Understanding the regulatory requirements for Gut IgA B cell responses and their potential role in mucosal vaccine development

Rathan Joy Komban

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

UNIVERSITY OF GOTHENBURG

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Cover illustration: Peyer's patch germinal center with GFP positive B cells

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Rathan.joy.komban@gu.se

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“The secret of life is to fall seven times and to get up eight times”

Paulo Coello

ABSTRACT

It is important to understand gut B cell differentiation, from the activation of cells at inductive lymphoid sites to the formation of gut plasma and memory B cell, to be able to develop efficient oral vaccines but the process is incompletely defined. To address this we developed an adoptive transfer system based on B1-8^{hi}/GFP^{+} NP-specific B cells and NP-CT, a hapten-carrier complex that allows us to follow antigen-specific IgA responses following oral immunization. In paper (I) we provide evidence for early migration of activated B cells via draining lymph and blood to germinal centers (GC) in Peyer's patches (PP) spatially distinct from where the cells originated, thus explaining how synchronization between PP may be achieved. We address the requirements for activated PP B cells to re-enter GC, and demonstrate the necessity of antigen and expression of CD40 on B cells. Gut IgA plasma cells did not form from B cells lacking CD40 in a competitive bone marrow transfer experiment, suggesting that the GC pathway dominate their formation. In paper (II) we show that activated PP B cells interact with M cells and antigen in the sub epithelial dome (SED) of PP. B cells in SED had an IgD^{-}, GL7^{-} and CCR6^{+} phenotype and migrated rapidly towards the GC after interacting with antigen. We hypothesize that during a PP immune response, B cells intermittently sample and transport antigen from the basal pockets of SED M cells and that this feeds antigen into the GC to maintain the response. Paper (III) demonstrates that gut memory B cells and long-lived plasma cells are not closely clonally related. We propose that the plasticity of PP allows these two classes of B cells to evolve in temporarily or anatomically separate GC processes, leading to diverse low-affinity memory B cells and clonally restricted high-affinity plasma cells. In this way, the plasma cells are focused on antigens currently in the gut whereas a broader repertoire of memory cells is able to recognize related antigens. On reactivation, the mucosal memory B cell response was dominated by clonally selected, high-affinity cells, leading to the formation of plasma cells of high affinity. Paper (IV) demonstrates that germ free (GF) mice largely lack IgA producing plasma cells despite having intact B cell expansion and differentiation in PP GC. This suggests that lack of bacterial colonization is associated with that the gut lamina propria (LP) cannot attract and/or host IgA producing plasma cells. An oral immunization with NP-CT did not only induce antigen-specific IgA plasma cells in the LP but also largely restored polyclonal IgA production in the gut. Thus, in GF mice, CT induced the LP effector site to attract polyclonal IgA producing plasma cells in a manner similar to that seen following bacterial colonization. Taken together, this thesis demonstrate several features that are unique to activated gut B cells, and show that these are relatively mobile and not as restricted to a single GC as during systemic responses.

Keywords: Germinal centers, Sub-epithelial dome, NP-CT, B1-8hi/GFP+ NP-specific B cells, Peyers patches, Lamina propria, Germ free mice

SAMMANFATTNING PÅ SVENSKA

Det är viktigt att förstå hur B celler utvecklas i tarmen för att kunna utveckla orala vacciner. Det finns dock många obesvarade frågor om denna process. Vi har utvecklat en metod som baseras på att föra över B1-8^{hi}/GFP^{+} B cells som känner igen NP till mottagarmöss och sedan immunisera dessa oralt med NP-CT, ett hapten/bärare komplex. I arbete (I) så visar vi att B celler kan lämna det organsystem där de aktiveras i germinalcentrum (GC) och sedan via lymfan och blodet ta sig in i GC i andra Peyerska plack (PP). Detta förklarar hur olika PP kan kommunicera med varandra under ett immunsvar. Vi visar att antigen och CD40 på B cellen är nödvändiga för att detta skall kunna ske. Genom att byta ut benmärgen i möss visar vi att CD40 är nödvändigt för att IgA producerande plasmaceller i tarmen skall bildas, vilket tyder på att den absoluta majoriteten bildas i GC. I arbete (II) visar vi att aktiverade B celler i PP interagerar med M celler och antigen i ett område som kallas den subepitelial domen (SED). Då IgD^{-}, GL7^{-} och CCR6^{+} B celler vid M celler känt igen antigen så rör de sig snabbt mot GC. Vi föreslår att under ett immunsvar så kommer B celler då och då besöka SED för att undersöka om det fortfarande förekommer antigen och transportera antigen till GC för att underhålla immunsvaret. Arbete (III) visar att minnesceller och lång-livade plasmaceller inte är nära besläktade vad gäller de gener för antikroppar som används. Baserat på detta föreslår vi att dessa celler kommer från GC som skiljer sig från varandra, antingen spatialt eller tidsmässigt, vilket leder till en större variation bland minnescellerna som också har antikroppar med lägre bindningsstyrka än plasmacellerna. Genom denna mekanism kommer plasmacellerna att fokuseras mot det antigen som för tillfället finns i tarmen, medan minnescellerna kommer att kunna känna igen en större bredd av olika närbesläktade antigen. I arbete (IV) studerar vi möss som växt upp sterilt i avsaknad av pathogener och finner att de i stor omfattning saknar IgA producerande plasmaceller trots att deras GC i PP verkar normala. Detta tyder på att slemhinnan i tarmen inte mognat färdigt och därför inte kan attrahera/upprätthålla IgA plasmaceller. En oral immunisering med NP-CT leder dock till att det inte bara bildas antigen-specifika plasmaceller utan också plasmaceller som inte känner igen detta antigen. Koleratoxin (CT) verkar därför kunna orsaka samma förändringar av slemhinnan som sker då bakterier växer i tarmen. Huvudfynden i denna avhandling kan sammanfattas som att vi definierar ett flertal mekanismer som är unika för aktiverade B cells i tarmen, och visar att dessa aktiverade celler är relativt rörliga och inte så bundna till ett enda GC som man normalt tror sker under ett svar mot ett antigen som tillförs kroppen via andra vägar.

LIST OF PAPERS

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

I. Komban R., Strömberg A., Jakob Cervin., Cervin J., Yrlid U., Johannes Mayer, Simon Milling., Mats Bemark., and Nils Lycke. **Orally activated B cells migrate via lymph to multiple Peyer's patches where they re-utilize germinal centres in an antigen and CD40-dependent fashion.** Manuscript, 2018.

II. Komban R., Strömberg A., Biram A., Cervin J., Yrlid U., Shulman Z., Bemark M., and Lycke N. **Activated Peyer's patch B cells sample antigen from M cells in the sub epithelial dome to maintain gut germinal center responses.** Manuscript under revision in Nature Communications.

III. Bemark M, Hazanov H, Strömberg A, Komban R, Holmqvist J, Koster S, et al. **Limited clonal relatedness between gut IgA plasma cells and memory B cells after oral immunization.** Nat Commun 2016, 7: 12698.

IV. Komban R., Bergqvist B., Strömberg A., Bemark M., and Lycke N. **Germ free mice exhibit poor gut IgA plasma cells responses but host intact and effective inductive sites in their Peyer´s patches.** Manuscript.

CONTENT

ABBREVIATIONS

PP	Peyers patches
GC	Germinal centers
MLN	Mesenteric lymph nodes
Sp	Spleen
LLPC	Long lived plasma cells
NP	4-hydroxy 3-nitrophenylacetic
CT	Cholera toxin
SED	Sub epithelial dome
TD	Thoracic duct
SI	Small intestine
LP	Lamina propria
Fi	Ficoll
TD	T-cell dependent
TI	T-cell independent
GALT	Gut associated lymphiod tissues
MALT	Mucosal associated lymphoid tissues

HSC	Hematopoietic stem cells
MPPs	Multipotent progenitors
CLPs	Common lymphoid progenitors
NGS	Next generation sequencing

1 INTRODUCTION

Without an immune system we would not survive. Our body is constantly under attack by many different microbial pathogens of which bacteria, viruses as well as parasites can cause infections, in worst cases leading to death [1] [2]. The immune system is composed of a vast network of tissues and cells that help distinguish between self from non-self. The innate immune system consists of many types of cells such as neutrophils, monocytes, macrophages, mast cells dendritic cells and many more [3]. The adaptive immune system, on the other hand, is composed of lymphocytes of only two types, B and T lymphocytes [4] [5]. The recognition of self and non-self is under the control of the T lymphocytes [6] [7] . Immune responses most often depend on both the innate and the adaptive immune system [8] [9] [10] [11]. The initial response to foreign antigen is provided by the innate immune system and activated through pattern receptor recognition (PRR), which is genetically an inherent set of receptors. An example of this type of receptor is the toll like receptors (TLR) [12] [13] [14] [15]. Lymphocytes have more complex receptors that are unique to specific antigens and which are continuously generated as new B and T lymphocytes are formed. This requires the recombination of receptor encoding genes. The innate immune response does not develop memory cells, whereas this is a hallmark of the adaptive immune system. Memory T and B cells are critical for a healthy life and vaccines are made to stimulate the development of strong protective memory lymphocytes [16] [17] [18] [19]. Moreover, activation of B lymphocytes also results in the formation of long-lived plasma cells, which reside in the bone marrow or at mucosal sites, where they maintain the presence of antibodies [20] [21] [22]. This thesis focuses on B lymphocytes, from now on termed B cells, in the gastrointestinal tract. I have studied how B cells are activated through oral immunization and how the gut IgA response is regulated and which sites are critical for the development of immune protection against attacking intestinal pathogens.

1.1 General concepts in B cell development

Being an integral part of the adaptive immune response, the B cells represent a separate function and lineage from the T cells [23] [24]. Starting with studies by Paul Ehrlich in Berlin in the 1890's it was identified that circulating antibodies, called antitoxins were important to protect against diphtheria and tetanus, but the existence of the antibody producing B cell came much later. Niels Jerne and others proposed that the antibodies were formed by B lymphocytes as late as in the 1950s [25]. Max Cooper and Robert Good in 1965, in an experimental setup using chicken were able to demonstrate that specific cells, B cells, develop in Bursa of Fabricus (bone marrow equivalent in birds) and were responsible for antibody production [26]. Apart from antibody production B lymphocytes have also been found to present antigen to T cells and for production of regulatory cytokines. Today B cells lineage differentiation and maturation has been widely studied and documented.

An outline of B cell differentiation is shown below,

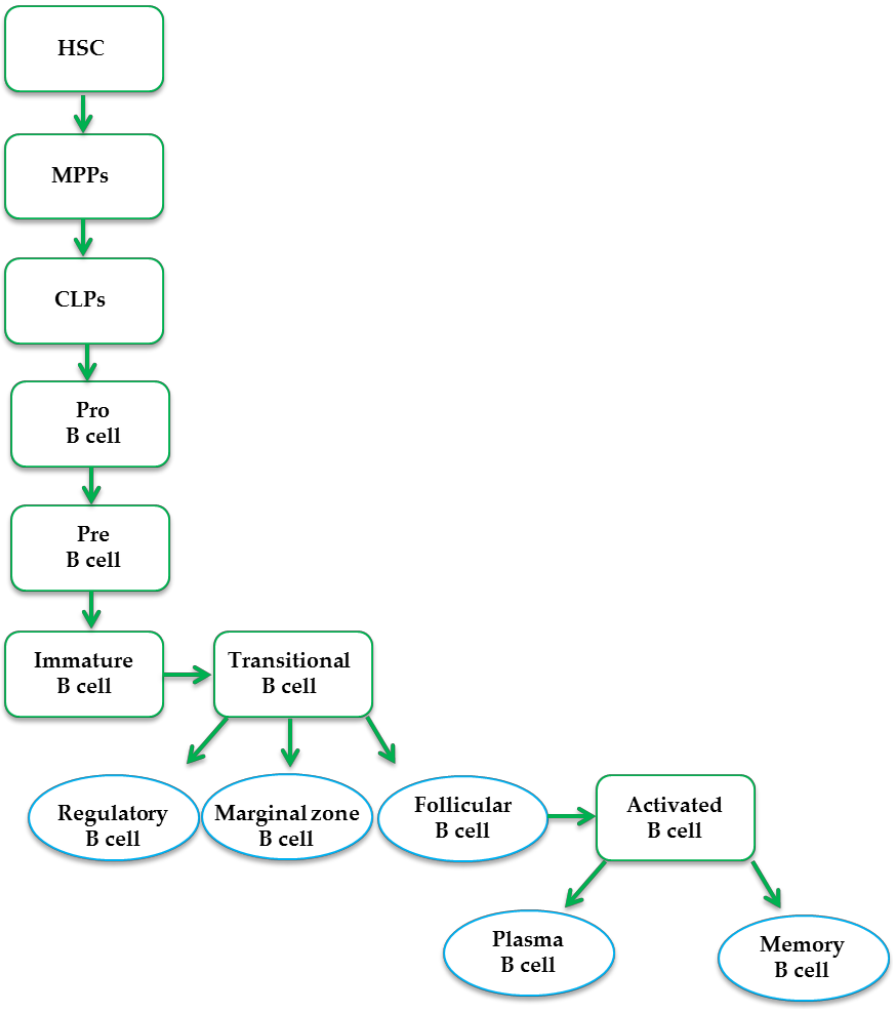

Figure 1. Overview of B cell Lineage differentiation: HSC (hematopoietic stem cells; MPPs (multipotent progenitor); CLPs (common lymphoid progenitors)

1.2 B cell Activation

Activation of B cells occurs when an antigen is recognized and bound to the B cell receptor (BCR), which is a membrane bound immunoglobulin of the IgD and IgM class. Once activated, B cells undergo massive expansion and finally they can differentiate into plasma cells and memory B cells. There are mainly two types of immune responses, those that are dependent on CD4 T cells [27] and those that can undergo with B cells alone, which are called T cell-independent immune responses [28] [29]. Most immune responses are against protein antigens and these all require the involvement of CD4 T cells. However, some lipids, and carbohydrates can stimulate a strong B cells response in the absence of T cells. This kind of activation is termed thymus independent (TI), while responses to protein antigens are named thymus dependent (TD).

1.2.1 T cell independent B cells responses

This route of B cells activation is divided into two subclasses, type 1 and type II [30] [31] [32] . For example, lipopolysaccharides (LPS), are type I antigens and have the ability to induce cell division through cross-linking of the BCR. LPS and certain nucleic acids like CpG can also activate B cells through TLRs, which leads to proliferation and differentiation of B cells with many specificities and, thus, called polyclonal activation. Immune responses against these types of antigens are dominated by IgM antibodies and are most often of low affinity as these B cells do not undergo isotype switching and affinity maturation processes.

Type II antigens are typically polysaccharides, involving strong BCR cross linking [33]. Epitope density is crucial for B cells activation by type II activation as it is achieved though cross-linking of multiple BCRs. A low density is insufficient to stimulate a response while a high density can result in unresponsiveness, also termed anergy [34] .

TI responses are faster than TD responses and days after a TI challenge a significant amount of plasma cells can be seen in the extra follicular regions

of the lymph node or spleen [35, 36]. A majority of these plasma cells die within a couple of weeks [33].

1.2.2 T cell dependent B cells responses

My thesis project is focused on studies of how to stimulate naive B cells (IgD^{hi}) in the gut. We have a reporter mouse that expresses GFP^{+} in all B cells that are specific for the NP-hapten. After adoptive transfer of these B cells into naïve recipient mice we study both T cell-dependent and T-cell independent IgA responses in the gut and at other locations. The T cell-dependent responses take longer time to develop than TI responses [37] [38]. They have two phases an early and late phase. In the early phase B cell expansion occurs in germinal centers (GC) that develop in the B cell follicle in the lymph node and this phase is followed by isotype switching and affinity maturation where also memory cells and long-lived plasma cells are formed [39] [40] [41].

Dendritic cells (DC) present antigen to naive T cells in the T cell area which results in the activation of the specific T cells. Although T cells and B cells recognize the same antigen, T cells react to peptides presented on MHC class II molecules on the DCs, while the B cells recognize conformational epitopes and do not need assisting DCs to get activated. Both types of activated cells move from the B cell follicle to T cell-B cell border region, where cognate antigen-recognition occurs, and prior to the formation of the GC, witnin 3-7 days [42]. Both activated B cells and the activated CD4 T cells participate in the GC reaction, which is established in the B cell follicle area around a network of follicular dendritic cells (FDC).

1.3 Mucosal Immune system

A major site for exposure to microorganisms and food antigens is the gut mucosa. This is because the intestinal mucosa constitutes of large surface area often exceeding 300 m^2 in humans [43]. To protect against pathogens the gut mucosal immune system produces IgA antibodies and the production of IgA in the gut is indisputably the largest amount of antibody produced per day in the whole body [44] [45] [46] [47] [48].

The mucosa-associated lymphoid tissue (MALT) [49] and the gut-associated lymphoid tissues (GALT) [50] are highly organized lymphoid tissues, which in the gut includes Peyer's patches (PP), cryptopathces (CP) and isolated lymphoid tissues (ILF) [51]. MALT and GALT are divided up into an inductive site and an effector site. In GALT the major inductive site is PP while the effector site is the lamina propria (LP) of intestine. This site hosts effector T cells, memory T cells and plasma cells that emanate from the inductive site [43]. Associated to GALT is the draining lymph node, *i.e* the mesenteric lymph nodes (MLN). Much remains to be understood about the function and development of these tissues [52]. My thesis work is an attempt to better define certain regulatory parameters that are essential for a functional gut IgA system.

1.4 The Gut Immune system

The intestinal mucosal surface has small finger link projections known as villi, which hosts the immune cells. The PPs are protruding dome like structures in the mucosa and represents the organized lymphoid tissue dominated by naive B and T lymphocytes. But, also macrophages, dendritic cells, and other stromal cells are abundant in this tissue. The follicle associated epithelium (FAE) that separates the lumen from the tissue hosts microfold (M) cell [53], which is specialized in taking up antigens from the lumen [54] [55].

To understand the function and organization of the gut IgA system it is critical to know where immune responses are initiated [56] [57]. Micro anatomical studies on IgA B cell responses have identified the PP as the major site for immune induction, but at times also the ILF and MLN could be involved [58] [59] [60, 61].

Table 1: Structures and tissues of Gut immune system

Structures of gut Mucosa	**Location**	**Function**
Peyer's patches	Seen in the Ileum region of the small intestine	Major site for plasma and memory B cell development. Also the key site for the coordinating immune response to pathogens in the gut.
Mesenteric Lymph nodes	Seen close to the wall of the small intestine	Specialized structure for the initiation of immune response.
Lamina propria	Beneath epithelium	Absorbs digestive products through network of blood vessels to the rest of the body.
Crypts	Within lamina propria in small intestine around villi	Location for replicating stem cells, Paneth cells and goblet cells
Villi	Outer part of gut wall facing lumen	Region of epithelial cells
Cells of gut Mucosa	**Location**	**Function**
Stem cells in crypts	Seen in the bottom of crypts between Paneth cells	Active self-renewal of the epithelium
Goblet cells	Intestinal crypts	Secretes mucous layer
Paneth cells	Intestinal crypts	Produce anti-microbial peptides
Intestinal effector T cells	LP and Intraepithelial cells (IEC)	Mediate immune homeostasis

Intestinal T regulatory cells	PP, LP, MLN	Suppresses immune response to commensal microbes. Maintain immune homeostasis
Intestinal B cells	PP, ILF, MLN	Source of IgA in LP
Intestinal dendritic cells	PP, LP, MLN	Regulates T cell homing to small intestine. Present microbial peptide to T cells for activation
Intestinal Macrophages	LP	Regulates inflammatory responses to bacteria and other harmful pathogens. Scavenge dead cells and foreign debris
Microfold (M cells)	FAE in PPs	Responsible for the uptake of antigen and present then to MALT
Intestinal epithelial cells (ECs)	Lining of small intestine	Secrete anti-microbial peptides, cytokines in response to microbes, recruit DCs. Present antigens to T cells and maintain immune homeostasis
Innate lymphoid cell type 2 (ILC 2)	LP	Induces mucous production and contributes to immune response to helminth worms
Innate lymphoid cell type 3 (ILC 3)	LP	Development of intestinal lymphoid organs, gut homeostasis

1.4.1 Peyer's patches (PP)

The PPs have been named after Johann Conrad Peyer, who defined them as lymph nodules in the small intestine as early as in 1673 [57, 62]. These structures are the major inductive sites for IgA responses and have been well described already 50 years ago, but their detailed immune function still remains to be investigated further [63] [64]. PPs can be divided into 4 main areas, i) the B cell follicle, where the GC develops, ii) the overlying follicle associated epithelium (FAE) and iii) the sub epithelial dome (SED) and located between the follicles & the T cell zone [57]. The PP is richly provided with lymphatic and blood vessels. The FAE and the M cells transport luminal antigens from the lumen to the SED. At this site the DC are known to take up antigen and migrate to the T cell zone, but also macrophages can take up antigen in the SED [65] [66] [67] Whether B cells at this location have an antigen transporting function is not known, but a few reports have indicated that could be the case. Much remains to be understood about the SED region and its functions in the IgA response. In the follicle, activated CD4 T cells and GC B cells interact and this occurs through the CD40L-CD40 molecules on the surface of the respective cell subsets [68] [69] [70]. However, the exact mechanism by which B cell responses are stimulated and regulated in the PPs is incompletely understood [57].

1.4.2 Mesenteric lymph nodes (MLN)

The lymph from the small and large intestines drains to the MLN [71] [72]. In fact, today, we have precise knowledge about which nodes that drain the small and large intestines. Also, GC and B cell responses are observed in MLN, but to what extent these are important for the IgA plasma cell responses in the LP are not completely clear. But, MLN could be a complementing site for IgA B cell responses [73]. Rather, the MLN is the major site for T cell tolerance induction to food proteins, constituting a protective wall against microbial invasion from the intestine [71] [74]. This way the MLN helps preserve antigenic ignorance to commensal bacteria [71] [74]. The migratory $CD103^+$ DCs in the LP take up antigen and carry antigen

to the MLN where naive CD4 T cells are primed. These T cells regulate immune reactions by dampening unwanted reactions and this way contributes to homeostasis in the gut [75] [71].

1.4.3 Lamina propria (LP)

The LP is a network of loose connective tissue in the mucosa. It is the effector site in the gut immune system, where plasma cells reside and plasma blast differentiate into plasma cells. Microscopic analysis of tissue sections of the gut show that the LP of the villi is filled with IgA producing plasma cells. The plasma cells produce dimeric IgA which is bound to a joining chain (J-chain). The complex binds to the polymeric Ig receptors (pIgR) on the basolateral membrane of the epithelial cell and is then taken up and transported through the cell into the gut lumen [76]. Activated B cells from GALT acquire homing receptors that enable them to migrate back to the gut LP. These cells migrate via the lymphatics to the MLN into the thoracic duct and after circulating in the blood they enter the LP [77]. These B cells are imprinted with homing receptors specific for the gut, i.e. $\alpha 4\beta 7$ integrin, CCR9 and CCR10 [78] [79]. At the effector site the endothelial cells in the blood vessels carry specific addressin cell adhesion molecules, such as moleculae-1 (MAdCAM-1). This is the key attractant for the integrin $\alpha 4\beta 7$ to the LP [80] [81] [82] [83]. In addition, other homing receptors are also required for final homing to the gut tissue and TECK (CCL25), expressed in the crypt epithelium of the small intestine interacts CCR9-expressing lymphocytes and CCL28 (MEC) attracts CCR10-expressing cells to large intestine [84] [85] [86].

1.5 Micro-anatomy of PP

1.5.1 Antigen uptake in PPs

We lack a detailed understanding of the mechanism for antigen uptake by M cells. These cells express Dectin-1 which facilitates the uptake of glycosylated bacteria [57] [87], but is not required for the uptake of IgA coated bacteria [88]. Also other molecules are expressed by M cells such as gangliosides which enables cholera toxin to access the tissue [89] and certain lectins facilitate up-take by M cells [90] [91]. The M cell, contrary to epithelial cells expresses glycoprotein-2 (GP2), which recognizes FimH (type of pili) carried by certain bacterial commensals and pathogens. The basal side of the M cell hosts large pockets that can harbors different types of cells, including IgD+ and IgD- B cells, DCs, Macrophages and T cells [90] [92] [66] [93] [94] [62]. The function of these pockets is still poorly known and it has been speculated to be important for antigen transport to the B cell follicle and T cell zone.

1.5.2 Sub epithelial dome (SED)

The sub-epithelial dome (SED) is a specialized structure that is in close proximity with the M cells in the PP's. There are several theories regarding the function of the SED [95] [96] [97] [98] and which cells traffic to and from this site [99] . My thesis work has taken a special interest in the B cells of the SED (Paper II in thesis). Apart from activated B cells the SED hosts a high density of classical DCs, $CD11b^{+}CD8^{-}$DCs and $CD11b^{-}CD8^{-}$ DCs [100]. However, also naive B cells along with macrophages, T cells, $ROR\gamma t^{+}$ ILCs have been identified in the SED [98]. It has been well documented that CCR6 is required for cells to appear in the SED region and both T and B cells express this membrane molecule.

1.5.3 Germinal centers (GC)

The GC reaction seen in B cell follicles during a T cell-dependent B cell response is complex [101]. GC was first defined by Walther Flemming in 1884 [102], as he observed sites where lymphocytes undergo mitosis in the follicular regions of the lymph nodes [103]. Today we know much more about the GC reaction and we know that it is critical for the affinity maturation of the antibody response, which is achieved through somatic hyper mutation (SHM) [104], and class-switch recombination (CSR) is also achieved in the GC [105] [101] [103] [106] [107]. Micro anatomical studies of GC show it is divided into two zones, a dark zone (DZ) and a light zone (LZ). The DZ is the active site for cell division, and the LZ harbors the FDC network which promotes the SHM and CSR. High levels of activation-induced cytidine deaminase (AID) are a hallmark of B cells in the DZ, also called as centroblasts. In the LZ the B cells are called centrocytes, as this site promotes contacts with CD4 T follicular helper cells (Tfh) [108] [109]. Within the germinal center, B cells move between dark and light zone in response to CCL12 (DZ) to CXCL13 (LZ) [109] [110]). Inside the GC multiple rounds of selection and mutation occurs [106], during this process many B cells undergo apoptosis and are removed by macrophages [105]. The result of the GC reaction is also memory B cells and long lived plasma cells, which are components that T-cell independent antigens do not stimulate.

1.5.4 Special features of PP GC and gut LP

This study is addressing which regulatory functions that govern gut IgA responses following oral immunization. The study dissects five important themes that we have discovered in the mouse model. The first is whether we develop memory B cells following oral immunizations. The second is the site and kinetics for B cell responses in GALT following oral immunization. The third is the function of Ag-specific B cells in the SED region and what relationship these have to the GC B cells. The fourth theme is the question of how re-utilization can occur of already existing GC in PPs and if this can be the basis for synchronization of the IgA LP response to high quality IgA

antibody responses. The fifth theme is the impact of germ free (GF) conditions on the gut IgA response and what role the CT adjuvant can have on the IgA response in GF mice. PP GC is a result of continuous exposure to the microbiota and food antigens. Hence, the PPs consistently host germinal centers (GCs) [110, 111]. The FDC network is a hall mark of the LZ while the network of $CXCL12^{+}$ reticular cells (CRCs), which attract CXCR4hi cells, is seen in DZ region. Of note, the CRCs distribution is more extensive in PP GC than found in LN or spleen [110] [112].

2 AIM

The general aim of this thesis work was to investigate B cell development in the gut; with focus on Peyer's patches and Mesenteric lymph nodes as the inductive site and small intestine lamina propria as the effector site.

2.1 Specific aims:

- To study the kinetics of B cells expansion in the gut
- Effects of migration of B cells within lymphoid tissues
- Germinal centers expansion of B cell and the markers associated with it, importance of CD40-CD40L interactions
- Antigen sampling by activated B cells from M cells in sub epithelial dome to maintain gut germinal center responses
- Long lived memory B cells development by oral immunization
- Ability of Cholera toxin in normalizing gut immune response in germ free animals

3 EXPERIMENTAL PROCEDURES

This section provides an overview of the methods used in the study. A detailed description of all the experimental methods can be found in the attached papers.

This study on IgA B cell biology has been thoroughly carried out using different mouse models. They were on C57BL/6 background and were housed in SPF or germ free conditions it our experimental biomedicine (EBM) facility at the University of Gothenburg, Sweden. To better control the bacterial microflora majority of the mice used in the experiment were bred at our facility. Mice were either used from experimental biomedicine facility, Gothenburg University or bought from Taconic farms (M&B, Lille Skensved; Denmark). The mice were all age and sex matched. The core of the thesis work has been based on adoptive transfer model developed in the research group. This transfer model we used NP-specific B-cell experiment mice generated through crossing C57BL/6 mice with homozygous B1-8^{high} GFP mice [58] [113] [114] (a gift from M Nussenzweig, Rockefeller University, New York, NY). For bone marrow chimeric studies and transfer studies, we generated GFPCD40$^{-/-}$ mice and NPGFPCD40$^{-/-}$ mice by crossing B1-8^{high} GFP mice with CD40$^{-/-}$ for several generation and mice for experiment were generated. mT/mG (tdTomato) mice used in chimeric transfers were purchased from Jackson laboratory and are maintained at our animal facility. The germ free mice were acquired from our own EBM facility at the University of Gothenburg (Fredrik B).

Immunizations

Unless indicated immunizations with NP-CT (4-hydroxy-3-nitrophenylacetyl conjugated with cholera toxin [58] [115]) p.o was given at 10μg/mice and booster immunization of NP-KLH (500μg, Bio search technologies) was also administered along with NP-CT to get better NP specific responses. Response against NP has been well documented over the years and NP being a hapten cannot elicit an immune response all by itself but need a carrier

protein. Systemic response with NP-CT was obtained with a dose of 4 µg i.p immunizations. TI systemic response was achieved using 4 µg NP-Ficoll (Biosearch technologies) immunizations. For ligated loop assays NP-PE (5070-1 LGC Biosearch technologies) was administered to the loop at around 200µg/loop. For blocking internal migration, gut homing and GC disruption FTY720 (0.1mg/dose), CD40L (Bio X cell) and $\alpha 4\beta 7$ (Bio X Cell West Lebanon, NH, 250µg/dose) blockade were used. More details on conjugation protocols and immunization dose and intervals can be found on the attached manuscripts.

As the study is based on B cell activation and development we use the liberty of technologies like Microscopy, ELISPOT, Sequencing and FACs sorting to unravel the biology behind B cells activation.

Work model

A major part of the study has been carried out using adoptive transfer model system where NP-specific B1-8^{hi} IgH knock-in λ-expressing GFP^{+} B cells transferred into normal recipient mice and then orally immunized the mice with NP-hapten conjugated to cholera toxin (NP-CT). This transfer system was redesigned in papers attached depending on the study, either changing the route of immunization or the antigen or by retransferring activated cells after this transfer procedure. Fig 2 shows typical response on D10. (Detailed information's on protocols used can be found in the attached papers).

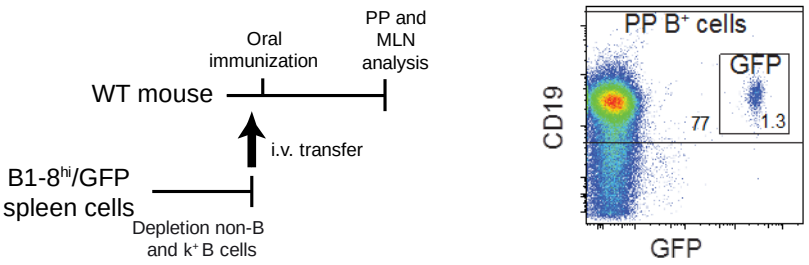

Figure 2. Work model for the analysis of early and late expansion kinetics of NP-specific B cells following oral immunizations is represented in the left-hand side and the gating strategy of NP-specific B cells from CD19+ lymphocytes is shown in the right-hand side.

Bone marrow chimeras

The role of CD40 proficient and CD40 deficient B cells in generation IgA plasma cells has not been studied; hence a bone marrow transfer system was designed after generation GFPCD40$^{-/-}$ mice by cross breeding, the experimental procedure shown at Fig 3 were performed. The advantage of such a system is, one essentially transfers a genotype of interest to the hematopoietic compartment of the recipient mice. Thus this system allowed us to compare the contribution of CD40 proficient and CD40 deficient B cells in developing IgA plasma cell responses under steady state.

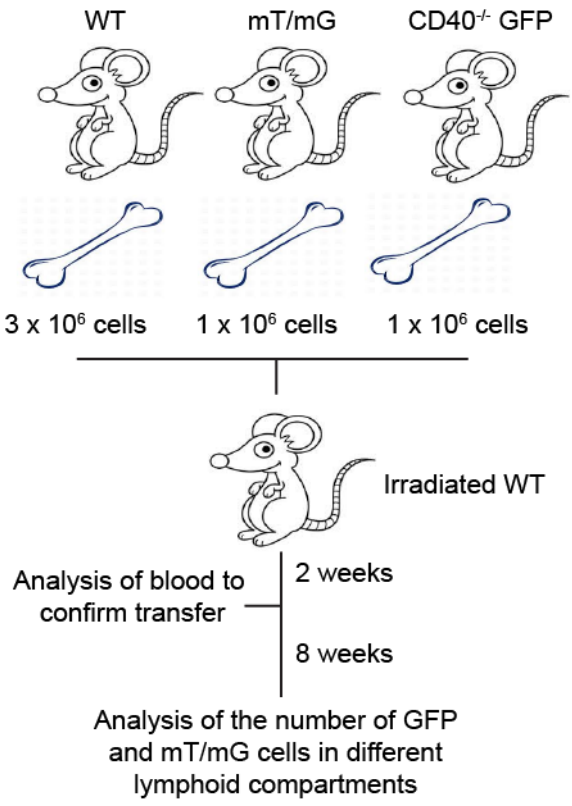

Figure 3. Bone marrow chimeric transfer model

Flow cytometry and cell sorting

Flow cytometry along with immunohistochemistry was the central part of this thesis. FACs was used to study different marker expression on B cells and was mainly used to quantify the findings. In theory differently labelled antibodies were used to identify different extracellular and intracellular markers. Using unique laser settings in the FACs relative size and granularity can also be determined. In principle a single cell pass through a set of laser beams and the respective fluorochrome on the cell emits fluorescence. This fluorescence is detected by photo multiplying tubes (PMT). These signals are further converted to electronic signals which software converts to a numerical value on an analysis plot. By comparing specific fluorescent signals on each cell, one can study the expression level of different markers. B cell at different stages of activation was compared and quantified using this approach in the project. In FACs sorting cell with specific fluorescent profile can be sorted into tubes or plates. I could use this method to separate B cell expressing certain markers and further stain them or retransfer them into a host.

Immunohistochemistry

Immunohistochemistry is an excellent tool for qualitative information about the distribution of the cells and components in the tissue. This technique has been pivotal to this study as it enabled us to study cell activation, location at an early or later stage, cell migration, cell interactions etc. Major findings of this PhD thesis are based on this technique. In theory this technique has similarities with FACs as we use antibodies differentially labelled with fluorescent compounds are used to target specific markers on the surface or within the cell. The images of these fluorescently labelled cells in tissues has been acquired using the Zeiss LSM 700 inverted confocal microscope.

RNAseq analysis

RNA was isolated from the FACs sorted cells from spleen and PPs. This was sent to BGI for transcript quantification using the RNA sequencing after amplification using a SMARTer PCR cDNA Synthesis Kit (Takara Biomedical

Technology, Beijing, China). Comparing the expression was carried out by bioinformatics workflow (BGI Genomics, Hong Kong, China). Samples from PP (GL7+ or GL7- activated GFP+ and GL7+ germinal center or GL7- GFP- naïve B cells) were used. For expression analysis of specific transcripts, the PP populations were compared to $GL7^{-}$ GFP^{-} naive splenic B cells; comparisons with individual splenic sample were made and had been prepared in parallel with the PP samples.

Cannulation

Thoracic duct cannulation provides an excellent tool to study the migration of lymphocytes. We used this approach to understand populations of B cells that migrate in the system. Protocols were carried out using standard established procedures [116]. Lymph was collected and FACs analysis was carried out to study these lymphocytes based on phenotypic markers they express.

Ligated loop study

One of the classical finding was conducted using loop study. Antigen sampling by activated B cell was visualized using this protocol. In short after transfer of cells on day 10 an incision was made along *linea alba* of the abdomen to expose the intestine. PPs were identified and ligated loops of intestine were made according to the protocol [117]. Antigen was injected inside the loop and mice were kept under anesthesia for different time duration to study Ag uptake mechanisms.

In vivo Imaging

Studies on activated B cell migration in the PP was conducted using in vivo imaging technique. A loop with one PP was surgically exposed and immobilized and analyzed under the microscope. This method helped us in understanding the internal migration of PP B cells within the micro anatomic structures of the PP.

Cloning and Sequencing

Cloning and sequencing part was carried out to study memory B cells or LLPC from spleen, MLN, PP or BM. RNA was isolated followed by cDNA conversion and PCR using NP specific primers were used. This PCR product was later transformed and identified using colony hybridization protocol. Plasmid was isolated and either traditional Sanger sequencing was used where clones was analyzed using staden package. Clones were classified as NP binding if their CDR3 region was between 9 and 11 amino acids long, they had a tyrosine at position 99, and there were at least two more tyrosine residues in the following three amino acids. When NGS of NP-binding gene sequences were undertaken, the Ion Torrent platform was used. Here nested PCR with FRW1 and IgA primers with barcodes was used to amplify purified DNA after eluting the DNA from NP specific PCR. After purification, of PCR product was loaded at 40 pM concentration onto Ion 314 v2 Chips using an Ion Chef and was sequenced. Sequences were aligned to V, D and J segments using IMGT/HighV-QUES [58].

4 RESULTS AND DISCUSSION

Paper I

4.1 Biology of B cell expansion

It is important to understand how immune responses in the gut are initiated following an oral immunization so that we may develop more effective oral vaccines [56] [118] [119]. Studies have suggested that the PPs are the critical sites for induction of gut IgA responses, but to what extent MLN, ILFs or even the LP contribute to the response is poorly known [57, 58] [59] [61]. To answer these questions we developed an unique mouse model based on the adoptive transfer of NP-specific B1-8^{hi} IgH knock-in λ-expressing GFP^{+} B cells into normal recipient mice and then orally immunized the mice with NP-hapten conjugated to cholera toxin (NP-CT) [58] [115] (*M&M Fig 2*). Using this adoptive transfer system it was found that after per-oral (p.o) immunization a dramatic expansion of NP-specific GFP^{+} cells were observed in the PP already on day 4 and the response peaked on day 10. Furthermore, we found a gradual engagement of proximal PPs on day 5 to involve also more distal PPs on day 10. Interestingly, whereas GL7 is commonly thought of as a marker for GC, the expression level of GL7 in PPs remained around 20%, while this was higher than 80% in the spleen after an i.p immunization with NP-CT. Immuno-histochemical sections of PPs showed, however, that the GFP+ B cells were located to the GC in both the spleen and PPs. Of note, we observed a clearly less dense distribution of NP-specific GFP^{+} cells in PP GCs compared to that observed in MLN or in SP (Fig 4).

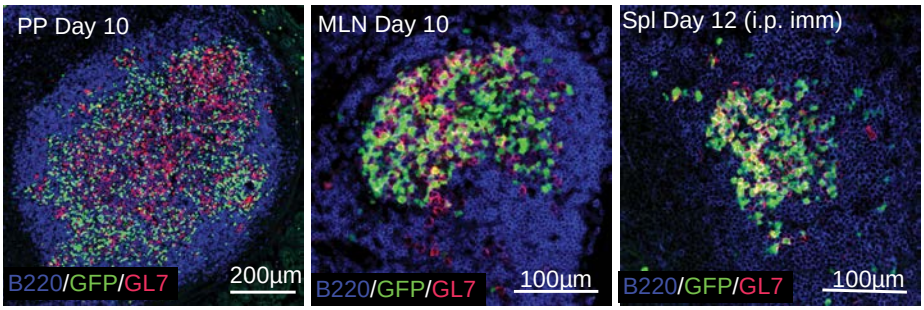

Figure 4. PP, MLN and SP D10 sections after immunization. Green-GFP⁺NP specific B cells, Blue-B220⁺Bcells, Red-GL7⁺ germinal center cells

4.2 Migration of activated B cells

We have shown in a previous study [115] that activated B cells from one PP can migrate into multiple PPs where they appear to undergo differentiation. Here we studied the role of B cell migration following oral immunization in greater detail. We used FTY720 to block egress of activated B cells from the inductive sites in the PPs [120] [121] [122] [123]. We administered FTY720 to achieve complete or partial blockade following oral immunization with NP-CT. We observed after FTY720 treatment from day 0 a dramatic drop in NP-specific GFP⁺ cells in the PPs, but when FTY720 was given from day 3 we observed an increase in NP-specific GFP⁺ cells in PP and , in particular, in the MLN, strongly arguing for an early migration from inductive sites in PP to other PPs and the MLN. We observed a 3-fold increase in NP-specific GFP⁺ cells in MLN. Immuno-histochemical sections showed a reduction in NP-specific GFP⁺ cells in the GC in PPs after FTY720 treatment from day 0. It is interesting to notice FTY720 treated group showed a decrease number of GFP+ plasma cells in villi close to PP and the opposite finding in the untreated group. Our findings suggested that FTY720 treatment prevented early migration of B cells from PP-inductive sites to multiple PPs at more distal sites. It appears that the PP B cell affects the NP specific B cells in MLN, because the delay in FTY720 treatment to day 3 strongly augmented the presence of NP-specific B cells in MLN [60] [121] [122].

The studies suggested an early migration of activated NP-specific GFP^+ cells from PPs to MLN and then into the blood to finally arrive at distal PPs. The activated PP B cells could have left via the draining lymph at an early time point after immunizations. To investigate this possibility we undertook experiments with surgical cannulation of the ductus thoracicus. Two groups of animals were used, one with intact MLN and another with the MLN removed (MLNX).

In both groups we identified activated B cells; i.e $CD19^+$ IgD^- $GL7^+$ cells around (0.1-0.15 %) as well as $CD19^+$ IgD^- $GL7^+$ $CD73^+$ cells, which suggested a GC origin. These findings suggested that irrespective of the MLN, GFP^+ cells could pass into the draining lymph and migrate into the blood to home back to multiple PPs in the distal gut. In order to test this notion we performed surgical cannulation of the thoracic duct after the transfer of NP-specific GFP^+ cells and oral immunizations. An early (D5) and a late time point (D8) was studied, GFP+ B cells and GFP+ plasma cells were both identified at these time points, further strengthening the idea of migration of NP-specific GFP^+ cells at an early time point to other PPs and the MLN via the draining lymph. This way we could explain how synchronization of gut IgA plasma cell responses can occur [115] [56].

4.3 Requirements for the re-utilization of germinal centers

To study the conditions required for the re-utilization of already existing GC in multiple PPs we postulated that presence of antigen could be a critical component. We speculated that antigen had to be delivered 1 day before the transfer of activated GFP+ PP B cells to allow the cells to accumulate in the PPs of recipient mice. We administered NP-CT orally 1 day before or 1 day after the adoptive transfer of activated PP B cells. While naïve GFP+ B cells expanded in PPs even when antigen was given 1 day after transfer the activated PP B cells only accumulated in PPs when antigen was given 1 day before transfer. Thus, antigen is critical for the re-utilization of pre-existing GC in PPs of the recipient mice. When recipient mice were given anti-CD40L Mab to eliminate existing GCs the accumulation of B cells in PPs was interrupted. There was a dramatic loss of GFP^+ B cells in PPs and a severe

reduction of plasma cells in the LP (Fig 5). This explains how we can achieve a synchronized gut IgA plasma cell response.

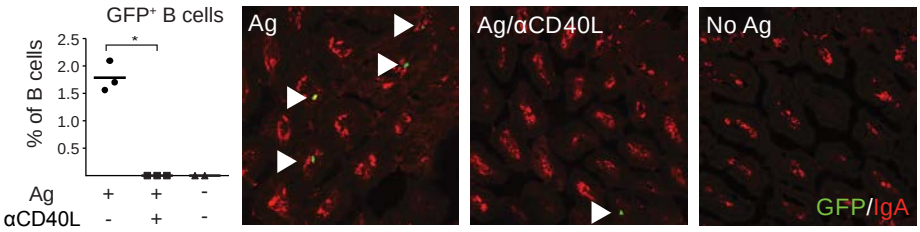

Figure 5. The graph shows adoptively transferred activated GFP⁺ B cells from PP were, indeed, capable of repopulating recipient PPs in the presence of antigen while in CD40L treated recipient group, GFP+ B cells were not able to differentiate. Right panel shows adoptively transferred activated GFP⁺ B cells from PP are capable of entering the germinal of the recipient mice while CD40L treatment destroyed the germinal center structure, Non-immunized group is kept as the control

We further studied the role of CD40-expression on activated NP-specific GFP⁺ cells for the ability to re-utilize existing GC in multiple PPs. NP-specific GFP⁺ cells from different sources were compared. Splenic cells from CD40-deficient mice or wild type (WT) mice were compared following NP-Ficoll (T-cell independent) immunizations or after oral NP-CT (T-cell dependent) immunizations. As a positive control we used oral priming with NP-CT. All recipient mice received an oral dose of NP-CT 24h before the B cell transfer. We observed that both splenic and PP cells activated by NP-Ficoll (TD) or NP-CT accumulated in the PPs irrespective of if they represented TD or TI-type of responses. By contrast, CD40 deficient B cells from NP-Ficoll immunized mice did not accumulate in the PPs of the recipient mice. Hence, it can be concluded that B cells activated by TI antigens appear also to accumulate in PP and could possibly also benefit from exploiting the GC. Of note, no cognate interaction was required in this process, but the presence of Ag and CD40L –CD40 interactions were critically needed. It, thus, appears that both type of B cell responses, TD and TI-type, can re-utilize already existing GC in the PPs. Interestingly, cognate interactions with antigen-specific T_{FH} cells seemed not to be required by already activated B cells when

re-utilizing an already existing GC in multiple PPs. Whereas the T_{FH} population is critical for the GC reaction, its prime function in PP GCs appears to be CD40L expression to support B cell affinity maturation and IgA CSR.

4.4 Bone marrow chimeric transplant confirming the role of CD40-expression in gut IgA plasma cell generation

To study the role of TD and TI type of B cell responses in the gut we generated chimeric mice by transplanting bone marrow from GFP^+ $CD40^{-/-}$ or mT/mG (tdTomato) WT mice into irradiated WT mice. The aim was to test whether CD40-proficient or CD40 deficient B cells contributed to the IgA response in the gut LP. Strikingly, we observed a dramatic loss of the GFP^+ gut IgA plasma cells, while the proportion of mT/mG BM derived WT IgA plasma cells was unperturbed, indicating that B cells need CD40-expression to be competitive in the GALT (Fig 6). The complete loss of GFP^+ B cells was obvious in the gut LP, whereas it was not seen in peripheral blood, MLN or the PPs (15-20% GFP+ and 20-25% mT/mG B cells). This distribution of B cells was also reflected in the distribution of CD4 T cells of the GFP+ or mT/mG+ origin in the different tissues. In contrast, the gut IgA plasma cells were exclusively derived from CD40-proficient B cells with minimal numbers of CD40-/- GFP cells, arguing that CD40 expression is critical for the B cells to enter GC in the PP to undergo proliferation and affinity maturation prior to homing to the gut LP as IgA plasma cells. In conclusion, this experiment proved that CD40-expressing B cells, even activated with TI antigens, can re-utilize already existing GC in multiple PPs. CD40-expression is required for B cells to undergo expansion and differentiation in PP GC.

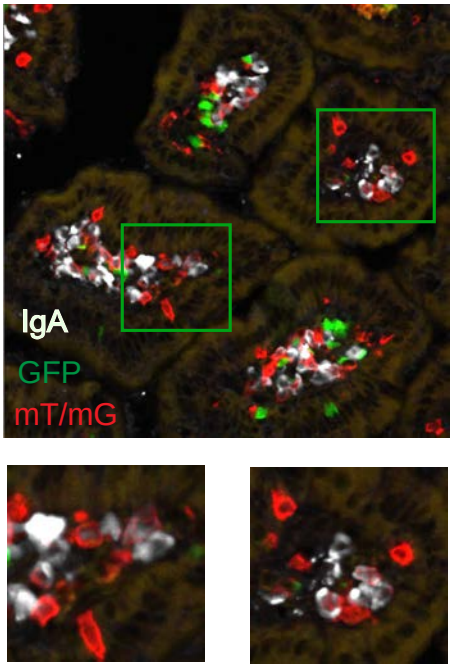

Figure 6. Representation of LP after the transfer of GFP and mT/mG cells. IgA positive cells were exclusively from the CD40 proficient origin. IgA-white, mT/mG CD40 proficient- Red, GFP CD40 deficient- Green.

Earlier studies suggest gut IgA antibodies must be of sufficient quality to effectively protect against pathogen [124]; this is achieved by re-utilization of germinal centers resulting in a clonal expansion of B cells [115]. In this study we demonstrates that re-utilization of pre-existing unrelated GC, in multiple PP can occur and is achieved through an early migration from the inductive site in proximal PPs, via the draining lymph and blood. For the accumulation of activated B cells in the PP GCs two important factors are required, presence of antigen and CD40-expression on the migrating B cells, suggesting that perhaps cognate interactions in the PP GC are not required for further propagation and differentiation of activated PP B cells. Bone marrow chimeric studies were undertaken and indicated that B cells, even when activated by luminal TI antigens, can enter PP GC to further differentiate.

Paper II

4.5 Germinal center dynamics

Germinal centers in PPs are different from GCs in other lymphoid tissues in several ways [125] [126] [127]. One important difference is that they are constantly present in PPs due to the gut microbiota. This makes it difficult to study the induction of a specific GC response in the PPs following oral immunization. Whereas antigen is travelling with the lymph or on migrating DC through the afferent lymph to peripheral lymph nodes, the way antigen gets access to the PP is through the FAE and more specifically the M cell uptake of antigen from the lumen [128] [129]. However, we still lack precise information about how antigen is transported from the M cell to the B cell follicle. It has been demonstrated that DCs play a critical role for this transport, but also B cells have been implicated in this regard. Yet, another question to answer is whether there is any selection of antigen from the myriad of antigens present in the gut lumen to promote a specific response. In this regard the B cells with their specific receptors would serve such a function if they were involved in antigen transport in the PP. Using our B1-8^{high} adoptive transfer model we investigated the role of the B cells in the SED for an antigen transport function. Following oral immunizations with NP-CT, a majority of NP-specific GFP^{+} B cells were found to be in the GC. A FACS analysis of these cells demonstrated that most of the GFP^{+} cells were activated IgD^{-} (> 90%), An unexpected finding was that only 20% of these B cells expressed GL7, which is traditionally viewed as a marker for GC B cells. This contrasted with the fact that more than 80% of the activated IgD^{-}GFP^{+} B cells in the spleen were GL7^{+}. Moreover, in the spleen GC the GFP+ B cells were dense, whereas in PP GC they appeared much less densely packed with GFP^{+} B cells. These findings indicate that the PP GCs regulatory microenvironment may be different from that found in other secondary lymphoid organs and spleen. A detailed analysis on the tissue sections of PP GCs showed proliferating (Ki67^{+}) GFP^{+} B cells that were dominated by GL7^{-} cells (Fig 7). Their GC nature was confirmed by BCL-6-expression in both GL7^{+} and GL7^{-} GFP^{+} B cells. The GL7-reactive antibody used to define GC B

cells detects α2,6-linked N-acetylneuraminic acid on glycan chains, an epitope highly expressed in GC B cells due to lost expression of CMP-Neu5Ac hydroxylase (CMAH) [130]. Therefore, in mice that lack CMAH all B cells express high levels of the GL7, which results in a state of hyper-responsiveness to Ig-crosslinking.

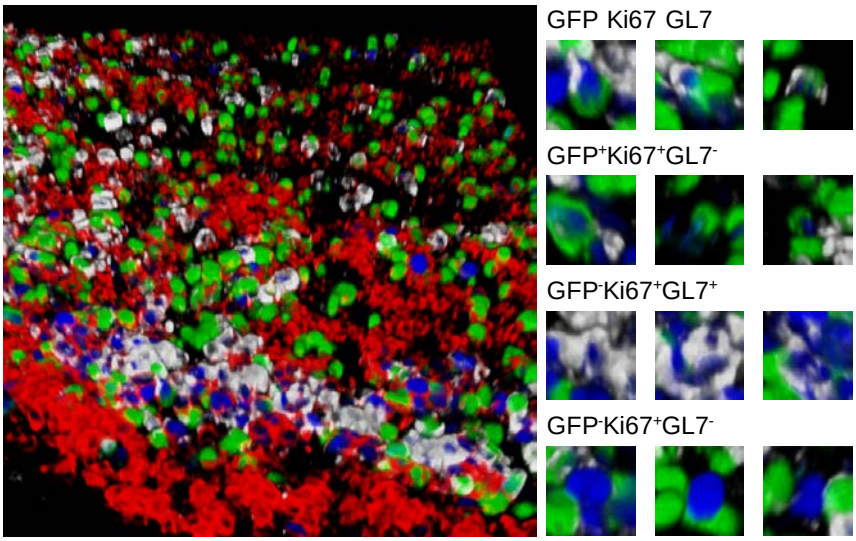

Figure 7. Close-up confocal microscopy image on a GC in PP with GL7$^+$ (white) or GL7$^-$ proliferating Ki67$^+$ (blue) GFP$^+$ (green) B cells B220$^+$(red) and examples of labelling patterns of activated NP-specific GFP$^+$ or non-specific GFP- B cells in the GC image.

The GC can be divided into a light and a dark zone [131] [132] [133]. To understand the role of the GL7$^-$ GFP$^+$ B cells we analyzed their distribution in these GC zones, to see if they could be associated with a functional stage of GC B cell differentiation. However, we observed no clear pattern as the GL7$^-$ GFP$^+$ B cells appeared to be equally present in the light and dark zones. Furthermore, we performed an extended phenotypic analysis by FACS of the GL7$^-$ and GL7$^+$ GFP$^+$ B cells. We found that the GFP$^+$ IgD$^-$ cells were CD38$^-$ PP B cells and shared all of the activation markers that we tested for. Hence, it appeared that the two GC B cell populations were similar and identical to the endogenous GC population (GL7$^+$B220$^+$GFP-), but phenotypically different from naive B cells (GL7$^-$IgD$^+$GFP$^-$ B cells). To investigate whether the GL7$^-$ phenotype was stable we isolated GFP$^+$ GL7$^+$ and GFP$^+$ GL7$^-$ cells by FACS

sorting and transferred them into hosts that were given NP-CT 24hr prior to the transfer. We found that both populations accumulated in the PP GC and both populations gave rise to a distribution of 20% $GL7^+$ and 80% $GL7^-$ GFP^+ IgD^- B cells in the recipient PP. This suggested that the GL7 phenotype in PP GC is not stable, but rather appears reversible as B cells can upregulate and downregulate GL7 during the different stages of differentiation.

4.6 Gene expression profiles of $GL7^+$ and $GL7^-$

The studies on GFP^+ B cells in PP GC showed that the GL7-marker could be expressed at different stages of GC development. To better understand if the GL7-expression could identify a distinct stage of differentiation we undertook an RNASeq analysis of the sorted PP B cells. We found that the gene expression pattern in both GFP^+ B cell subsets were largely similar to the one obtained from the endogenous $GL7^-$ GFP^- PP B cell population. The global gene expression heat maps showed $GL7^+$ and $GL7^-$ B cells from activated NP-specific GFP^+ cells to be similar to endogenous $GL7^+$ B cells. This was further confirmed in the principle component analysis (PCA) of the global gene expression profile that was found with activated $GL7^+$ GFP^+ cells, showing that, indeed, $GL7^-$ and $GL7^+$ GFP^+ B cells were very similar, albeit not identical .For example, the EBI2 gene has been linked to B cells migrating to outer follicular regions (OFR) [134], and, hence, repression of this gene supports a GC localization and relates to the upregulation of BCL6 [130, 134]. In our RNASeq analysis EBI2 was seen differentially expressed in GC $GL7^-$ and the $GL7^+$ B cell subsets. Hence higher EBI2 gene expression on $GL7^-$ population could be a requirement for $GL7^-$ GFP population to leave the GC and migrate to the subepithelial dome (SED) region.

Gene expression levels of CD83, CD86 and CXCR4 were similar in both $GL7^+$ and $GL7^-$ GFP^+ B cells, supporting the notion that there should be no major differences in light/dark zone distribution between $GL7^+$ and $GL7^-$ B cells. Similar expression levels of CXCR4 in both the populations, perhaps, associated with a similar tendency to leave the PP [108]. More importantly, the PP $GL7^-$ GFP^+ B cell population showed a gene expression prolife often

associated with memory B cell development with higher expression of EBI2 (Gpr 138), CD38, CCR6, which indicated differentiation towards a precursor memory or memory B cells stage [135] [136] [137]. The two populations also differed with regard to CCR6 gene expression, which was recently shown to be linked to a pre-memory stage of B cell differentiation [138] [139] [134]. Alternatively, the higher expression of CCR6 and CCR1 mRNA could indicate trafficking of $GL7^{-}$ GFP^{+} B cells to the SED [99].

4.7 Distribution of $GL7^{-}$ phenotype

We have previously reported that IgA CSR is effective in the GALT of CD40-/- mice. The B cells undergoing IgA CSR in CD40-/- mice were found to be $GL7^{intermediate}$ and it was thought to be at a stage of differentiation prior to a manifest GC stage. This IgA CSR could have occurred in the SED as B cells express AID in SED [140] [139]. In the PP there are two major compartments where the activated NP-specific GFP^{+} cells are found following oral immunization, namely in the GC and the SED region. Therefore we examined GC and SED regions in detailed for $GL7^{+}$ and $GL7^{-}$ GFP^{+} B cells. We observed dividing $Ki67^{+}$, GFP^{+} B cells in both the GC and SED regions (Fig 8). On further examination we observed that the GFP^{+} B cells in the SED region were $GL7^{-}$ and expressing CCR6. Of note, B cells undergoing IgA CSR in SED were reported to be mainly IgD^{+} $CCR6^{+}$ and, thus, different from the $GFP^{+}IgD^{-}CCR6^{+}$ B cells we observed [99], the proportion of GFP^{+} cells that expressed AID in SED was significant, which argues against a memory phenotype. It appeared that some GFP^{+} cells had switched to IgA, but IgA-expression was low, speaking against differentiation of plasma blasts in the SED. The most striking and unexpected finding in the SED region was the presence of GFP^{+} B cells in close proximity to GP-2 expressing M cells. It was clear that the B cells were not just randomly distributed close to the epithelium as few cells were close to $EpCAM^{+}$ epithelial cells. We interpreted this location to be important and reflecting a function of $GL7^{-}$ GFP^{+} cells in the SED and we hypothesized that these B cells acquired luminal antigens taken up by the M cells.

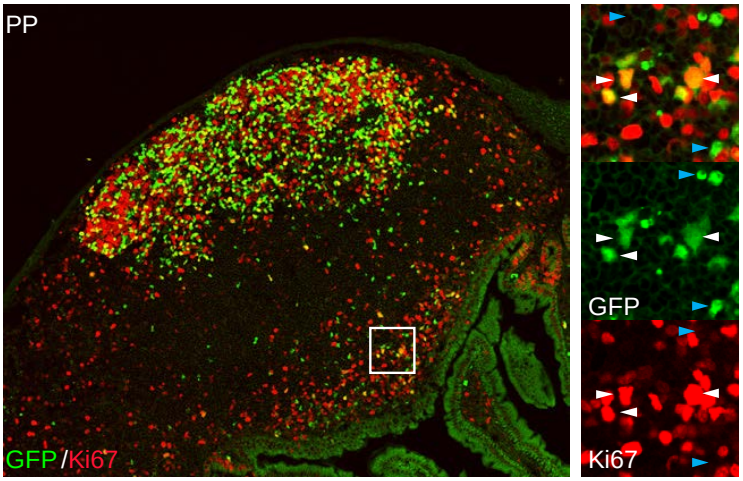

Figure 8. Representative microscopy images showing activated proliferating Ki67⁺ (red) GFP⁺ B cells (green) in the SED and GC on day 10 following oral immunization with NP-CT

4.8 Antigen uptake by GFP⁺GL7⁻CCR6⁺ B cells

Activated GFP⁺GL7⁻CCR6⁺ B cells were seen in close proximity to the M cells of the FAE in PP. The presence of activated antigen specific B cells in both SED and GC suggested that the B cells could move from one region to another within the PP. To address this question we initiated collaboration with Ziv Shulman at the Weissman Institute in Israel. Together we could demonstrate that photoactivatable (PA-GFP) B1-8^{hi} B cells migrated from the SED to the GC in the PP at day 8 followed by oral immunization with NP-CT [141] [142]. Photo-activation sites were marked by co-transferring B1-8^{hi} B cells expressing cyan fluorescent protein (CFP) to AID-Cre tdTomato mice to visualize SED and GC regions. Tracking of the photoactivated B cells showed that 40% of SED B cells appeared in the GC area, while movement of GC cells to the SED was less clear. Hence it was concluded that during an ongoing response in PPs the B cells move from SED to GC, and, perhaps, also from the GC to the SED region. Further studies are required to analyze whether IgA CSR occurs in the SED region.

Because the GFP⁺ B cells were in close contact with the M cells in the SED region and could move towards the GC, we speculated that they could

perform an antigen transporting function in the PP. For trafficking of B cells from SED to GC or GC to SED the expression of migratory markers like CCR6 and CXCR5 will be critical. To test whether the B cells could acquire antigen from the M cells we injected NP-PE into ligated loops of the small intestine 10 days after the transfer of cells and oral immunization with NP-CT. We found that at 1hr following injection of antigen a significant number of GFP^+ B cells carried NP-PE. The percentage increased even more and after 2hr following injections into the ligated loops nearly 50% of $CCR6^+$ $GL7^-$ SED NP-specific GFP^+ cells were PE^+, while around 20% of $CCR6^-GFP^+$ B cells had bound PE labeled antigen. By contrast, the endogenous B cells (GFP-), or GFP^+ B cells from PPs outside the loop, or inside a loop injected with PE alone all had few PE-labeled cells, indicating that only NP-specific B cells could capture the luminal NP-PE antigen. Also the frequency of PE^+GFP^+ B cells was highest in SED compared to GC ($CCR6^-GL7^+$), supporting the notion of an antigen-transporting pathway from SED to GC. An immuno-histochemical analysis confirmed that M cells had, indeed taken up NP-PE from the lumen of the loops (Fig 9). NP-PE was bound to the GFP^+ B cells at the basal pockets of the M cells (Fig 9). Taken together it appeared that antigen-specific B cells in SED interact closely with the M cells to bind antigen taken up from the gut lumen. The SED B cells migrate with antigen to the GC, which could be a mechanism for perpetuating the GC reaction for as long as the B cells can bind luminal antigen in the SED. It can be speculated that if this antigen uptake pathway is disrupted or M cell function is lost it can severely affect IgA antibody responses in the gut [143] [129].

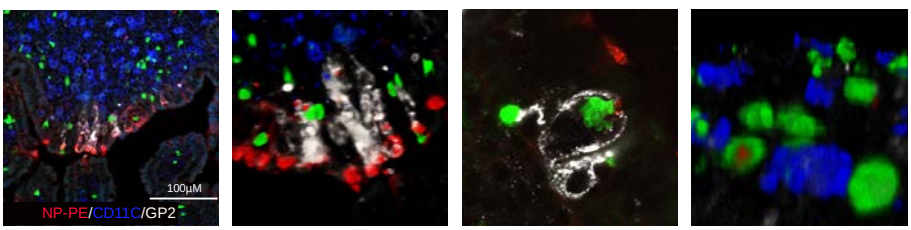

Figure 9. Representative confocal microscopy image of antigen accumulation (NP-PE; red) to M cells ($GP2^+$; white) in the FAE of a PP at 30 min. following inoculation of a loop with NP-PE. Also depicting dendritic cells (CD11c; blue) in SED (panel 1). NP-PE antigen (red) located to M cells ($GP2^+$; white) and not to the $EPCAM^+$ epithelium. GFP^+ B cells (green) in close proximity to or even surrounded by M cells (panel 2 and 3) and GFP^+ B cell in SED carrying NP-PE and DC ($CD11c^+$; blue) (panel 4).

Paper III

4.9 Poor clonal relatedness of long lived plasma cells and memory B cells

A critical question to answer is whether oral immunization can stimulate a long lasting memory [144] [145] [146]. This is essential if oral immunizations are to be considered for long term protection against invading pathogens. Evidence from several clinical vaccine trials have indicated that memory may be short lived following oral vaccination. Therefore we studied the development of long lived plasma cells and memory B cells after oral immunization. Following three oral immunizations with NP-CT at 10 days apart, clonal relatedness of long lived plasma cells and memory B cells were investigated. In the first part of the study we used the traditional Sanger sequencing technique to sequence the V_H186.2 heavy chain V region gene, encoding NP specificity. We found that long lived IgA and IgG plasma cells in the bone marrow (BM) were clonally closely related and exhibited a similar frequency of mutations. Nearly 50% of the sequenced IgA genes carried the high-affinity mutation, characteristic for the NP-response. Moreover, IgA and IgG NP-binding B cells in the small intestine (SI) LP and BM were also clonally related.

We established clonal trees to picture clonal relatedness in the NP-specific memory B cells and long lived plasma cells. We found that the memory B cells and long lived plasma cells that resulted after oral priming were clonally poorly related as determined by a global gene analysis using NGS of sorted IgA+ cells from different tissues. This was a surprising finding and suggested that memory B cells had, in fact, left the GC at an earlier time point than the plasma cells or was derived from a uniquely different inductive site. But upon reactivation of memory B cells it was observed that NP-specific memory B cells in PP and plasma cells in the LP exhibited strong clonal relationships, arguing that reactivation of memory B cells initiated a second round of strong selection and maturation into plasma cells. These expanding

memory B cells in PP and the IgA plasma cells in the gut LP were closely related. It appears rational that memory B cells following oral priming should have a broader, less mutated, repertoire of IgM^+ and IgA^+ cells and that upon re-exposure to antigen the memory B cells undergo somatic mutations to acquire a less broad more effective protective population of high quality IgA antibody producing plasma cells in the gut

4.10 Memory B cells from oral immunization

NP-specific memory B cells were extremely rare at 1 year after oral immunizations, numbering somewhere in the ratio 1:100,000 B cells. Yet a booster dose at 1 year after the mice were orally primed resulted in a rapid increase in antigen-specific IgA antibody forming cells (AFCs) in the SI LP. From 1:100,000, the NP-specific B cells made up to 15% of all IgA plasma cells in the SI LP after 1 week following the oral booster immunizations. This increase was also seen in IgA and IgG AFCs in the spleen and BM, as well as an increase in serum NP-specific antibodies. Most interestingly, though, was the observation that NP-specific IgA memory B cells carried more mutations than was found in the long-lived plasma cells, suggesting that they were clonally unrelated. However, NP-binding IgA sequences shared clonal origin irrespective of if they were isolated from (SP, BM or SI LP) arguing for a highly effective clonal selection process following a booster immunization. Thus, it can be concluded that oral priming immunizations effectively promoted the development of antigen specific memory B cells at mucosal and systemic sites.

Next we analyzed if systemic immunizations could stimulate a gut mucosal IgA response or not. The route of immunization has been suggested to be critical for a strong gut IgA response. To this end we undertook to i.p prime mice, which was then followed by an oral boost with NP-CT to stimulate both an anti-NP and anti-CT IgA response in the SI LP and in serum. However, we failed to observe any gut IgA AFC response after a p.o boost to i.p primed mice. By contrast, an i.p. boost to p.o. primed mice was effective at eliciting a gut IgA AFS response, comparable to those seen after a p.o boost to p-o primed mice. Noteworthy, oral and systemic priming with NP-CT

were similarly effective at promoting serum anti-NP and anti-CT IgG responses. Hence it can be concluded that p.o immunizations are much more effective than systemic immunizations at generating memory B cells, that provide both a gut mucosal IgA and a systemic IgG antibody response. Only oral priming imprinted gut homing properties in memory B cells, while systemic priming did not affect the expression of CCR9, required for homing to the SI LP

A unique feature of the PP is the continuous presence on GCs because of the gut intestinal microbiota. IgA memory B cells dominated in the PP after oral immunizations, but at all other sites these cells were predominantly IgM. Hence, in SP and MLN a majority expressed IgM, while 20-40% expressed IgG and very few were IgA memory B cells. Memory GFP^+ B cells in these organs had a resting phenotype (GL7-CD38+) and were distinctly different from activated B cells ($GL7^+CD38^-$). Interestingly, the memory GFP^+ B cells (B2220+, GL7-CD138-) were located to the B cell follicles close to GC in all the organs that we analyzed. We think this may be critical for the effective selection process in the secondary GC when reactivated by a booster immunization. Upon re-activation of memory B cells (with a very low frequency among all B cells in the lymph node or PP ; <0.02%) memory B cells exhibited an exceptional expansion and differentiation upon reactivation. The presence of memory B cells expressing IgA in PP contrasts with previous reports, showing that mostly IgM+ memory B cells should be expected in lymph nodes following immunization [147].

Also oral priming of NP-specific GFP^+ cells resulted in expression of significant levels of the gut homing receptor $\alpha 4\beta 7$ in PP and spleen, while i.p priming did not result in $\alpha 4\beta 7$ expression. When an anti- $\alpha 4\beta 7$ monoclonal antibody (Mabs) was used to block gut homing, we found significantly fewer NP-specific GFP^+ plasma cells in the SI LP after p.o or i.p booster immunizations. This observation confirmed the requirement for $\alpha 4\beta 7$ expression for gut homing of plasma cells to the SI LP. . Both splenic and PP memory B cells expressed the $\alpha 4\beta 7$ homing receptor following oral immunization, but CCR9 was acquired only in the PP upon a booster immunization and not in the spleen. Collectively, these observations

suggested that memory B cells following oral immunizations acquire gut homing receptors and are recirculating cells residing in both systemic lymphoid tissues and the GALT. Upon reactivation they effectively can home back to the gut LP provided the memory B cells enter the PP or, perhaps the MLN, where they acquire CCR9 expression which enables them to home to the gut LP. So mucosal memory B cells require both $\alpha 4\beta 7$ and CCR9 to successfully home to SI LP

Another important observation was that NP-specific GFP^+ memory $B220^+$ cells expanded close or within GC in the PP when responding to recall antigen. Resting NP-specific GFP^+ memory cells ($CD19^+B220^+CD138^-$ B cells) co-express CD73 and CD80 while the PD-L2 marker was downregulated on a majority of responding memory B cells after reactivation. Therefore, we believe CD73 is an excellent marker that identifies resting and activated mucosal memory B cells. We observed that B cells with a memory CD73+ phenotype increases with age in PP, which is supported by the finding of Linder *et al.*, that B cells in GALT progressively accumulate somatic mutations in IgA V region genes [148] [149]. Hence, the PP GC is important for the development of memory B cells following oral immunization and when the GC is disrupted by administration of anti-CD40L Mab during an ongoing immune response, a significant reduction of memory B cells was observed.

Paper IV

4.11 Inductive sites (PP) of Germ free mice appears normal while the effector site (LP) exhibit poor IgA plasma cell response

It is well known that the microbiota is important for the gut microenvironment and critical for allowing guts IgA responses to TD and TI antigens [150] [151] [152]. Previously, we showed that $CD40^{-/-}$ mice had near normal levels of IgA^+ plasma cells in the gut, despite no GCs and that they

did not respond to oral immunizations with TD antigens [140]. The hypothesis is that the microbiota is critical for the performance of the IgA inductive site in the PPs [153] . But, it has been incompletely investigated and it could equally well be that the effector site (SI LP) in the absence of the microbiota does not support and maintain a plasma cells response. For this study we used germ free (GF) mice to better understand the impact of the commensal flora on IgA plasma cell responses. We investigated both the inductive (PP) and the effector sites (LP). We could confirm previous observations of a near complete lack of IgA plasma cells in the LP [154]. Unexpectedly, however, we found the PP compartment exhibited normal frequencies and sizes of GC as well as an unperturbed FDC network (CD35), normal AID expression and IgA B cell frequencies mimicking what can be observed in conventionally reared mice. This drop in LP IgA plasma cells was detectable as 90% reduction in IgA AFC in GF compared to normal mice. Serum IgA levels were also reduced whereas serum IgG and IgM were not significantly altered. Using the adoptive transfer model with B1-8hi NP-specific IgH knock-in GFP^+ B cells we found intact GC formations and strong GFP^+ B cell responses in the PPs following oral NP-CT immunizations [58]. Thus it appeared that the PP inductive sites were unperturbed and functionally normal despite the lack of microbiota.

4.12 IgA CSR environment appears normal in GF mice

Next we investigated if we could observe IgA CSR in the PPs and so we analyzed if the GF mice expressed normal levels of genes encoding factors that are required for IgA CSR. To this end we analyzed by qPCR a set of genes known to influence IgA production. However, we found in GF mice that PPs expressed normal levels of most of the genes, including genes encoding BAFF, APRIL, IL-5, IL-6 and IL-10. The exception was mRNA expression of the Nod2 encoding gene, which showed an upregulated expression and the gene encoding iNOS gene which was down modulated compared to that found in conventional mice. This is in line with previous observations showing that iNOS-/- mice have lower levels of IgA producing cells in the gut LP [155]. Thus, our analysis of the PP-inductive site in GF and conventional mice revealed no major alterations in the expression levels of

genes directly or indirectly involved in IgA CSR or IgA B cell responses. Moreover, the level of AID mRNA expression in PPs did not differ from that in conventional mice. Most importantly direct evidence for IgA CSR was obtained from PP from GF mice as we could detect unaltered levels of germline α-transcripts and post-switch α-circle transcripts,. These findings indicated that in GF mice the inductive site for IgA responses appears normal. Thus, food antigens or endogenous non-bacterial antigens could clearly activate IgA B cell responses in the PP of GF mice.

4.13 The gene expression profile of the LP effector site is altered in GF mice

Because we did not find a crippled IgA B cell inductive site in the PPs of GF mice we extended our analysis to include whether B cells left the inductive site to travel to the effector site. We used a technique by which we cannulated the thoracic duct lymph in GF and conventional mice and followed the drainage of B cells and plasma blasts coming from these. We observed no major change in the composition or numbers of activated B cells or plasma blasts that left the PP inductive site for the LP effector site in GF mice. Hence, similar frequencies of PP B cells were observed in GF and conventionally reared mice.

Whereas the PP-inductive site and the egress of activated B cells and plasma blasts in the draining lymph were unperturbed in GF mice we focused our attention to the effector site in the LP of the SI. Therefore, the next step was to investigate whether we had normal conditions at the effector site to host the gut homing B cells and plasma blasts. To identify possible signalling pathways involved in such a process we screened SI LP biopsies for a set of genes known to be involved in plasma cell differentiation and survival using a qRT-PCR. We found that the PCR- array revealed that genes encoding iNOS, IL-5, IL-6, and IL-10 were clearly reduced as well as genes encoding bacterial recognition, such as the Nod1, Nod2, TLR4 and TLR9 encoding genes. Notably mRNA expression of BAFF and APRIL mRNA genes were not significantly different to conventionally reared mice. These finding lead

us to conclude that the microenvironment in the gut LP effector site appeared dramatically altered from that observed in conventionally reared mice.

4.14 The effector site can be restored by oral administration of CT

Oral administration of CT adjuvant could potentially restore the defect microenvironment seen in the LP effector site in GF mice. Following oral CT we observed a strong increase in total IgA AFC in the LP. This increase was only partially explained by the presence of CT-specific IgA AFC, and suggested a pharmacological effect of CT on the microenvironment of the LP effector site in GF mice. Immune histochemical analysis of gut tissue sections from CT-treated GF mice or re- colonised GF mice showed comparable levels of IgA plasma cells in the LP. Also, the levels of CD4 T cells were restored compared to that observed in conventionally reared mice.

Thus, the change in CD4 T cells might play a role in restoring IgA plasma cell levels in the LP of GF mice. Also there was an expansion of CD11c population in CT-treated GF mice as also seen in re-colonized GF mice.
Retinoic acid (RA) has been shown to have an important role in imprinting gut tropism of IgA committed B-cells [77, 78, 156]. In the GALT DCs produce RA, which is an important element for the expression of gut homing receptors $\alpha 4\beta 7$ and CCR9 on activated B and T-cells? It is possible that a lack of RA in the DC population in GF mice can be restored or compensated for by treatment with CT adjuvant. However, we did not find a reduction in CCR9-expression in PP B cells, hence this notion is unlikely. Rather, the DC at the effector site could be lacking RA, but whether this can account for 90% reduction of gut IgA plasma cells in GF mice waits to be further investigated.

5 CONCLUSION

Paper 1: Following oral immunization NP-specific GFP+ B cells enter the proximal region of PP and starts to proliferate and later clonally related PP B cells are evenly distributed in the Gut associated lymphoid tissues (GALT). This occurs in the presence of antigen and by CD40-dependent fashion. Migration of activated cells occurs via blood and lymph. Later stages they are seen in the small intestine lamina propria (SI LP) as plasma cells.

Paper 2: In an ongoing immune response to an oral antigen activated NP-specific GFP+ B cells can take part in antigen sampling. They are seen either in close proximity or interacting with the basal pockets of M cells carrying antigen. These cells can move towards germinal center to present antigen and presumably for an effective germinal center (GC) response.

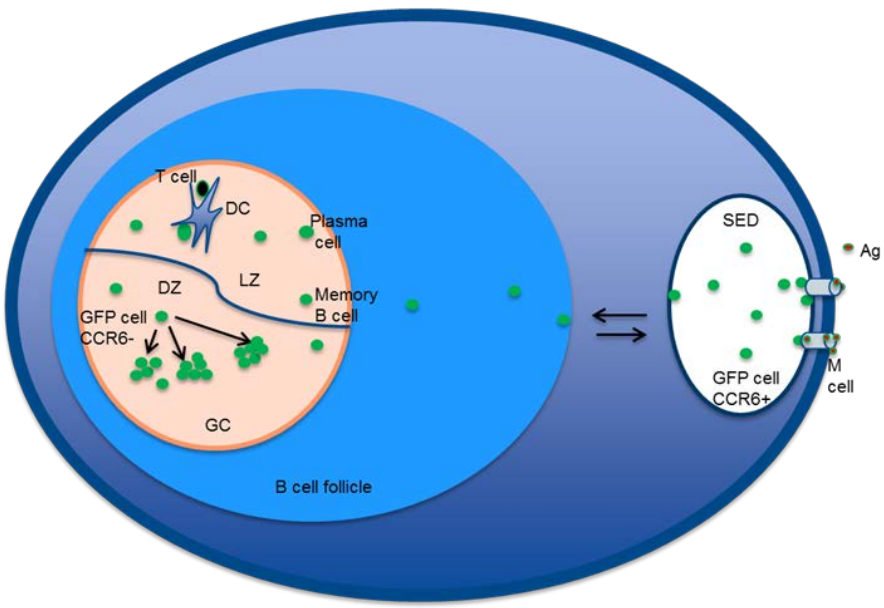

Figure 10. Antigen sampling by activated B cells from FAE region. GC- germinal center, SED-sub epithelial dome, LZ-light zone, DZ-dark light, DC-dendritic cell, M cells-microfold cells, Ag-antigen.

Paper 3: Following immunization we hypothesis the plasticity of GALT allows the generation of memory B cells and long-lived plasma cells (LLPC), but involving temporarily separate processes in the GC or even anatomically separate GC, leading to lower-affinity maturation in memory B cells. This allows for a broader, less mutated, repertoire of both IgM^+ and IgA^+ mucosal memory B cells following oral immunization than that ultimately used for host protection. On re-exposure to antigen, gut memory B cell clones have migrated to multiple sites in the GALT and the response can be synchronized and clones strongly selected, leading to clonal relatedness in the GALT and SI LP with only high-affinity and strongly mutated clones being represented.

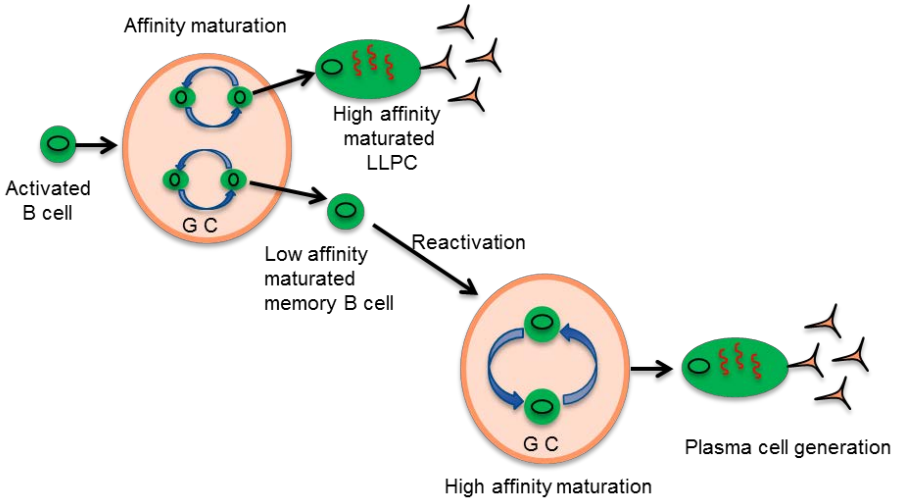

Figure 11. Activated B cell undergoing two separate pathways for becoming high affinity matured LLPC and low affinity matured memory B cells, on reactivation, these memory B cells can undergo further maturation and arises to clonally relate synchronized response in GALT.

Paper IV. The effector site lamina propria (LP) of GF mice has low levels of secretory IgA (SIgA), while colonization or oral immunization with CT restores IgA plasma cell responses. Hence we propose GF mice host a defective effector site and that oral CT or bacterial colonization provide compensation for lack of environmental cues necessary for plasma cell maintenance or survival in LP

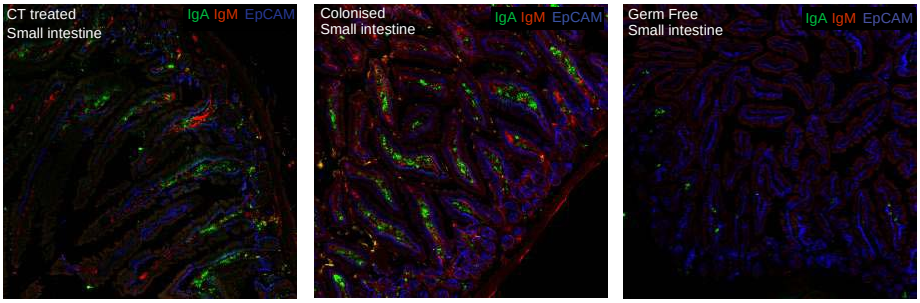

Figure 12. Oral immunization of GF mice with CT or bacterial colonization restore plasma cell in the gut LP, IgA- green, IgM-red, EpCAM-blue

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