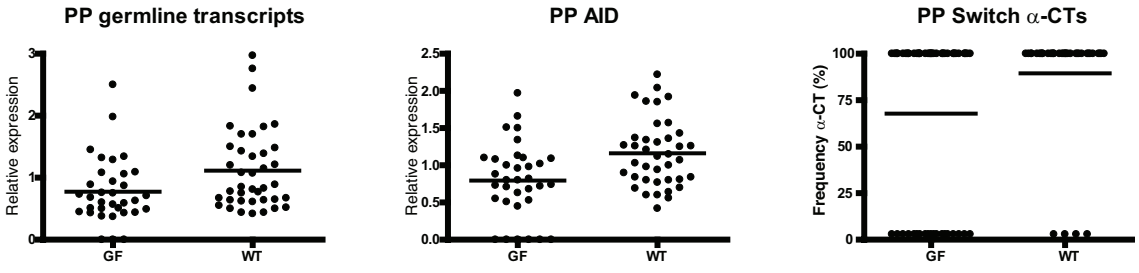

Figure 7
Analysis of molecular markers of IgA CSR in GF mice. The analysis shows relative expression of a germline transcripts and AID mRNA analyzed by qRT-PCR. Switch α-CTs were detected by nested PCR as described in manuscript II. Every dot represents one individual PP.

Expression of gut homing receptors in GF mice

Poor expression of gut homing receptors could explain the lack of LP IgA plasma cells. Gut homing properties of the B-cells are dependent on CCR9 mRNA expression in the PP and MLN. GF mice the expression exhibited unaltered mRNA expression of CCR9 compared to WT mice, arguing against that the homing properties of B-cells were changed in GF mice. However, it has previously been shown that the MAdCAM-1 expression in the LP was lower in GF mice compared WT (202) which could result in a decreased attraction of $\alpha 4\beta 7^+$ B-cells. Thus, it is possible B-cells acquire the necessary homing properties in the organized GALT but that homing to the LP is abrogated by the lack of attraction molecules in the LP itself. These theories are currently being addressed.

Bacterial colonization of GF mice restores the number of intestinal IgA plasma cells without significantly altering IgA CSR

Gut IgA producing cells rapidly develop in GF mice after colonization with a SPF microbiota. However, the IgA CSR activity is not increased during colonization. Ina fact, there is a slight decrease in the expression of α-germline transcripts and AID transcripts in the PP and MLN compared to GF and WT mice. This decrease could reflect an influx of naïve B-cells into the GALT, which would alter the expression of the housekeeping gene in the analysis. Thus the frequency of cells expressing α-germline transcripts and AID transcripts appears lowered, but the total number of cells expressing these markers is in fact increased. However, the expression of α-switch CTs were increased in mice during colonization and reached WT levels. Furthermore, GC formation and the frequency of $GL7^+$ cells is not altered subsequent to bacterial colonization.

Big changes in the mesenteric lymph nodes following bacterial colonization

The most dramatic changes during bacterial colonization were found in the MLN where most of the genes analysed were induced. This could be explained by bacterial translocation to the MLN during colonization before protective IgA levels in the intestine was restored (114, 166, 171). Additionally, bacterial colonization restored iNOS expression in the small intestine, which could be involved in the IgA homeostasis. Further studies are required to address the roles of these effects in generating IgA plasma cells in the gut.

TD IgA responses are elicited by NP-CT conjugates

Thus far our studies have focused on TI IgA responses. Next we addressed TD responses, using the mucosal antigen cholera toxin (CT) (56). Typically CT specific IgA responses are evaluated by ELISPOT and/or ELISA which gives a good end point analysis of the elicited response, but here, we explored an IgA response towards an orally administered TD antigen on a molecular level. However, it is not known which genes are involved in a CT specific IgA response, and the pattern of SHM during a CT response is not elucidated which makes it difficult to follow CT specific responses. Therefore, we conjugated the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) to CT. NP has been extensively studied and evaluated in systemic responses and the V_H genes and NP specific mutations are well characterized (192, 194, 195, 203, 204). This allowed detection of NP specific B-cells in situ, both by immunohistochemistry, and by molecular markers. Furthermore, SHM and affinity maturation of NP responses are characterized by a $W_{33} \rightarrow L_{33}$ (107 G→T) replacement mutation in the V_H gene which enhances the affinity for the antigen roughly 10 times (196). Thus, selection and affinity maturation of IgA responses elicited by CT can be followed indirectly by analyzing the well known response towards NP.

The CT molecule consists of two subunits where the A subunit confers the enzymatic activity, and the five B subunits binds to the GM1 gangliosides on nucleated cells (56). Western blot analyses showed that NP bound to both subunits equally well. However, the enzymatic activity of the molecule decreased somewhat after conjugation, but the GM1 binding capacity remained intact. Additionally parenteral immunisations elicited a strong anti NP responses, showing that the immunogenicity of CT was not disrupted by NP conjugation. When orally administered, the IgA response was detected throughout the intestine by NP specific IHC. The NP specific cells were distributed similar to the IgA^+ cells in the intestine and no localized differences were detected. Interestingly, initial data suggested that the proximal intestine contained more NP specific plasma cells than the proximal part which could be due to a local effect of the oral administration route. On the other hand, the total number of IgA^+ cells correlated well with the observed distribution of NP specific cells, and when normalizing the number of NP^+ cells with the number of IgA^+ cells in different parts of the intestine, the relative frequency of NP^+ cells were comparable throughout the intestine. Strikingly though, the frequency of NP^+ cells in the intestine were as high as 15% after 3 oral immunizations, which highlights the plasticity and the ability of the intestine to respond to orally administered TD antigens.

High affinity clones are selected early during a TD antigen response

Molecular data showed that IgA plasma cells were efficiently generated after three oral immunizations and could be detected using molecular methods. However, the sensitivity of the assay requires further evaluation since three immunizations were needed to detect NP specific sequences whereas NP specific cells were found in the LP after one and two immunizations usig IHC. Strikingly, the PP hosted many NP specific B-cells after three immunizations, suggesting an expansion of NP specific B-cells following three oral immunizations. However, very few NP specific IgM cells are present in the PP and MLN after one and three immunizations, indicating that NP specific NP-cells are absent or rapidly undergo IgA CSR after antigen exposure. Additionally, no NP specific IgM cells were detected in the LP after one or three immunizations, indicating that the NP specific responses in the LP were exclusively IgA. By sequencing NP specific V_H genes we found that more than 50% of the NP specific sequences in the LP were affinity matured and selected for NP specificity as judged by the high affinity mutation $W_{33} \rightarrow L_{33}$ (107 G→T) after three oral immunizations. However, a single dose does not generate any NP specific IgA responses, neither in the PP, nor in the small intestinal LP. After two immunizations NP specific and affinity matured sequences were found in the PP and MLN but not in the LP. Three immunizations were needed to generate highly selected NP-specific IgA responses in the LP. Additionally, several complex clonal relationships were found in the small intestinal LP. First, the $W_{33} \rightarrow L_{33}$ (107 G→T) mutation was usually the first shared mutation, and B-cells acquired additional mutations subsequent the characteristic high affinity NP mutation. Second, the complexity of the clonality and the fact that clonally related cells were found at spatially separated locations in the intestine showed that the cells had a common clonal origin. Third, unmutated NP specific clones were found in the LP, indicating that IgA CSR precedes SHM, and seed the LP with IgA^+ plasma cells, much like the findings in manuscript II where TI independent IgA CSR precedes SHM in the GC. Thus, it appears that high affinity clones are selected early during a TD response and that TD IgA CSR occurs prior to entering a GC.

Discussion

IgA CSR in CD40$^{-/-}$ mice

The data presented in this thesis addresses one of the most striking features of the gut immune system; the ability to produce and secrete SIgA. Despite an extensive literature on this subject there are still several questions to be answered as to how and where IgA is generated in the gut. We have shown that CD40$^{-/-}$ mice are able to generate and produce near normal levels of IgA, despite the lack of visible GCs in the GALT. This finding prompted us to use sensitive molecular markers for detection of the sites of IgA CSR. Because GCs are not an absolute requirement for Ig CSR (205), we analyzed the PP in CD40$^{-/-}$ mice for evidence of IgA CSR. In paper I, we analyzed the expression of germline α-transcripts, AID mRNA and α-switch CTs in CD40$^{-/-}$ and WT mice using semi-quantitative RT-PCR in combination with Southern blotting. The data showed that the IgA CSR activity in CD40$^{-/-}$ mice was reduced compared to that in WT mice. To get quantifiable data of the reduction we developed quantitative real time qRT-PCR assays for detection of germline α-transcripts and AID mRNA in paper II. However, the rare α-switch CTs were assayed with a qualitative nested RT-PCR, and thus, was not suitable for qPCR analysis. Therefore, we analyzed the α-switch CTs in serial dilutions providing a semi-quatitative measure. With the combination of these assays we found that the IgA CSR activity in CD40$^{-/-}$ PP was roughly 25% of that in WT mice.

A highly debated concept is that IgA CSR can occur in the non-organized LP *in situ*, driven by the gut bacterial flora (115, 116, 119, 137, 206). Several studies have questioned these findings (36, 38, 120), and the data in our first two papers further support that this hypothesis is unlikely. Biopsies of ILF-free LP from the colon or the small intestine consistently lacked evidence for IgA CSR. Molecular markers for IgA CSR were detected only when ILFs were present in the biopsies. Furthermore, the LP does not host a significant number of IgM^{+} cells, or proliferating (Ki-67^{+}) cells, which argues against these cells as a source for IgA CSR in the LP. A likely explanation to the discrepancy between previous reports and the results in the present thesis work could be that ILFs were not removed prior to analysis in the former studies. In our study, we carefully excluded the ILFs from our LP samples, whereas Fagarasan *et al*. isolated B220^{+} IgM^{+} LP cells without considering the potential presence of ILFs (119). However, we and others have shown that ILFs are frequent and abundant in the small intestine, and that they, indeed host proliferating B220^{+} IgM^{+} B-cells which can undergo IgA CSR (143, 154, 162, 207). Therefore,

the IgA CSR detected in the LP preparations could have reflected IgA CSR in ILFs rather than IgA CSR in the non-organized LP. An alternative explanation could be the existence of species differences as the most extensive data suggesting IgA CSR in the LP is from human colon biopsies (115). We do not believe this is very likely, though, because Boursier, Spencer and Dunn-Walters have failed to find evidence for IgA CSR in non-organized LP in human intestinal tissues, and show that IgA responses are generated in the organized GALT (36-38).

ILFs are a documented source for IgA CSR in the intestine (127, 143, 154, 162). However, the relative contribution of ILF compared to PP to the overall generation of IgA plasma cells in the gut remains to be determined. Because the original site for IgA CSR under normal conditions may be different from that of gene knockouts or transgenic mice due to redundancy between pathways, misinterpretations or overstating the importance of results can easily be made. Our data show that, despite detecting molecular markers for IgA CSR in some ILFs, a majority of the follicles do not carry evidence of IgA CSR. This data suggest that the ILFs have a limited contribution to the overall gut IgA production under normal conditions in WT mice. To more conclusively address the role of PP relative to ILFs in our $CD40^{-/-}$ mice ongoing studies using *in utero* blocking of PP-development by treatment of the pregnant mothers with LTβ-Ig-fusion proteins are underway. Hopefully, these experiments will answer to what extent PP-deficient $CD40^{-/-}$ mice will generate IgA plasma cells in the LP, thereby exploiting the potential of ILFs as a source for TI IgA CSR.

Earlier studies have shown that SHM can occur without GC formation (208-211). To investigate this possibility in the $CD40^{-/-}$ mice we analyzed random V_H-IgA sequences isolated from three separate parts of the small intestine for mutations. WT mice readily showed a mutated repertoire, and most of the sequences were mutated. However, the VH genes in $CD40^{-/-}$ mice were unmutated, stressing the importance of GCs for developing SHM and high affinity Ig responses. Strikingly, we found clonally related sequences, i.e. identical CDR3 rearrangements and V_H genes, in separate parts of the small intestine, further supporting that IgA CSR is restricted to the GALT and does not occur in situ in the non-organized LP (36-38).

B1 cells from the peritoneal cavity are thought to contribute to the total IgA plasma cell numbers found in the gut LP (48). However, most studies reporting on the critical role of this axis have been performed in B-cell deficient or lethally irradiated mice, where empty niches for repopulation of B cells upon adoptive transfer have been present. Because of this fact, overinterpreting the importance of this axis is potentially possible when the results are translated into normal conditions in WT mice. In the present thesis work we observed that B-cells in the peritoneal cavity had an activated phenotype and expressed AID. However, at variance with previous studies, we were not able to detect α-switch CTs in these cells (212). This may be due to strain differences as these studies used BALB/C mice and not C57Bl/6. However, to better evaluate a possible B1-cell involvement in gut IgA plasma cell levels, we adoptively transferred GFP-expressing transgenic B-1 cells from peritoneal washings by i.p. injections into WT mice (65, 213, 214). Strikingly, we detected a substantial population of GFP-expressing B-cells (20%) only in the peritoneal cavity of the recipients and no cells were detected in the LP five weeks after transfer, suggesting that there was no traffic of B-1 cells from the peritotoneal cavity to the gut LP. As roughly 50% of the plasma cells are exchanged within a five week period (Pabst, O. Personal communication), we had expected to find significant numbers of GFP positive, B-1 derived IgA plasma cells in the intestine. Therefore, we believe that during steady state, peritoneal B-1 cells do not significantly contribute to the IgA plasma cell pool in the gut intestine. Continued studies in this transfer model may shed more light on this matter. Since we injected the cells i.p we could have restricted their potential of migrating to the gut LP in the WT mice, although, one would imagine that after antigen recognition B-1 cells would leave the peritoneal cavity. Perhaps the fact that we used transgenic B-1 cells may have lowered the probability of antigen activation in the peritoneal cavity and, thus, could explain why cells did not appear in the gut LP.

My work has clearly demonstrated that the PPs are the only sites which host substantial IgA CSR in $CD40^{-/-}$ mice. In fact, despite a lower IgA CSR activity in the PP compared to WT it was sufficient to generate close to normal levels of IgA plasma cells. The PPs hosted a relatively intact $GL7^{int}$ population which underwent IgA CSR. We analyzed the frequency of mutations in this population in both $CD40^{-/-}$ and WT mice, and found dramatically fewer mutations in the $GL7^{int}$ population compared to the $GL7^{high}$ population, indicating that IgA CSR preceded SHM prior to the formation of GC. As most of the sequences of IgA plasma cells in the LP of

CD40$^{-/-}$ mice were unmutated and these mice did not have GC we suggest that SHM follows on after IgA CSR and requires an established GC. However, in WT mice gut LP IgA plasma cells were mostly heavily mutated, suggesting that PP GL7^{int} cells also enter into GCs, and undergo SHM. We have to assume that in WT mice the GL7^{int} PP B cell population includes both the TI and TD IgA CSR pathways, arguing that in WT mice IgA plasma cells responding to the bacterial flora also acquire SHM prior to seeding the gut. How and why TI-antigen specific PP B cells undergo SHM in normal mice is unclear. Further studies should address this matter, but we can conclude that in CD40$^{-/-}$ mice these B cells undergo IgA CSR as GL7^{int} cells but few cells undergo SHM, probably because of the lack of GC.

IgA CSR in germ free mice

In GF mice, very few IgA plasma cells are found in the gut LP (107, 112). The reduction of IgA producing LP cells in GF mice is roughly 90%, which is supported by results from both ELISPOT and ELISA using isolated mononuclear cells and gut lavage , respectively.This dramatic reduction is perhaps best appreciated in IHC microscopic sections labelled for IgA, where the difference between conventional and GF mice is apparent. Based on this information we hypothesized that the commensal flora played a critical role in WT mice to promote IgA CSR activity in the GALT. Thus, we expected to find reduced IgA CSR activity these mice. However, to our surprise the PPs demonstrated near normal IgA CSR activity and also displayed clear and frequent GCs. We concluded that the PP-inductive site appeared normal with regard to IgA CSR and GC formations. This suggested that GC do not require the presence of the microbiota to develop, and that other luminal antigens appeared to be sufficient for GC formation. Whether TLR-signalling is critical for GC formation cannot be answered at this time, but it is probable that the food pellets may have contained e.g. LPS or bacterial flaggellin, or other TLR-ligands (215). It is possible that IgA CSR in the PP and gut LP IgA plasma cell levels reflect largely responses to TD-antigens. To experimentally address whether TD-antigen responses are intact and the IgA CSR activity in the PP is adequate in GF mice we will orally immunize mice with NP-CT and compare the PP IgA CSR activity and the generation of gut LP IgA anti-NP cells with that stimulated in WT mice. We hope that these experiments will give additional information whether GF mice mice have a defect in their effector site in the LP rather than a defect in the inductive site for IgA CSR. We therefore, speculate that the GF status is primarily affecting the ability of IgA plasma cells to home to and/or survive in the gut LP, rather than a defect in the IgA CSR avtivity in the PP-inductive site.

Because IgA CSR activity was not altered in the PP of GF mice compared to that observed in conventional mice we undertook a screening of different genes influencing IgA CSR in the PP. We found that iNOS gene expression was downregulated in the PP of GF mice compared to PP in conventional mice.. Although iNOS deficient mice have reduced levels of BAFF and APRIL, that directly could have affected IgA CSR activity in the PP, we were unable to demonstrate a reduction in BAFF and APRIL gene expression in GF mice (138, 139). Therefore, we concluded that except for iNOS gene expression other genes influencing IgA CSR were normally expressed in GF PP and we believe PPs were fully functional to support IgA CSR. As conventional and GF mice do not exhibit a differential IgA CSR activity we did not expect to find much different IgA CSR activity after colonizing the GF mice with an SPF-flora. In agreement with this notion this was also the case for the PP, whereas the genes influencing IgA CSR were dramatically increased in MLN. However, a corresponding alteration in molecular markers for IgA CSR did not accompany the increase in expression of BAFF, APRIL and other genes. Thel ack of gut anti-bacterial IgA in the GF mice had probably resulted in an increased translocation of intestinal bacteria to the MLN and caused the increased gene expression (114, 163, 171). The IgA CSR in the MLN remained low in GF and newly colonized mice, at a level comparable to that seen in conventionally raised mice, supporting the theory that MLN is a poor site for IgA CSR, but critical as a barrier for preventing bacteria from getting access to systemic tissues (166).

Knowing that the induction of IgA was unaltered we speculated that homing to the LP was defective in these mice. Previous studies have suggested a link between iNOS expressing DCs and the presence of retinoic acid which is capable of imprinting homing properties on IgA committed B-cells (138, 139, 172, 173, 216). Homing to the small intestinal mucosa depends on the expression of CCR9 and α4β7 on B-cells, and the corresponding CCL25 and MAdCAM-1 expression in the crypt epithelium in the mucosa (173, 182, 217). Hence, two scenarios were possible; the first possibility was that the imprinting of the B-cells in the GALT was deficient. This scenario would have generated B-cells with lower levels of CCR9, and/or α4β7, unable to home to the gut mucosa. The second possibility was that the ability of the LP in GF mice to recruit or maintain IgA plasma cells over time was decreased compared to what pertains in conventional mice. A decreased expression of MAdCAM-1 and/or CCL25 in the gut LP would fail to recruit the activated B cells. Moreover, a lack of factors released in response

to the microbiota that influence the terminal differentiation of IgA plasma cells or survival time, such as the effect of BAFF/APRIL in other tissues could explain the low numbers of IgA plasma cells. First, we analyzed PP and MLN B cells for reduction in CCR9 expression in GF mice, but found normal expression levels of CCR9 compared to those observed in conventional mice. Further analysis of $\alpha4\beta7$ as well as assessment of actual homing ability to the gut LP in adoptively transferred MLN and PP B cells will follow. A previous study reported that GF mice can have a decreased expression of MAdCAM-1 in the LP, which could lead to a deficiency in the ability to recruit $\alpha4\beta7^+$ B cells to the gut LP in GF mice (202). This would, in fact, reconcile the findings of lack of IgA^+ plasma cells in the LP with an intact IgA CSR in the PP in GF mice. Finally, we found several genes influencing IgA plasma cell differentiation and recruitment being poorly expressed in GF LP. Although, BAFF and APRIL gene expression levels were the least reduced in the small intestine other genes encoding critical factors, such as the IL-5 and IL-6 genes, may be responsible for the low level of IgA lasma cell levels in the GF LP. Ongoing studies will dissect these possibilities.

NP-specific T-cell dependent responses

In the first part of my thesis work I focused on TI IgA B-cell responses in the gut mucosa, driven by the bacterial microflora. The second part of the thesis is devoted to TD IgA B-cell responses in the gut LP following oral immunizations. We employed the well charcterized hapten, NP, for these studies as much information already exists as to which genes are involved in antibody binding and which mutations lead to to affinity maturation of the NP-specific response (192, 194-196, 203, 204, 218). We also took advantage of the much studied oral immunogen, CT, for effective stimulation of mucosal IgA immunity after oral administration of of antigen (56). The hapten NP was conjugated to CT, which enabled characterization of TD IgA B cell responses at the molecular level and detection of NP-specific plasma cells, by immunohistochemistry. Since no previous research has attempted to follow the build-up of an antigen-specific IgA response after oral immunizations using sensitive molecular markers we expected to get new insights into the kinetics of the induction of IgA responses in the different parts of the GALT and gut LP as well as understand if these responses underwent SHM and selection. We found that NP-specific plasma cells were readily detected already after a single oral dose, but the number of specific IgA anti-NP plasma cells increased dramatically with increased number of immunizations. The microanatomical distribution of NP-specific IgA plasma cells in the villi

was similar to the overall distribution of IgA plasma cells in the different parts of the small intestine and we observed a frequency around 10-15% of the total IgA plasma cells specific for NP in all parts of the small intestine. Thus, we observed no local variation of NP-plasma cells in the proximal or distal segements of gut LP, arguing that these cells had a common origin prior to homing to the gut LP. The proximal small intestine harboured most NP-specific plasma cells, but here also total IgA plasma cell levels were the highest, rendering a frequency of 10-15% NP-specific plasma cells. In fact, there may be a technical explanation to the relative difference in the distribution of total IgA SFC in the proximal and distal parts of the intestine, simply that much higher numbers of ILFs are present in the distal compared to the proximal small intestine , and hence, many more naïve monoculear cells diluted the NP-specific plasma cells in the samples taken from the distal small intestine (207). Thus, when isolating LP lymphocytes, the contribution of naïve B-cells would be greater in the distal part prior to the ELISPOT analysis, thus generating less spots in the analysis. Interestingly, as many as 10-15% of the IgA plasma cells in the gut were antigen specific following three oral immunizations with NP-CT, demonstrating the efficiency and the plasticity of gut mucosal IgA protective immunity.

We next used Ig V_H gene expression for assessing the presence of NP specific IgA and IgM cells in the LP of the small intestine and the colon, and in the PP and MLN after one and three immunizations. Three oral immunizations generated strong NP-specific IgA responses in the small intestine and in the colon. NP-specific B-cell responses were also observed in the PPs and MLN although the activity was dramatically weaker than in LP of the small intestine or colon. Of note though, because of a technical problem in discriminating PP from the surrounding LP the PP preparations could have contained contaminating mRNA from the small intestine. Thus, at present we cannot rule out that mRNA from plasma cells from the LP of the small intestine contaminated the PP preparations. Therefore, the IgM specific responses would be more conclusive, because there are very few IgM B-cells in the LP that could have contaminated the PP preparations. In addition, analysis of IgM specific V_H gene usage would provide information on whether more NP specific IgM responses were generated over time and if the kinetics in the GALT was different from that of the LP. As expected we found that IgM NP mRNA expression was in general weaker than IgA NP mRNA responses. The PPs generated the strongest IgM NP-specific mRNA expression of the different tissues. Only very weak NP specific IgM V_H gene expression was observed in the LP of the small intestine or the colon. Importantly, NP

specific IgM VH gene expression was only marginally increased after three oral immunizations. Possibly, this reflects that we detected naïve NP specific IgM^+ cells before they were activated, and when activated most NP-binding B cells undergo IgA CSR.

By analyzing mutations in the V_H genes from IgA or IgM cells, we found that the IgA response in the LP was highly mutated and also selected for high affinity NP binding after three immunizations. Based on identical CDR3 regions we could also establish the existence of clonally related sequences throughout the small intestine, suggesting that TD responses originated from a common source in the GALT. Additionally, the NP specific mutation was found in early appearing clones in the lineage tree analysis, which suggested that the mutations were introduced early during SHM and subsequently selected for their higher affinity. It may be that additional mutations could be generated by recirculation of the NP-specific B cells within the GC between the dark and light zone to generate more mutations, as been reported (65, 68, 102). At present we do not know whether these additional mutations are selected and render in even higher antigen affinity and bigger databases are required to detect such additional selection patterns. Importantly, we found that, one immunization did not generate affinity matured responses in the PP, MLN or the gut LP, whereas two oral immunizations resulted in many selected IgA VH genes both in the PP and MLN. Interestingly, at this time point strong NP specific IgA VH-chain gene expression/responses could be found in the small intestine but none of these sequences were mutated to exhibit the high affinity maturation towards NP. Three oral immunizations were needed for this to occur in the gut LP. These findings may suggest that TD IgA CSR can occur prior to SHM in a PP GC, much like TI IgA CSR in PP of $CD40^{-/-}$ mice. Future studies will investigate these findings in more detail. At the moment we can conclude that TD IgA B cell responses, as reflected in anti-NP-CT responses, following oral immunizations, are induced in the GALT and primarily in the PP, prior to seeding the effector site in the small and large intestine.

Conclusion

In this thesis we have provided evidence that TI IgA CSR occurs exclusively in the GALT prior to SHM in GCs. IgA CSR activity was never found in the non-organized LP, and peritoneal cavity B-cells do not significantly contribute to LP IgA plasma cells. Additionally, we show that the induction of IgA CSR is intact in GF mice, but subsequent IgA plasma cell development appears to be impaired, resulting in 90% reduction in gut IgA plasma cells in the small and large intestine. Finally we show that TD IgA responses are efficiently generated in the GALT and that the responses early on undergo mutational selection events that result in high affinity IgA plasma cells seeding the gut LP.

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