Enhancing mucosal B cell responses with all-*trans* retinoic acid

Verstärkung der humoralen Immunantwort an mukosalen Oberflächen mittels all-*trans* Retinsäure

Doctoral thesis for a medical doctoral degree
at the Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg,
Section Infection and Immunity

submitted by

Michael Christian Reinhart

from Würzburg

Würzburg, 2022

Submitted on: ……

Office stamp

Members of the Thesis Committee:

Chairperson:	**Prof. Dr. Nicolas Schlegel**
Primary Supervisor:	**Prof. Dr. Manfred Lutz**
Supervisor (Second):	**PD Dr. Götz Ulrich Grigoleit**
Supervisor (Third):	**Prof. Dr. Ulrich von Andrian**

Date of Public Defence: **……**

Date of Receipt of Certificates: **……**

Summary

Diarrheal diseases are a major cause of death in developing countries. Vaccinating against the causative pathogens could reduce mortality and morbidity in these countries. Unfortunately, only for some of the most common enteral pathogens are vaccines available. Some of these available vaccines have limitations in terms of effectiveness and duration of protection. There is therefore an urgent need to develop new vaccine strategies that can generate protection against enteral pathogens.

The presence of all-*trans* retinoic acid (ATRA) during lymphocyte maturation is known to imprint a phenotype on lymphocytes that enables them to home to the intestines. Additionally, ATRA is known to play a role in B cell class switch to IgA, which is the dominant immunoglobulin in the intestines.

The aim of this study was therefore to investigate whether the addition of all-*trans* retinoic acid (ATRA) or a retinoic acid receptor agonist (AM80) to a parenteral vaccination could provide protection at the intestinal mucosa against enteric pathogens.

C57BL/6 mice received s.c. priming and boosting immunizations with Ovalbumin followed by several s.c. injections with either ATRA, AM80 or the respective solvent as control substance. Feces, serum, saliva and vaginal lavage samples were collected and analyzed by ELISA for detection and relative quantification of antigen-specific antibodies. B cell populations in the draining lymph nodes were investigated after immunization using flow-cytometry. Antigen-specific antibodies producing cells were visualized in the small intestine of vaccinated animals using two-photon microscopy.

Animals that were vaccinated and were exposed to AM80, and to a lesser extent ATRA exposed mice, had higher serum, fecal, saliva and vaginal lavage antigen-specific IgA titers when compared to animals that were vaccinated but did not receive ATRA/AM80. Antigen-specific IgG titers were not altered in any of the investigated tissues. In the draining lymph nodes, IgA^+ and IgG^+ B cells were increased after vaccination and AM80 exposure at several time points within 14 days after vaccination. Antigen-specific IgA^+ cells were found in the small intestine of immunized and AM80-exposed but not control substance-exposed mice.

These results suggest that the addition of ATRA or AM80 to parenteral vaccine formulations increases the abundance of antigen-specific antibodies at mucosal surfaces,

and therefore have the potential to generate protective antibody titers at those mucosal surfaces.

Zusammenfassung

In Entwicklungsländern sind Durchfallerkrankungen noch heute eine Haupttodesursache. Impfungen gegen die Erreger dieser Erkrankungen könnten dort Morbidität und Mortalität reduzieren. Jedoch sind nicht einmal für alle der häufigsten Erreger von Durchfallerkrankungen weltweit Impfstoffe verfügbar. Zudem gibt es bei einigen verfügbaren Impfstoffen Einschränkungen bezüglich Dauer des Impfschutzes und Effektivität der Impfung.

Es ist bekannt, dass die Anwesenheit von all-*trans* Retinsäure (auch Tretinoin oder ATRA) während der Lymphozytenreifung einen intestinalen Phänotyp vermittelt. Weiterhin spielt ATRA eine Rolle beim Ig-Klassenwechsel der B-Lymphozyten zu IgA, dem dominanten Immunglobulin im Gastrointestinaltrakt.

Ziel dieser Arbeit ist es, herauszufinden ob der Zusatz von ATRA oder eines Agonisten des Retinsäurerezeptors (AM80) zu parenteral applizierten Impfungen einen Impfschutz gegen Erreger im Verdauungstrakt aufbauen kann.

C57BL/6 Mäuse erhielten eine subkutane Impfung mit Ovalbumin sowie zeitlich versetzt eine subkutane Booster-Impfung. Außerdem wurden mehrere Injektionen mit ATRA oder AM80 oder den jeweiligen Lösungsmitteln als Kontrollsubstanzen durchgeführt. Kot-, Serum-, Speichelproben und Proben vaginaler Spülung wurden mittels ELISA auf Antigen-spezifische Antikörper untersucht. Außerdem wurden die die Impfstelle drainierenden Lymphknoten hinsichtlich Ihrer B-Lymphozyten Populationen mittels Durchflusszytometrie untersucht. Weiterhin wurden Zellen, die Impfantigen-spezifische Antikörper produzieren, im Dünndarm geimpfter Tiere mittels Zwei-Photonen-Mikroskopie dargestellt.

Es konnte gezeigt werden, dass die Tiere, die neben dem Impfantigen auch AM80 oder ATRA subkutan erhielten, sowohl in Kot-, Serum- und Speichelproben, als auch in Proben vaginaler Spülung höhere Impfantigen-spezifische IgA-Titer aufwiesen als Kontrolltiere, welche anstatt AM80 oder ATRA nur das jeweilige Lösungsmittel erhielten. Hingegen wurde zwischen den Experimentalgruppen kein Unterschied in den Impfantigen-spezifischen IgG-Titern festgestellt. In den die Impfstelle drainierenden Lymphknoten konnte ein relativer und absoluter Anstieg der IgA^+ und IgG^+ B-Lymphozyten nach Impfung und subkutanen Gaben von AM80 im Vergleich zu

Kontrolltieren festgestellt werden. Im Dünndarm geimpfter Tiere, die auch subkutan AM80 erhielten, konnten Impfantigen-spezifische IgA^+ Zellen festgestellt werden.

Die hier vorgestellten Ergebnisse weisen darauf hin, dass der Zusatz von AM80 oder ATRA zu parenteral applizierten Impfungen zu erhöhten Titern von Impfantigen-spezifischem IgA auf mukosalen Oberflächen führt. Diese Antikörper können potentiell eine protektive Funktion im Rahmen von Durchfallerkrankungen oder anderen Erkrankungen mit mukosalem Infektionsfokus ausüben.

Table of contents

1.Introduction

1.1. Global burden and prevention of diarrheal diseases

Diarrheal diseases are a major cause for child deaths worldwide [1]. Pathogens that cause diarrhea include viruses, bacteria and parasites [2]. The Global Burden of Diseases, Injuries, and Risk Factors Study 2015 (GBD 2015) found that taken together, Rotavirus, *Cryptosporidium* spp. and *Shigella* spp. accounted for more than 50 % of diarrhea-associated deaths in children below the age of 5, followed by Adenovirus, *Salmonella*, *Campylobacter*, *Vibrio cholera*, ETEC and others [3]. *Shigella* spp. and *Vibrio cholerae* were leading causes of death in age groups 5 - 14 years and 15 - 99 years respectively [3]. However, there has been debate over the methods and models used by several similar studies as results differed substantially in some categories [4-6]. For example, the FERG study [7] estimates that in children < 5 years, more deaths are attributable to ETEC than to *Shigella* spp.

Severe differences in the abundance of different pathogens are also found when different geographical regions are compared [4, 8]. Regional differences and the uncertainty, which published estimates are the most accurate have to be taken into account when deciding on developing vaccines for specific pathogens [4].

A worldwide effort to decrease child mortality by diarrheal diseases is presently being taken [9] and much has been achieved in the last years: between 1990 and 2017 the mortality rate from diarrheal diseases decreased by 70 % in children below the age of 5 [10]. Still, it is estimated that in 2017 over 500 000 children below the age of 5 years have died from diarrhea [10]. The reduction in mortality has been attributed to the implementation of prevention and treatment programs [1, 11]. Educational programs have been shown to increase breastfeeding [12] as a preventive measure for diarrhea incidence and mortality [13]. Similarly, the implementation of sanitation, hygiene and water improvement programs have been able to reduce the incidence of childhood diarrheal mortality [14, 15]. Oral rehydration solution has been recommended by the WHO since the 1970s to treat diarrhea. Recent meta-data suggest that oral rehydration solution can

prevent up to 93 % of deaths caused by diarrheal diseases [16]. More recently, the addition of zinc treatment to oral rehydration solution has been recommended due to its beneficial effects as an additive [17, 18]. Oral rehydration solution cannot, however, mitigate symptoms of diarrhea or reduce morbidity [19].

There is growing evidence that diarrheal diseases in childhood cannot only lead to death but can also be associated with long-term morbidity such as stunted growth, organic gastrointestinal disorders and neurologic dysfunction [20, 21]. Potential consequences of such long-term morbidities are an increased social economic burden and impaired long-term intellectual function of the individual [22-24]. Therefore, a reduction in global diarrheal burden could also improve financial stability and lead to better school- and intellectual performance.

Large scale implementation of prevention programs however is strenuous due to infrastructural and funding difficulties. Therefore progress in disease prevention has been slow in recent decades [1]. The development of innovative vaccination strategies could potentially break this trend and reduce mortality and morbidity, as well as minimizing secondary effects associated with diseases. Hence, a major effort is being taken to develop vaccines targeting mucosal surfaces, in particular the gastrointestinal tract [25].

1.2. Mucosal surfaces and the mucosal immune system

The mucosal immune system regards to the branch of the immune system associated with mucosal surfaces. This includes, but is not limited to, the gastrointestinal tract, the respiratory tract and the urogenital tract. The mucosal immune system can be partitioned into inductive sites, where antigen uptake occurs, leading to activation of lymphocytes and into effector sites where activated immune cells then take action against pathogens [26]. In the small intestine, inductive sites are considered to be Peyer's Patches, mesenteric lymph nodes and isolated lymphoid follicles, whereas the effector sites are considered to be the lamina propria and the epithelium of the small intestine [27]. Secretory Immunoglobulin A has been identified to be the predominant mediator of the adaptive immune system at most mucosal surfaces [28]. Trafficking of lymphocytes from

inductive- to effector sites will be covered in more detail in the following chapter.

1.3. Lymphocyte homing to the small intestine mucosa and other mucosal surfaces

Effective immune surveillance relies on constant recirculation of lymphocytes through blood, lymphoid and non-lymphoid organs [29, 30]. To leave circulation, lymphocytes have to go through a multi-step adhesion cascade that eventually results in crossing the endothelium followed by extravasation of the cells [31]. Following antigen priming and activation, lymphocytes express a specific set of surface molecules that enables them to specifically home to specific tissues [32]. This tissue homing imprinting is dependent on the local microenvironment and the type of cells interacting during lymphocyte priming. A profound understanding of these homing mechanisms is fundamental for the development of new vaccination strategies that induce lymphocyte homing to e.g. the intestinal lamina propria and thereby having the potential to confer protection to intestinal surfaces.

In vitro co-culture of T cells and dendritic cells (DCs) from Peyer's patches has been shown to result in the upregulation of the integrin $\alpha_4\beta_7$ on T cells [33]. This observation contrasts to those of co-cultures using DCs originating from spleen or peripheral lymph nodes, where no significant up-regulation of $\alpha_4\beta_7$ has been observed. $\alpha_4\beta_7$ binds to the mucosal vascular addressin cell adhesion molecule (MAdCAM), which is expressed in endothelial cells of mucosal tissues, particularly in the small intestine [34]. Moreover, T cells co-cultured with Peyer's patch DCs are significantly more responsive to CCL25, the ligand for the chemokine receptor CCR9, whose interactions are known to play an important role in migration of lymphocytes to the small intestine [35]. When transferred to naïve recipients, these *in vitro* cultured T cells preferentially home to the small intestine [33], in contrast to T cells activated in the presence of splenic DCs. Interestingly, CCL25 expression in the mouse small intestine decreases from proximal to distal, as does CCR9 dependent T cell accumulation [36]. In humans, it has been shown that CCL25 expression is highly specific for jejunum and ileum, but not for other parts of the gastro-intestinal tract [37].

The imprinting of this intestinal homing phenotype on T cells has later been shown to be dependent on all-*trans* retinoic acid (ATRA), which is produced by Peyer's patch DCs, but not splenic DCs [38]. In fact, co-culture of T cells with splenic DCs and exposure to physiological levels of ATRA results in the up-regulation of the gut-homing receptors $\alpha_4\beta_7$ and CCR9 on T cells, similarly to what is seen when T cells are co-cultured with Peyer's patch DCs [38]. The up-regulation of these gut homing receptors by Peyer's patch DCs can be inhibited by a retinoic acid receptor (RAR) antagonist [38]. Together, these observations demonstrated that ATRA is both necessary and sufficient to induce gut-tropism on T cells [38]. These effects have been shown to be mediated by the retinoic acid receptor alpha (RARα), and have been reproduced by biological ATRA analogs, such as tamibarotene (also known as AM80) [38].
Similar to T cells, upregulation of the gut-homing receptors $\alpha_4\beta_7$ and CCR9 have also been observed on B cells activated in the presence of ATRA [39]. These cells also retain a preferential homing capacity to the small intestine when transferred to naïve recipients [39]. In practice, mice that were s.c. immunized with dead *Salmonella* in the presence of external ATRA were shown to have a reduced bacterial burden in Peyer's patches after oral *Salmonella* inoculation [40].

For other mucosal surfaces, lymphocyte homing mechanisms are much less well studied and understood. For the female genital tract, it has been shown in humans that asymptomatic HSV-2 infection is associated with an increased systemic $\alpha_4\beta_7$ expression on T cells and a positive correlation has been found between the number of $\alpha_4\beta_7^+$ blood T cells and activated $CD4^+$ T cells in the cervix [41]. Interestingly, individuals with HSV-2 infection are more susceptible to HIV infection. And in this context, an increased frequency of $\alpha_4\beta_7$ expressing T cells in a rhesus macaque *in vivo* model has been shown to correlate with simian-human immunodeficiency virus (SHIV) replication in HSV-2 infected tissues [42]. In line with these observations, an adoptive transfer mouse-model showed that the absence of β_7 or E-selectin on T cells reduces systemic cell recruitment to the female genital tract in infection [43]. Moreover, monkeys that were treated with anti $\alpha_4\beta_7$-mAb were less susceptible to simian immunodeficiency virus (SIV) after vaginal virus challenge [44].

However, one recent publication has shown that interference with $\alpha_4\beta_1$ rather than $\alpha_4\beta_7$ can impair CD4-T cell recruitment to the uterus in a mouse model of *Chlamydia* infection [45]. This is in line with results showing that VCAM-1, the ligand for $\alpha_4\beta_1$ is expressed in naïve uterine blood vessels and its expression has been found enhanced in uterus infected with *Chlamydia*. However, the ligand for $\alpha_4\beta_7$, MAdCAM, has not been detected [46, 47]. Confirming the upregulation of $\alpha_4\beta_1$ on $CD4^+$ T cells in women with bacterial vaginosis, systemic CD11c expression on $CD8^+$ T cells has also been proposed as a marker for mucosal immunity in women [48].

In regard to the salivary glands, it has been shown that both integrin $\alpha_4\beta_1$ and the chemokine receptor CXCR3 are crucial, at least in uninfected mice, for homing of $CD8^+$ T cells to these tissues [49, 50].

1.4. Compartmentalization of the mucosal immune system

For a long time, the mucosal immune system has been seen as a homogenous unity. More recently, however, it became apparent that mucosal defense relies on different compartments and regions (reviewed in [51]). In order to design new vaccination strategies, it is crucial to understand this compartmentalization and the immune responses that can be elicited from a mucosal vaccination at different mucosal surfaces.

Table 1: Mucosal IgA-antibody response in mucosal tissues (left column) after different mucosal immunization routes (top row) in humans and non-human primates; Adapted from [52], and extended with information from indicated references

	Immunization route				
Expression site	nasal	sublingual	oral	rectal	vaginal
upper respiratory	+++	+++	-	-	-
small intestine	-	+++	+++	-	-
colon	-	?	+	++	+
rectum	-	?	(+)	+++	-
blood	+++	+++	+	++	+
female reproductive tract **[51]**	++	+++	-	-	+++
saliva	+ [53, 54]	+ [55]	++ [54, 56]	+ [57]	?

Common s.c. or i.m. immunization protocols perform poorly in inducing protection at mucosal surfaces. Therefore, many mucosal immunization routes and their effect on antibody production at other mucosal surfaces have been studied in recent years. Results are summarized in Table 1.

As shown in Table 1, the strongest immune response is usually found at the original site of vaccination. Nonetheless, some mucosal vaccinations exhibit an antibody response at a distant mucosal tissue, suggesting similar homing cues between the different mucosae. Moreover, all mucosal immunization routes can also exert a systemic antibody response ("blood" in Table 1), albeit in different strength depending on the vaccination site.

Notably, nasal (and tonsillar) vaccination has been shown to be able to induce an antibody response in the female genital tract [58]. Nasal vaccination against *Chlamydia trachomatis* has also been shown to be protective upon intra-uterine challenge with the same pathogen. This has been attributed to a formation of protective memory T cells [59]. As opposed to i.n. immunization, oral vaccination is not capable of inducing antibody response at the level of the female reproductive tract.
It is known that immune responses to oral antigens can lead to the presence of antigen-specific antibodies in the saliva [56, 60]. In the case of antibodies of the IgA-isotype, this has been attributed to intestinal B cells being able to home to the salivary glands [61]. However, also tonsillar and nasal antigen delivery is capable of inducing salivary antigen-specific IgA antibodies [62, 63].
The oral vaccine delivery route has been studied extensively to enable protection of the intestinal tract against enteric pathogens. To date, vaccines against enteropathogens such as *Vibrio cholera* or Rotavirus are licensed and being used in clinics. However, although initial results from animal models were promising, many studies in humans have not been able to reproduce pre-clinical findings [25]. More recently, the sublingual vaccine delivery route and its protective effect on the gastrointestinal tract has received more interest. This immunization route has been suggested to induce small intestinal IgA responses equally to what is seen for oral vaccine delivery [51].

1.5. Protection mechanisms against pathogens on mucosal surfaces

Table 2 lists vaccines targeting mucosal tissues that are approved in Germany and recommended by the STIKO at Robert-Koch-Institut (RKI). Some of the listed vaccinations are reserved for specific indications (e.g. vaccination against *S. typhi* for travelers), whereas for some other listed vaccines a general recommendation has been declared (e.g. vaccination against Rotavirus for infants).

Table 2: representative selection of vaccines recommended by the STIKO at RKI in Germany (8/2020) that either have a mucosal application site or target mucosal surfaces such as the gastrointestinal tract

Target	**Composition**	**Route of administration**	**Standard administration protocol**	**Trade name**
V. cholerae	Killed whole-cell *V. cholerae* O1 and recombinant CT B subunit	oral	Two doses in an interval of 1-6 weeks	Dukoral®
Human papilloma virus 16 and 18	Recombinant L1-protein of HPV 16 and HPV 18	i.m.	Two doses in an interval of 5 months	Cervarix®
	Recombinant L1-Protein of HPV 6, 11, 16, 18, 31, 33, 45, 52, 58	i.m.	Two doses in an interval of 5 months	Gardasil®
Influenza	Four heat-inactivated influenza strains	i.m.	One dose; new vaccine every year	Infusplit Tetra®
	Four live-attenuated influenza strains	i.n.	One dose; new vaccine every year	Fluenz® Tetra
Pneumococcal disease	Adsorbed 13-valent conjugate vaccine	i.m.	one or several doses, depending on age	Prevenar 13®

	23-valent polysaccharide vaccine	i.m. or s.c.	Single dose	Pneumovax® 23
Poliovirus	inactivated poliovirus type 1, 2, 3	i.m.	4 doses in different intervals	IPV Merieux®
Rotavirus	Live attenuated Rotavirus RIX4414	oral	Two doses in an interval of < 1 month	Rotarix®
S. typhi	Vi-Polysaccharide from *Salmonella typhi* (Ty 2)	i.m.	Single dose	Typhim Vi®

Even though there has been intensive research in the field of mucosal vaccines for decades, only few vaccines are available in Germany and internationally [64] that can be administered through a mucosal route or that target mucosal surfaces (esp. the intestines). Especially for some of the pathogens that very frequently cause diarrhea and go along with high mortality such as *Cryptosporidium* spp., *Shigella* or *Campylobacter*, currently no effective vaccination is available.

One of the reasons for the development of mucosal vaccines being slow is that different pathogens require different defense mechanisms for protection. A selection of approved mucosal vaccines will be discussed here to point out defense mechanisms against enteric pathogens, focusing on humoral protection mechanisms.

Cholera is a diarrheal disease that is characterized by rapid dehydration affecting millions of people, mostly in developing countries [65]. It is transmitted in a fecal-oral way through fecal contaminations in drinking water and food. Out of over 200 serogroups of *V. cholerae*, only the groups O1 and O139 can cause cholera disease as they are capable of producing a toxin. This cholera toxin (CT) consists of one A subunit that is associated with five B subunits. After ingestion and passage of the stomach, *Vibrio cholera* colonizes the small intestine and secretes CT. The B-subunits of CT bind to small intestinal epithelial cells followed by translocation of the A subunit into the epithelial cell. This A subunit then activates a G protein through blockade of its GTPase activity. As a result,

intracellular levels of the second messenger cAMP rise, followed by changes in the activity of ion channels at the cell membrane. This results in secretory diarrhea, which is characterized by a loss of electrolytes and water to the small intestinal lumen [66].

Many trials with oral *V. cholera* vaccinations have been performed. Oral *V. cholera* vaccination has been shown to result in a serum antibody (mainly IgG) response and an intestinal IgA response. Antibodies of the IgA-isotype have also been found in the saliva of vaccinated individuals [67]. Antigen specific T cells with gut homing characteristics have been found in older children and adults after oral cholera vaccination [68]. The oral vaccination has been shown to be efficient with a protective efficacy of 50 - 67 % over 2 - 3 years, however declining to baseline after 3 - 4 years [69, 70]. When comparing serum antibody titers after cholera vaccinations in children from Sweden and Nicaragua, Swedish children have higher titers after oral cholera vaccination [71]. This observation is likely associated with factors known to affect oral vaccinations, such as diet, hygienic conditions and existence of recurrent intestinal infections.
The mechanism of protection conferred by the vaccine is subject of discussion. Most authors attribute the protective effect of the oral cholera vaccine to the high anti-CT and antibacterial IgA titers in the small intestine given the non-invasive and more superficial mechanism of infection for *Vibrio cholerae* [52]. For others, however, vibriocidal serum antibodies are the major effector in a series of factors including mucosal IgA [72].

Other than cholera, typhoid fever caused by *Salmonella typhi* is not only mediated by a toxin, but also by the replication of the bacteria itself [73]. After ingestion, the bacteria enter and replicate in intestinal M cells [74]. Subsequently, bacteria rapidly disseminate systemically and cause the typical clinical symptoms like fever, influenza-like symptoms, nausea and others [75]. Due to the rapid systemic involvement, it has been proposed that common parenteral vaccination can be effective in stopping systemic dissemination and thereby preventing clinical symptoms [52]. Indeed, the Vi Polysaccharide vaccine (Table 2) has a protective efficacy of 55 % in the first 2.5 to 3 years after single i.m. injection [76]. Serum anti-Vi IgG antibodies have been identified as correlate of protection [77]. These likely different mechanisms of protection resulting from

vaccinations against cholera and typhoid fever are in line with the different mechanisms of infection comparing *Vibrio cholera* and *Salmonella typhi*.
Additionally, the internationally (though not in Germany) approved oral Ty21a live vaccine also shows a similar three-year cumulative efficacy of 53 % [76]. Whereas the protective mechanism of this vaccine is unknown, its efficacy suggests that mechanisms other than serum IgG antibodies can play a role in protection against *Salmonella typhi* infections. All in all, there is a lack of conclusive studies as to which subset of the immune system plays the leading role in conferring immunity in infections with *Salmonella typhi*.

Rotavirus is a double-stranded RNA virus of the family Reoviridae. Transmission occurs through the fecal-oral route and more than 50 % of cases occur in children below 1.5 years of age [78]. The RNA encodes for 6 structural and 5 to 6 non-structural proteins. The outermost layer of the virus consists of the structural proteins VP4 and VP7. After infection, neutralizing antibodies in the host against VP4 and VP7 can be found and therefore are considered crucial for host immunity against rotavirus [79]. The understanding of the pathogenesis of rotavirus infection is limited. Rotavirus mainly infects epithelial cells at the top of the villi in the small intestine, the relevance of extra-intestinal spread is subject of ongoing debates [79]. Correlates of protection are not well established. Even though for the currently licensed rotavirus vaccines, serum anti-rotavirus IgA level has been used to measure immunogenicity [80], this marker does not perform well in all settings [81].
Several live attenuated oral vaccines have been licensed and proven to perform well in developed countries. Rotarix® for example has a 1-year protective efficacy of 87.1 % against rotavirus gastroenteritis and 100 % against hospital admission due to rotavirus infection in Europe [82]. However, the same vaccine has only shown a 1-year protective efficacy of 30.2 % in a trial in Malawi and South Africa [83].

This differing immunogenicity of live and killed orally, but not i.m. or s.c. administered vaccines between developing and developed countries has been named “tropical barrier” and has been described for several vaccines including vaccines against Rotavirus, Polio [84] and Cholera [52]. Multiple causes for that differing responsiveness to vaccines have been proposed including maternal antibody transfer, training of the innate immune

system, malnutrition, difference in gut microbiota and ongoing infections or diarrhea [84]. The cause or the interplay of causes that lead to a diminished responsiveness in developing countries could also depend on compounds of the vaccine.
Overall, as most mortality from diarrheal diseases prevails in developing countries, there is an urgent need to develop new vaccination strategies that provide protection at mucosal sites without relying on the oral vaccine administration route.

1.6. IgA class switch, transport and functions of secretory immunoglobulins

Immunoglobulins are part of the adaptive immune system and are produced by B cells. Five heavy chain isotypes exist in humans. Whereas IgD and IgE isotypes appear in external secretions only in very low concentrations and their function there is largely unknown, IgA, IgG and IgM isotypes seem to be more relevant in external secretions [85]. Out of these three, IgM levels are lowest at mucosal surfaces in humans [86]. Whereas in the genital tract and in bronchioalveolar lavage IgG- are generally higher than IgA-levels, in the gastrointestinal tract and saliva, IgA levels exceed IgG levels [85].

Antibodies of the IgG isotype can be transported through the small intestinal epithelial barrier via the neonatal Fc receptor for IgG (FcRn) [86]. This receptor is well known to be crucial for the transport of maternal IgG to the offspring in rodents and humans [87, 88]. The leading mechanism, however, is different between these two species. In rodents, transport of maternal IgG occurs predominantly postnatally through absorption of IgG from maternal milk via FcRn in the proximal small intestine of the pups [89]. In humans, IgG is predominantly transferred prenatally from mother to child through FcRn in the placenta [88].
Whereas in rodents, levels of FcRn as measured via mRNA expression in the intestines decline rapidly after weaning [90], in human fetal and also adult small intestinal samples, FcRn can be detected [91]. Moreover, whereas in adult mouse enterocytes of crypts, no FcRn can be detected via immunohistological staining, strong expression of FcRn is present in apical enterocytes [92]. Another study suggests a role of FcRn in the transfer of IgG across mucosal epithelia also in adult rodents. Here, after i.n. immunization with a HSV-2 antigen, wildtype mice were protected from vaginal challenge with HSV-2 virus, whereas FcRn knockout animals showed a lower protection after challenge [93].

The exact production site of antigen-specific IgG antibodies that can be found in the feces after vaccination [94] is not fully clear. However, it is known that pig small intestinal mucosal explants can produce not only IgA but also IgG antibodies suggesting a local production by B cells [95]. On the other hand, i.v. injected IgG antibodies in newborn calves have been found to be predominantly eliminated through the intestines. Thus, also serum IgG can be transported to the small intestinal lumen [96].

In humans, unlike serum IgA which is mainly monomeric, secretory IgA is predominantly polymeric (mostly dimeric) and linked with the so-called J-chain [97, 98]. In the small intestine, secretory IgA is produced locally and is transported to the lumen via the polymeric immunoglobulin receptor (pIgR). This has been proven in pIgR knockout mice, where no secretion of IgA to the small intestinal lumen has been found [99]. IgM is also transported across mucosal epithelial barriers with the polymeric immunoglobulin receptor [99].

In order for B cells to produce IgA antibodies, they have to undergo class switch. In the small intestine, T cell dependent IgA class switch takes place at inductive sites (Peyer's patches, mesenteric lymph nodes and isolated lymphoid follicles in the lamina propria) [100]. *In vitro* experiments with splenic and Peyer's patch B cells have identified transforming growth factor-β (TGF-β) to be crucial for IgA class switch [101]. Interleukins 2 (IL-2), IL-4, IL-6 and IL-10 were found to have a positive effect on IgA class switch in various *in vitro* settings [102]. Knock out of the TGF-β receptor II in mice *in vivo* leads to decreased serum IgA titers and the lack of an IgA^+ B cell population in Peyer's patches [103] underlining the importance of TGF-β for IgA class switch.
Further *in vitro* data have identified two members of the tumor necrosis factor family, BAFF and APRIL to be able to induce IgA class switch. This class switch has been found to be independent of TGF-β [104]. BAFF and APRIL are widely expressed by various cell types including follicular dendritic cells, monocytes or activated T cells [105]. A subset of $CD4^+$ T cells (Th3 cells) has been identified that secretes TGF-β and can provide help for IgA class switch [106]. However, TGF-β is widely expressed and secreted in the body.

Consequently, there has to be another reason why IgA class switch is so specific for the mucosal immune system. Retinoic acid has previously been shown to induce IgA class switch *in vitro* in synergism with IL-5 or IL-6 upon co-culture with dendritic cells [39]. Additionally, dendritic cells from lymphoid tissue in the intestines, other than dendritic cells from other lymphoid tissues are capable of synthesizing retinoic acid [39]. Therefore, retinoic acid has been proposed to be crucial for the local specificity of IgA class switch in the (intestinal) mucosal immune system [107].

1.7. Hypothesis and aim of the study

As percutaneous immunizations are unable to provide protection at mucosal surfaces and oral immunizations underperform in developing countries, there is an urgent need to develop new vaccination strategies to protect the intestines from pathogens.

We hypothesize that the addition of ATRA/AM80 to common parenteral vaccination strategies can lead to homing of antigen-specific T and B cells specifically to the small intestine, thereby conferring protection against enteric pathogens.

The aims of this project are:

- to quantify long-term mucosal and systemic antigen-specific antibody titers after vaccination with and without ATRA/AM80 exposure
- to investigate the mechanism of antigen-specific IgA induction in the small intestine
- to determine the breadth of other mucosal surfaces influenced by parenteral immunization with ATRA/AM80

The aim of the overall project and all collaborators is to develop a nanoparticle-based vaccine platform that can protect against enteric pathogens and to transfer preclinical findings into clinics.

2.Materials and Methods

2.1. Buffers and reagents

PBS	1:10 dilution of 10x PBS (VWR, Cat. No. 119-068-151) in ddH_2O
cOmplete protease inhibitor solution	one tablet cOmplete protease inhibitor (Sigma, Cat. No. 11873580001) in 50 ml PBS
ELISA wash buffer	20 ml tween-20 (Sigma, Cat. No. P1379-1L) in 5 l PBS
FACS buffer	5 % FBS (Gemini, Cat. No. 100-106) in PBS
FACS blocking solution	1:500 Fc-Block (anti CD16/CD32; Bio X cell, Cat. No. CUS-HB-197-A) + 2 % goat serum (Gibco, Cat. No. 16210064) in FACS Buffer
cRPMI	5 % FBS + 10 mM HEPES buffer (VWR, 25-060-CI) + 50 µM 2-mercaptoethanol (Sigma, Cat. No. M3148-25ml) in RPMI (VWR, Cat. No. 45000-398)
Microscopy blocking solution	4 % rat serum (Jackson Immunoresearch, Cat. No. 012-000-120) and 1:500 Fc-Block (anti CD16/CD32; Bio X cell, Cat. No. CUS-HB-197-A) in Triton X-100 (VWR, Cat. No. AC327371000)
HBSS/PS/2.5 % FBS	100 ml 10x HBSS (Corning, Cat. No. 20-021-CV), 900 ml ddH_2O, 30 ml FBS, 10 ml penicillin:streptomycin solution (Gemini, Cat. No. 400-109)
Fixation buffer solution	4% Paraformaldehyde (Sigma, P6148) in ddH2O

2.2. Antibodies and fluorescent proteins

Table 3: antibodies and fluorescent proteins used

Label	**Antibody/Protein**	**Clone (if antibody)**	**Company**	**Catalog number**
Alexa Fluor 488	Gl7	GL7	Biolegend	144603
Alexa Fluor 488	Ovalbumin	-	Thermo Fisher	O34781
Alexa Fluor 647	anti-mouse IgG	polyclonal	Southern Biotech	1030-31
Biotin	$\alpha_4\beta_7$	DATK32	BioLegend	120612
Biotin	anti-mouse IgA	C10-1	BD Biosciences	556978
Brilliant Violet 421	Anti-mouse Ig light chain κ	RMK-45	Biolegend	409511

Brilliant Violet 510	CD45	30-F11	BioLegend	103138
Brilliant Violet 650	IgD	11-26c.2a	BioLegend	405721
FITC	CD43	S7	BD Biosciences	553270
Pacific Blue	CD45	30-F11	BioLegend	103126
PE	Streptavidin	-	Invitrogen	S-866
PE	IgA	11-44-2	SouthernBiotech	1165-09
PE	anti-mouse IgA	mA-6E1	eBioscience	12-4204-81
PE/Cy7	CD19	6D5	Biolegend	115520
PE/Cy7	Streptavidin	-	eBioscience	25-4317

2.3. Animal Procedures

2.3.1. Mice

C57BL/6J (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were 6 - 8 weeks upon purchase and were 6 - 12 weeks old when experiments were started.

Mice were kept under specific pathogen-free conditions at the NRB animal facility at Harvard Medical School. Holding conditions and the experimental protocol were according to Institutional Animal Care and Use Committee at Harvard Medical School.

2.3.2. Immunizations

2.3.2.1. Stock preparations

Ovalbumin (Sigma, Cat. No. A5503) was diluted in PBS and a stock was kept at 4 mg/ml. The stock was stored at 4 °C. CpG-B #1668 (Invivogen, Cat. No. tlrl-1668-1) was diluted in PBS. A stock solution was prepared at 500 µM and stored at -80 °C. All-*trans* retinoic acid was purchased from Sigma (Cat. No. R2625). A stock solution was prepared either in DMSO (50 mM) or in ethanol (25 mM) and stored at -80 °C. Tamibarotene (AM80; Selleckchem, Cat. No. S4260) was diluted in either DMSO or ethanol (100 mM) and stored at -80 °C.

2.3.2.2. Preparation of reagents

Unless otherwise noted, mice were immunized with 100 µg Ovalbumin and 5µM CpG-B #1668 in 100 µl PBS. ATRA or AM80 were diluted to 5 mM in PEG-400 (J. T. Baker, Cat. No. U216-07) in a final volume of 100 µl per injection. Control animals received an equivalent amount of the respective solvent for ATRA or AM80 (DMSO or ethanol) in PEG-400.

2.3.2.3. Immunization procedure

Subcutaneous immunizations were performed in the scruff using 1 ml insulin needles (VWR, Cat. No. 329424). During immunization, mice were anesthetized using isoflurane (Patterson Veterinary Supply Inc., Cat. No. 07-890-8540).

2.3.2.4. Common immunization scheme

Animals were injected with ovalbumin (Ova) and CpG on day 0 and received either ATRA, AM80 or DMSO/ethanol on days 0, 1, 2, 3, 6, 10, 13 as published previously [40]. After 4 weeks, this pattern was repeated once followed by one or several oral challenges with the antigen (Figure 1).

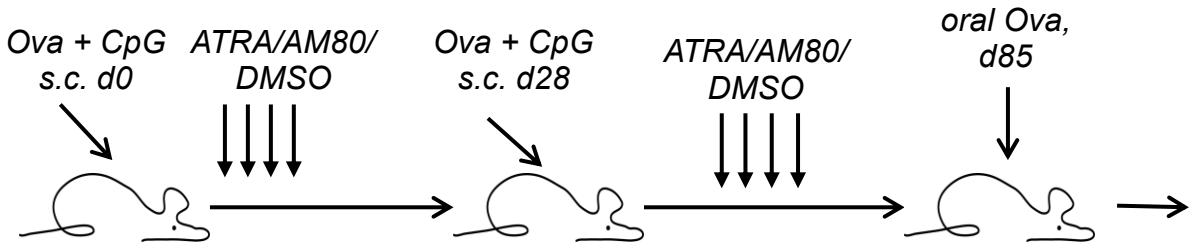

Figure 1: Illustration of the used vaccination scheme

2.3.3. Oral gavage

Ovalbumin (Sigma, Cat. No. A5503) was diluted in PBS and a stock was kept at 50 mg/ml. To mimic an infection by delivering the antigen to the small intestine, mice were orally challenged with Ova (100 µl of the 50 mg/ml solution unless noted otherwise)

using disposable feeding needles (Fisher Scientific, Cat. No. 01 208 88). For that purpose, mice were restrained manually, the tip of the feeding needle was pushed forward through the mouth into the stomach and the appropriate volume was released.

2.3.4. Intra-uterine antigen challenge

Mice received 10 µl of a 50 mg/ml solution of Ovalbumin diluted in PBS. Intra-uterine antigen challenge was performed using a non-surgical embryo transfer device (ParaTechs, Cat. No. 60010). In detail, mice were allowed to sit on top of the cage and grabbed by the base of the tail to ensure access to the vagina. A speculum was inserted into the vagina and the tip of the device was entered into the uterus. Subsequently, the solution was released.

2.3.5. Sample collection and preparation

2.3.5.1. Blood samples

Blood samples were obtained via facial vein bleeding into 1.5 ml microcentrifuge tubes. 100 µl – 200 µl of blood was collected from one animal. The blood was left to clot for 1 hour at room temperature. Clotted blood was spun down for 15 min at 1000 g and serum was collected in a 96-well storage plate (ThermoFisher, Cat. No. AB0787) followed by sealing with an appropriate sealing mat (ThermoFisher, Cat. No. AB0674). Serum samples were stored at -20 °C until analysis.

2.3.5.2. Fecal samples

Animals were separated into cardboard containers and an appropriate time was waited until animals had defecated. Feces were collected into manually pre-weighed 1.5 ml microcentrifuge tubes. Fecal samples were brought into solution thereafter or stored at - 20 °C until processed. For solubility, feces were resuspended in 1x cOmplete protease inhibitor solution at 10 µl solution per 1 mg feces or 5 µl solution per 1 mg feces. The suspension was vortexed vigorously for 15 min and spun down for 10 min at 10,000 g. The supernatant was collected into a 96-well storage plate (ThermoFisher, Cat. No. AB0796) followed by sealing with an appropriate sealing mat (ThermoFisher, Cat. No. AB0674). Processed fecal samples were stored at - 20 °C until further analysis.

2.3.5.3. Vaginal lavage

Non-anesthetized animals were restrained manually and were allowed to urinate. 50 µl PBS was instilled in the vagina using a pipette. 10 cycles of aspiration and release were performed with the pipette. The recovered fluid was pipetted into a 1.5 ml microcentrifuge tube and put on ice immediately. After sample collection, lavage fluid was briefly centrifuged at 10,000 g and the supernatant was collected into a 96-well storage plate (ThermoFisher, Cat. No. AB0796) followed by sealing with an appropriate sealing mat (ThermoFisher, Cat. No. AB0674). Processed vaginal lavage samples were then stored at - 20 °C until further analysis.

2.3.5.4. Saliva collection

Salivation was induced by intraperitoneal injection of 500 µg pilocarpine (Abcam, Cat. No. ab141301) in 50 µl of PBS in non-anesthetized mice. Mice quickly started salivating and saliva was aspirated with a pipette, carefully not to damage the oral mucosa thus avoiding contamination with blood.

2.3.6. Splenectomy and sham surgery

Animals were anaesthetized using ketamine/xylazine (50mg/kg and 10mg/kg respectively). Fur was shaved on the left lateral side of the abdominal cavity. The skin was incised and opening the abdominal cavity allowed to visualize the spleen. In animals that received splenectomy, splenic vessels were cauterized and the spleen was removed. In animals that received sham surgery, the spleen was mobilized, but not removed. The resulting wound was closed with two small sutures. Vaccination protocols did not start until seven days after the surgery, allowing animals to recover.

2.4. ELISA

ELISA plates (Biolegend, Cat. No. 423501) were coated overnight at 4 °C with 50 µl per well of a 2 µg/ml solution of Ova (Sigma, Cat. No. A5503) in PBS. Coating solution was removed, and plates were blocked with 100 µl per well of a 2 % (w/v) solution of BSA (EMD, Cat. No. 2960) in PBS for 1h at room temperature. Plates were washed using a semiautomatic microplate washer (Molecular Devices, AquaMax 2000) with a 96-well

platewash head as follows: To make sure the tubes in the machine were filled with ELISA wash buffer, the machine's priming program was used prior to every run. Then each plate was washed three times using ELISA wash buffer.

Fecal, serum and vaginal lavage samples were pre-diluted in PBS (1:2 for fecal anti-Ova IgA and IgG, 1:500 for serum anti-Ova IgG and 1:20 for serum anti-Ova IgA and 1:2.5 for both vaginal anti-Ova IgG and IgA) and 75 µl of each sample was pipetted onto the first row of the ELISA plate. As standard, a monoclonal anti-Ova IgA antibody (Chondrex, clone no. 2G12E12) or a monoclonal anti-Ova IgG2b antibody (Chondrex, clone no. 4B4E6) was added to each plate in a starting dilution in PBS of 500 ng/ml. For both standard and samples, two technical replicates were performed, except for vaginal lavage samples due to the limited sample volume. For standard and samples, six steps of a 1:3 dilution series were performed (1:3 to 1:729) from the original dilution stated above and plates were incubated overnight at 4°C (final volume of 50 µl/well). After incubation, plates were washed as described above and detected with 50 µl of a 1:5000 dilution of HRP-coupled goat anti-mouse IgA (SouthernBiotech, Cat. No. 1040-05) or IgG (Southern Biotech, Cat. No. 1030-05) antibody per well for 30 min at room temperature. Plates were then washed 5 times with ELISA wash buffer using the microplate washer. TMB substrate (Biolegend, Cat. No. 421101) was prepared as a 1:1 mixture of two components according to the manufacturer's manual. 50 µl of TMB substrate was added to each well. After approximately 10 min, the reaction was stopped using 1 M sulfuric acid. Absorbance was measured rapidly after development using a semiautomatic plate reader (BioTek, model Synergy Mx) at 450 nm and 570 nm. Background absorbance values at 570 nm were subtracted from values at 450 nm and resulting values were averaged by their technical duplicates. Data were plotted against the respective dilution factor. All data points on the linear part of the curve were picked manually and corresponding concentrations were calculated from the respective standard curve using a nonlinear fitting algorithm (GraphPad Software, Prism, nonlin fit algorithm). The concentration of antibody given here was calculated as the average of dilution multiplied by the concentration for all data points of one sample that were extracted from the linear part of the curve mentioned above.

2.5. Microscopy of whole mount sample of small intestine

Mice were euthanized according to the experimental protocol approved by the Institutional Animal Care and Use Committee at Harvard Medical School. Immediately after euthanasia, the complete small intestine was removed, cut into three pieces and washed with ice cold HBSS/PS/2.5 % FBS using a syringe with a 18G blunt end needle (BD Biosciences, Cat. No. 305180) attached to remove fecal residues. Tissue was immediately transferred to and kept in ice cold HBSS/PS/2.5 % FBS whenever not handled. Small-intestinal pieces were cut open longitudinally and washed again in ice cold HBSS/PS/2.5 % FBS. Pieces were transferred to tissue embedding cassettes and immediately incubated in fixation buffer solution overnight at 4 °C followed by a 30 min washing step in 1.5 ml microcentrifuge tubes filled with PBS on a rotation incubator at 4 °C.

Washed small intestinal pieces were stained overnight at 4 °C with DAPI (1:20000; Invitrogen, Cat. No. D1306), Alexa Fluor 488 conjugated Ovalbumin (1:200) and Biotin conjugated IgA (1:200) in microcentrifuge tubes in microscopy blocking solution. Three washing steps with PBS were performed, 30 min each, on a tube rotator at 4 °C. The second staining step with PE-coupled streptavidin (1:200) was again performed in microscopy blocking solution at 4°C overnight on a tube rotator followed by five washing steps as described above.

The tissue was mounted between microscope slide and coverslip with the villi facing the slide with ProLong Gold antifade mountant (ThermoFisher Scientific, Cat. No. P36930) and were imaged the following day using two-photon microscopy.

2.6. Flow-cytometry

2.6.1. Sample preparation and data acquisition

Mice were euthanized as described above and brachial and inguinal lymph nodes were harvested in PBS into 1.5 ml micropipette tubes. All steps were performed with ice-cold reagents and on ice unless noted otherwise. Per individual, lymph nodes from the right and left side of the body were pooled and single cell suspensions were prepared by mechanic dissociation of the tissue on a stainless-steel wire mesh (WS Tyler, Cat. No. 238136-60) using a syringe plunger. The resulting solution was centrifuged (400 g,

4 min). The supernatant was then discarded, followed by resuspension of the cell pellet in 1 ml of PBS. Isolated cells were subsequently counted using the Accuri C6 Cytometer (BD Biosciences). A maximum of 1 million cells per sample were transferred to a 96-well V-bottom plate (Sarstedt, Cat. No. 83.3925.500). Cells were centrifuged as described before and 15 µl of FACS blocking solution was added and incubated for 10 min on ice. Antibodies (see Table 3) were diluted in FACS buffer to a final dilution of 1:200 (1:400 for viability dyes, Streptavidin-coupled dyes and MHC-II antibodies) and added to the cells followed by 15 min incubation on ice. Cells were washed with FACS buffer and a secondary antibody was added in FACS blocking solution and incubated for 15 min on ice. After another washing step, cells were fixed for 30 min at room temperature using fixation buffer (BD Biosciences, Cat. No. 554655). Cells were washed and resuspended in FACS buffer and stored overnight at 4 °C for analysis.
A CytoFlex (Beckman Coulter) instrument was used for data acquisition followed by analysis with FlowJo software (Treestar).

2.6.2. Gating strategy

In order to quantify the B cell subpopulations in the draining and non-draining lymph nodes, a gating strategy was used that includes single cells via two doublet-exclusion gates. Dead cells were gated out by including only cells that have a negative fluorescence in the dead cell stain, a dye that interacts with free amine groups from proteins, and is cell membrane impermeable, unless the cell is no longer viable. As most resting B cells, but not T cells, monocytes or granulocytes are negative for CD43 [108], B cells were distinguished from non-B cells by being positive for CD45, but negative for CD43. This population was then plotted by its IgD and IgG or IgD and IgA surface fluorescence and total IgG^+ and IgA^+ signals as well as IgD^-IgG^+ and IgD^-IgA^+ subpopulations were quantified (Figure 2).

In order to quantify the proportion of $Gl7^+$ cells in B cell subpopulations, a different gating approach was chosen (Figure 3). First, a cell gate allowed to extract lymphocytes and larger cells based on their sideward scatter and forward scatter. A subsequent doublet exclusion via forward scatter area vs. forward scatter width was followed by a live cell gate. Next, a B cell gate was selected by gating on $CD45^+CD19^+$ cells. Equivalent to what

has been described for the previous gating strategy, total IgG^+ and IgA^+ cells as well as IgD^-IgG^+ and IgD^-IgA^+ subpopulations were identified. For all subpopulations, the percentage of $Gl7^+$ cells was then determined.

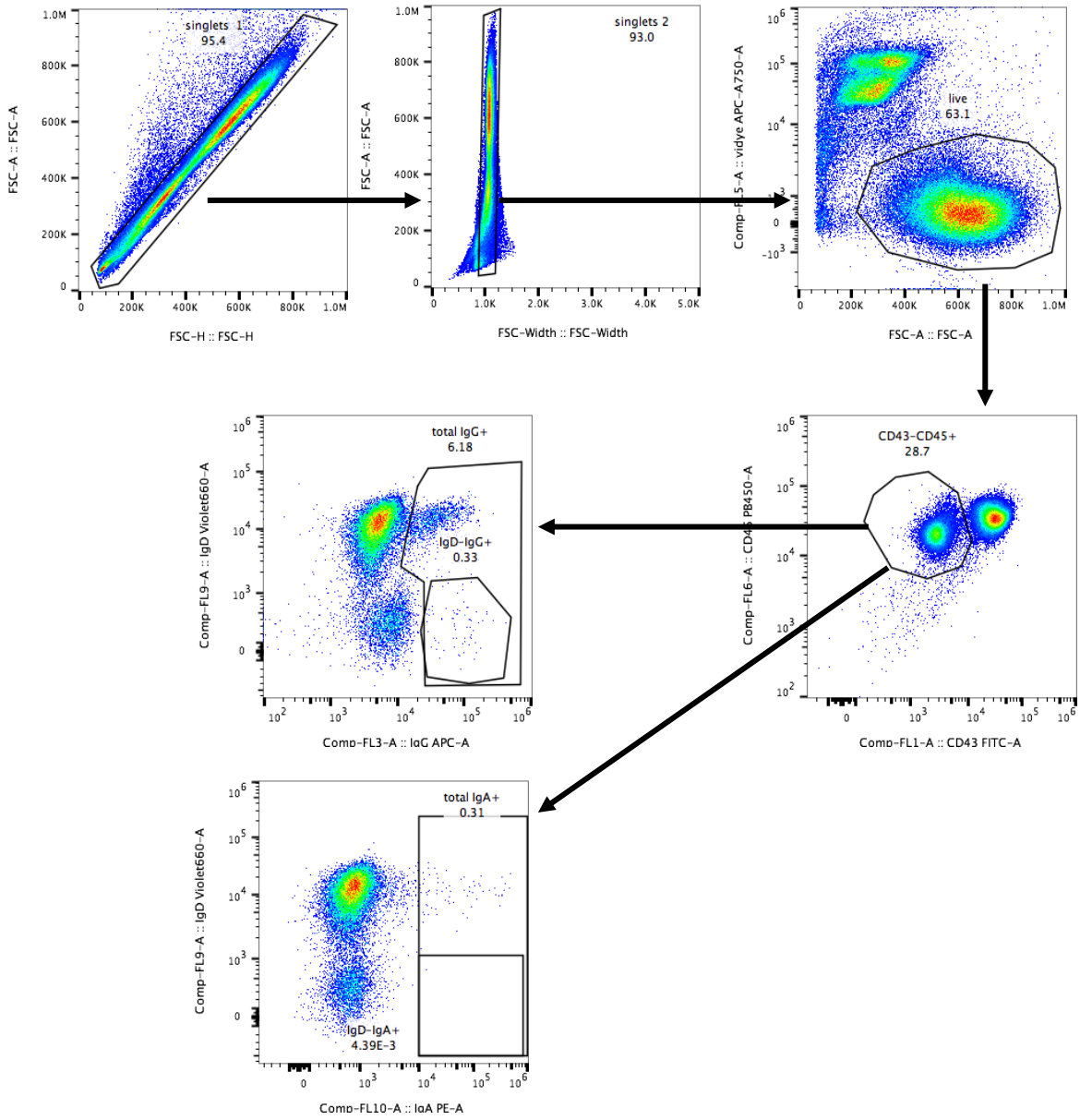

Figure 2: gating strategy for quantification of B cell subpopulations in draining and non-draining lymph nodes

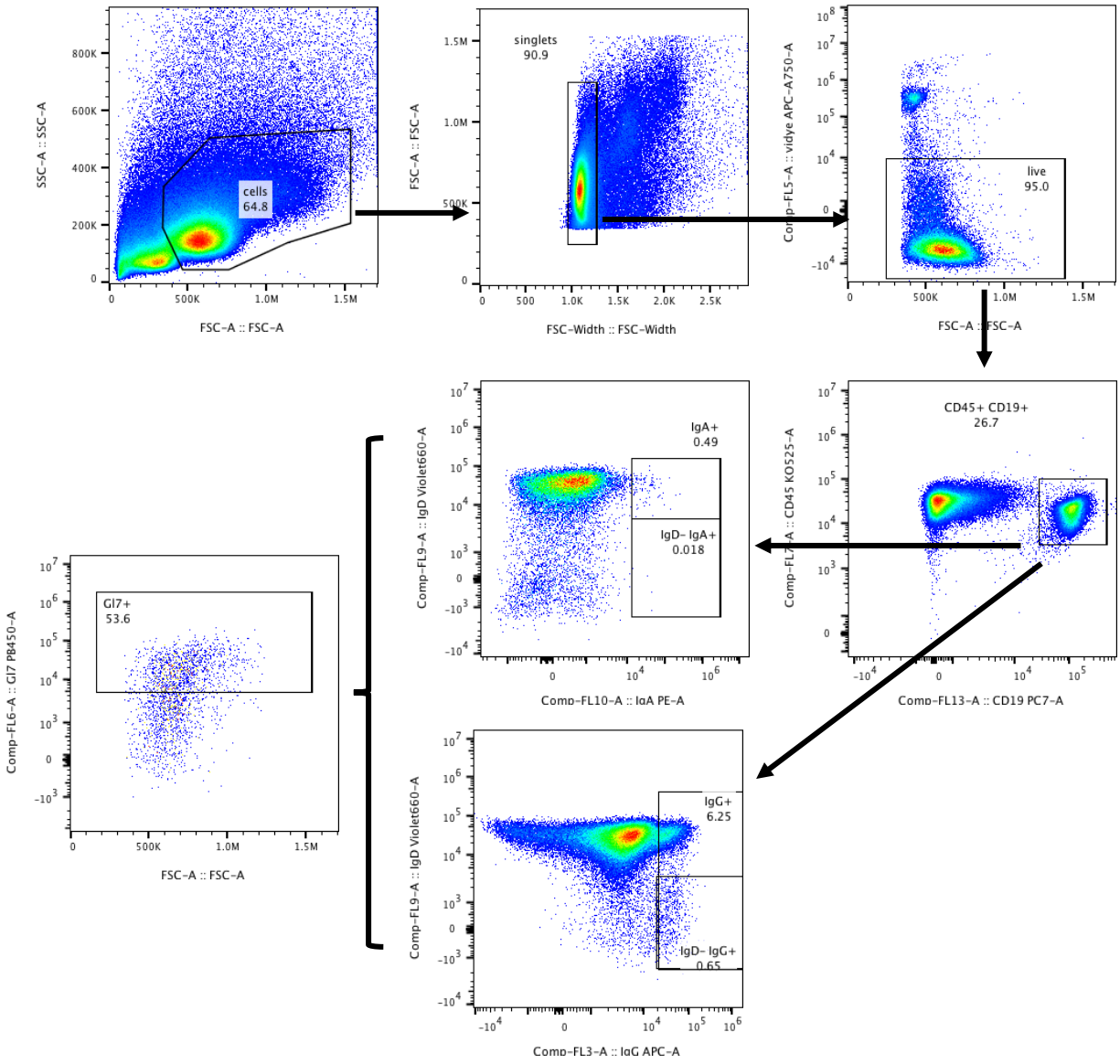

Figure 3: gating strategy for determination of Gl7$^+$ percentage in subpopulations

2.7. Measurement of cytokine production from lymph nodes

Animals were euthanized as described before and brachial lymph nodes were harvested into ice cold PBS in 1.5 ml microcentrifuge tubes. Per individual, lymph nodes from the right and left side of the body were pooled, 250 µl of cRPMI was added and lymph nodes were mechanically dissociated in a 12-well plate (Westnet, Cat. No. 3513) between two layers of a stainless-steel wire mesh (WS Tyler, Cat. No. 238136-60) with a syringe plunger. The cell suspension was transferred to a 96-well round bottom plate (Sarstedt,

Cat. No. 83.3925.500) and incubated at 37 °C with a CO_2 level of 5 % for 20 hours. Cells were centrifuged (400 g, 4 min) and the supernatant was stored at -20 °C until analysis.
For analysis, the LEGENDplex™ Mouse Th Cytokine Panel (13-plex) was used (Biolegend, Cat. No. 740005) and the manual was followed strictly. The only exception being the incubation conditions which were done on the laboratory bench rather than in a plate shaker. For acquisition, CytoFlex (Beckman Coulter) was used and analysis was performed using FlowJo software (Treestar). Results are given as the difference in MFI (ΔMFI) of the sample with an average MFI registered for 4 control wells. In control wells, cell-free cRPMI was used.

2.8. Statistical methods

Statistics were calculated using Prism 7 (GraphPad Software, La Jolla, California, USA). For comparison of experimental groups over more than one time point, two-way ANOVA was used, correcting for multiple comparisons using Tukey's multiple comparisons test. The same test was used to compare flow-cytometric data from several experimental groups from one or two organs or over several time points or within sub-populations.
For comparisons of more than two experimental groups at one time point, multiple t-tests were used correcting for multiple comparisons using the Holm-Sidak method. For comparison of two experimental groups at a single time point, Mann-Whitney test was used. One-way ANOVA correcting for multiple comparisons using Tukey's multiple comparisons test was used to analyze cytokine production. Significance levels are given as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), for a 95 % confidence interval.

3. Results

3.1. Antibody titers in feces and serum

In order to test the hypothesis, that addition of ATRA or the RARα agonist AM80 to parenteral vaccinations can lead to homing of antigen-experienced B cells to the small intestine, antibody titers were measured in both fecal pellets and serum at different time points after immunization.

Animals received s.c. injections of Ova and CpG-B #1668 on day 0 and day 28. On day 0, 1, 2, 3, 6, 8, 10, 13, 28, 29, 30, 31, 34, 38 and 41 mice received either DMSO as a control, 10 mM ATRA or 5 mM AM80, all diluted in PEG-400. On day 85, mice were orally challenged with 5 mg Ova. On day 13, 28, 42, 57, 85 and 133, fecal and serum samples were taken from all animals. One animal from the AM80 group died at day 36 and one animal from the ATRA group at day 102. The first animal was excluded from statistical analysis, as were all data points from day 133 to ensure statistical evaluation that takes multiple comparisons into account.

No difference in fecal anti-Ova IgG was found between control (DMSO) and treatment groups (ATRA and AM80; $p = 0.88$ and $p = 0.80$). All animals, however, had measurable anti-Ova IgG in their feces on day 57. On day 42, 14 days after boost, only one animal per group had measurable anti-Ova IgG in the feces (Figure 4 A, left panel). Fecal anti-Ova IgA titers behaved differently than IgG. Whereas it could be detected in the DMSO-group only after booster injection, in the ATRA- and AM80 group already 13 days after primary vaccination anti-Ova IgA could be measured in the feces. This fecal anti-Ova IgA response was boosted by a second antigen delivery. Animals receiving subsequent injections of ATRA or AM80 after each antigen delivery had significantly higher fecal anti-Ova IgA levels than animals receiving only DMSO ($p < 0.0001$). When comparing animals that received AM80 to those that received ATRA, animals that received AM80 had higher fecal anti-Ova IgA titers ($p = 0.04$). Anti-Ova IgA could be robustly measured in all animals that have received ATRA or AM80 for more than 3 months after booster injection (Figure 4 A, right panel). The magnitude of fecal anti-Ova IgA detected in the AM80 group was at least 100 times higher than that of fecal anti-Ova IgG at all time points after booster injection (Figure 4 A).

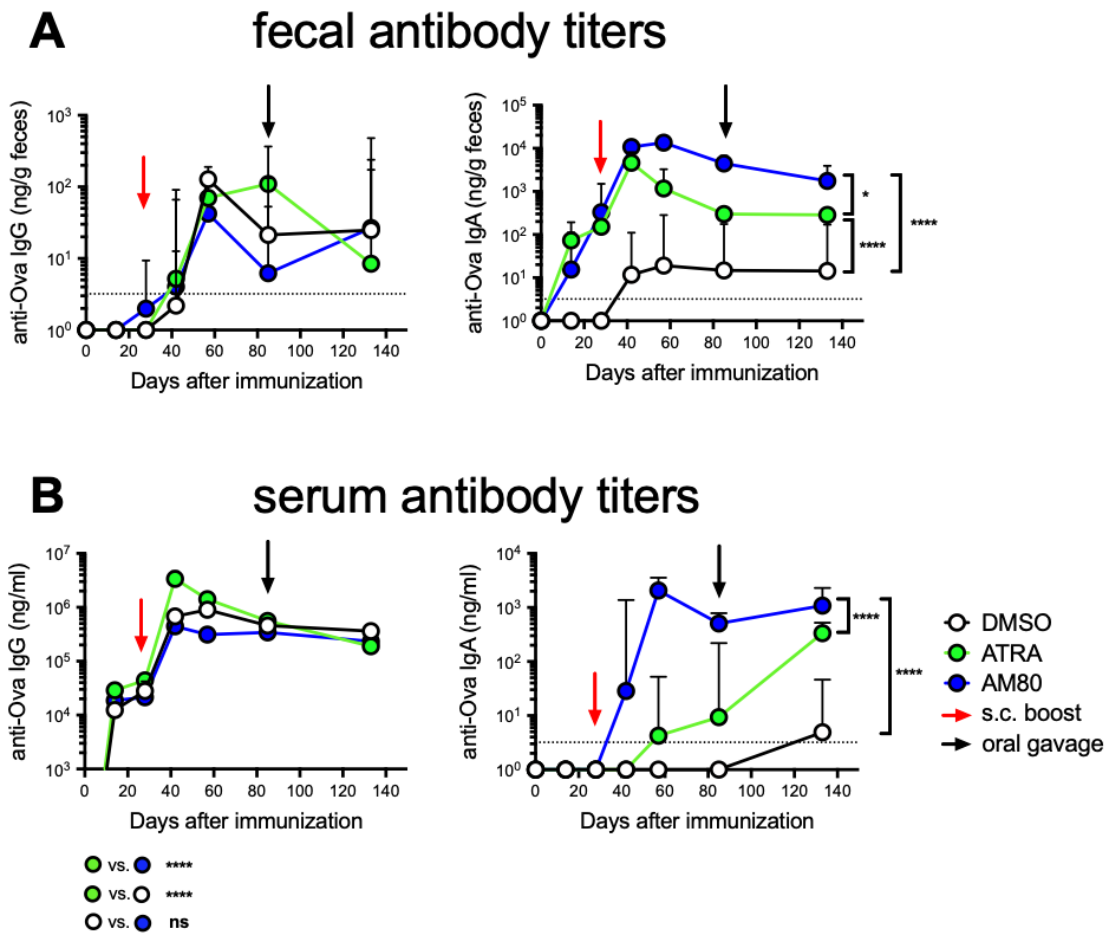

Figure 4: Time-course of fecal and serum antibody titers after vaccination with and without ATRA/AM80. Fecal (A) and serum (B) Ova-specific IgG- (left panels) and IgA (right panels) -antibodies after the above described vaccination scheme were measured by ELISA. Results are shown as geometric mean + geometric SD with 3-5 animals per group and are representative of more than 3 independent experiments. For statistical analysis, data from time point day 133 were excluded. * $p < 0.05$, **** $p < 0.0001$.

Serum anti-Ova IgG kinetics was seen to be different when comparing the group receiving ATRA to AM80 or DMSO treated animals ($p < 0.001$). However, when seen over the complete time course, curves from all experimental groups behaved similarly. Also, no group stably presented with maximal or minimal antibody titers at all time points. After boost, titers in all groups increased to at least 10-fold of their level before boost and remained high when compared to pre-boost for more than three months after booster injection (Figure 4 B, left panel). Anti-Ova IgA could be detected in the serum only after boost. Animals that received AM80 showed significantly higher ($p < 0.001$) serum anti-Ova IgA titers than ATRA or DMSO group (Figure 4 B, right panel).

3.2. Dependency of time point of ATRA/AM80 injection on antibody titers

Since ATRA and the RARα agonist AM80 were seen to induce significantly higher fecal IgA antibody titers after immunization than the control DMSO when administered at days 0, 1, 2, 3, 6, 8, 10 and 13 after vaccination and after boost, it was to be determined whether fewer administrations of ATRA or AM80 could also lead to similar results. Therefore, a similar experiment as described above was started (booster injection was performed on day 35 and DMSO/AM80 injections after booster injection were shifted accordingly) and groups were added that received AM80 on day 0, 1 and 2 after vaccination and boost (3x 5 mM AM80) or only on the day of vaccination and boost (1x 5 mM AM80). As AM80 was seen before to outperform ATRA in induction of fecal IgA titers, animals did not receive ATRA in this experiment.

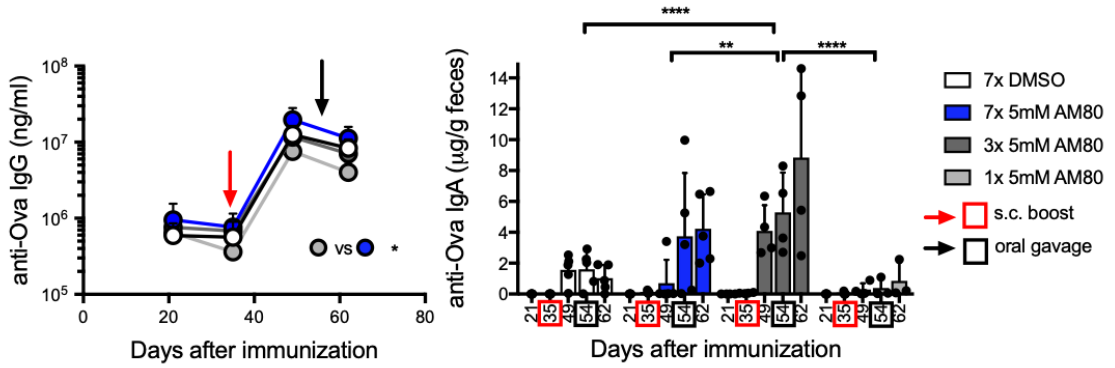

Figure 5: Dependency of time point of ATRA/AM80 injection on antibody titers. Antigen-specific serum IgG (left panel) and fecal IgA (right panel) titers after different vaccination schemes were measured by ELISA. Results are shown as mean + SD with 4-5 animals per group. For statistical analysis, two animals from group 1x 5 mM AM80 were excluded. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Serum anti-Ova IgG titers (Figure 5, left panel) were similar to those seen previously (Figure 4 B, left panel). All groups had measurable titers of serum anti-Ova IgG before booster shot and titers increased by at least 10-fold after antigen boost. Animals that received AM80 only with the original immunization and with boost (1x 5mM AM80) had lower titers of serum anti-Ova IgG than animals that received seven injections of AM80 after immunization and boost (Figure 5, left panel; $p = 0.03$). Fecal anti-Ova IgA titers were detectable in all groups after booster immunization. Animals that had received three injections of AM80 after immunization and boost had higher fecal anti-Ova IgA titers

than animals that had received only one injection of AM80 ($p < 0.001$) or only DMSO ($p < 0.001$). This group (3x 5mM AM80) even presented significantly higher titers than animals that received AM80 seven times after immunization and boost (Figure 5, right panel; $p = 0.009$). For statistical analysis, two animals from the group 1 x 5 mM AM80 were excluded as they died between day 35 and day 54 to ensure multiple comparisons could be considered.

3.3. Antibody titers on other mucosal surfaces

As fecal anti-Ova IgA was induced by the delivery of AM80 after vaccination, it remained to be determined whether this effect was limited to the gastrointestinal mucosal immune system or whether it could also be observed at other mucosal sites. As sampling from the female genital tract can be done easily, this mucosal surface was chosen as an example for mucosal surfaces other than the intestinal tract. Samples were taken from the same animals that are represented in Figure 5, albeit other time points for sampling were used. In lavage from the female genital tract, anti-Ova IgA titers showed a similar pattern to that of fecal anti-Ova IgA titers (Figure 6). 14 days after boost, animals that have received seven injections of AM80 had higher titers of vaginal lavage anti-Ova IgA than animals that have only received a single injection with AM80 ($p = 0.046$) or have received seven injections with DMSO only ($p = 0.002$). This trend could still be observed 27 days after boost but was not found to be statistically significant with the test used (Figure 6 A; $p = 0.31$, $p = 0.24$ respectively). Similar to what was observed previously regarding antigen-specific IgG in the feces (Figure 4 A), vaginal anti-Ova IgG titers were not different between the groups that have received injections containing AM80 or DMSO, 65 days post vaccination (Figure 6 C; $p = 0.65$). Notably, in the same specimen from the female genital tract, anti-Ova IgG titers were at least one order of magnitude higher than anti-Ova IgA titers (Figure 6 A, 6 C).

To confirm that other mucosal surfaces can be targeted by AM80 containing vaccinations, yet another mucosal surface was studied. On day 65 after vaccination, salivation was induced, and saliva anti-Ova IgA was measured by ELISA. All animals that have received AM80 had measurable titers of saliva anti-Ova IgA at that time point, whereas in none of the control animals, anti-Ova IgA could be detected (Figure 6 B; $p = 0.008$).

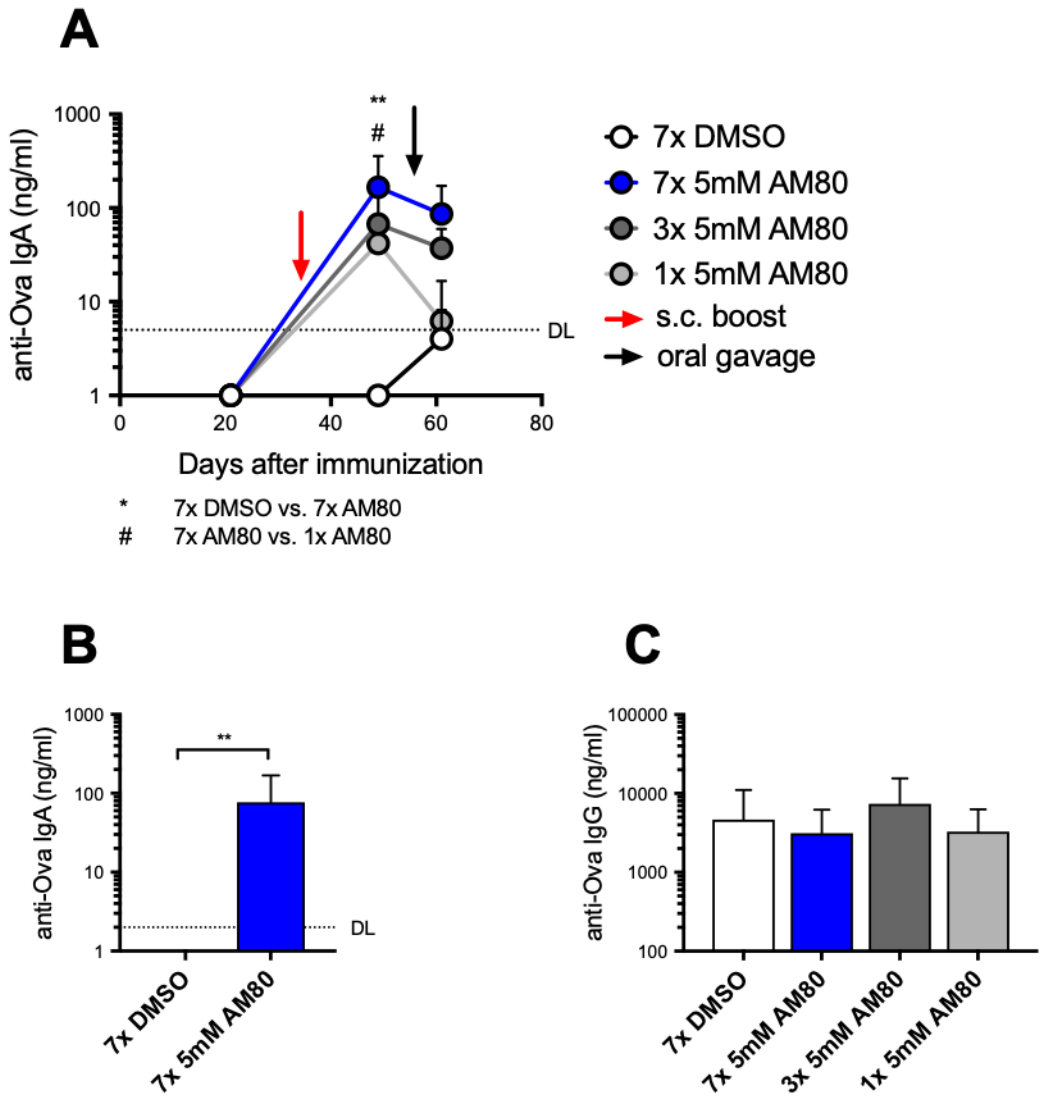

Figure 6: Anti-Ova IgA and -IgG titers in vaginal lavage and saliva. Vaginal anti-Ova IgA (panel A), saliva anti-Ova IgA (panel B) and vaginal anti-Ova IgG (panel C) after different vaccination settings were measured by ELISA. Results are shown as mean + SD with 4-5 animals per group. * $p < 0.05$, ** $p < 0.01$. DL = detection limit.

The observations above were not found when ATRA was used instead of AM80 (Figure 7). Here, animals received Ova and CpG on day 0 and day 28 and ATRA or DMSO on days 0, 1, 2, 5, 7, 9, 12, 28, 29, 30, 33, 37 and 40. Serum anti-Ova IgG titers were similar to those seen in all previous experiments. No differences between DMSO and ATRA receiving animals could be observed (Figure 7, left panel; $p = 0.91$). However, when anti-Ova IgA titers in the female genital tract were measured, no difference was found between animals that had received DMSO or ATRA injections ($p = 0.14$). From the eight time points, were antibody titers in vaginal lavage were measured, only at day 56 and 91 had

one animal in the ATRA group a measurable titer (Figure 7, right panel). Intra-uterine antigen challenge with Ova did not result in measurable vaginal anti-Ova IgA titers seven days after challenge (Figure 7, right panel).

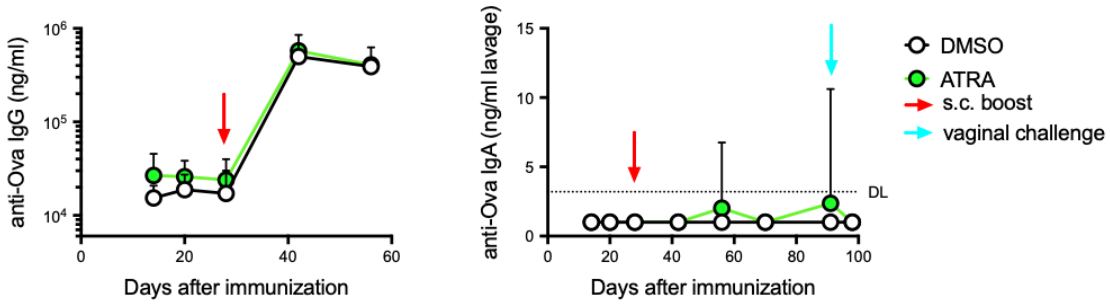

Figure 7: ATRA delivery does not induce measurable anti-Ova IgA titers in the female reproductive system in the doses delivered in this experiment. Serum anti-Ova IgG (left panel) and vaginal anti-Ova IgA (right panel) after vaccination and delivery of DMSO or ATRA were measured by ELISA. Results are shown as geometric mean + geometric SD with 3-4 animals per group. No significant differences were found between DMSO and ATRA groups by 2-way ANOVA. DL = detection limit.

3.4. Role of the spleen in the vaccination approach

Since we had previously observed many IgA^+ B cells in the spleen of animals that received ATRA after vaccination, we wanted to determine the involvement of the spleen in IgA class-switching when using this vaccination approach. Hence, one group of animals was splenectomized whereas another group received sham surgery 14 days before vaccination. Animals were then immunized with Ova and CpG on day 0 and day 35 and received AM80 on days 1, 2, 3, 6, 8, 10, 36, 38, 40, 42 and 44. Animals were also orally challenged with Ova on day 54. As an internal experimental control group, a third set of animals not submitted to surgery was immunized and received DMSO instead of AM80 at the same above-mentioned time points.

Both, splenectomized and non-splenectomized animals receiving AM80 had higher fecal anti-Ova IgA titers when compared to animals that received DMSO (splenectomized $p = 0.03$, non-splenectomized $p = 0.03$). However, no difference was found in fecal anti-Ova IgA titers between splenectomized mice and mice that had received sham surgery after immunization and exposure to AM80 (Figure 8 A, left panel; $p = 0.99$). No difference in serum anti-Ova IgG was found between the experimental groups (DMSO vs. splenectomized $p = 0.77$, DMSO vs. sham $p = 0.74$, splenectomized vs. sham

p = 0.39) and the time course of anti-Ova IgG resembled what was observed before and described previously (Figure 8 A, right panel).

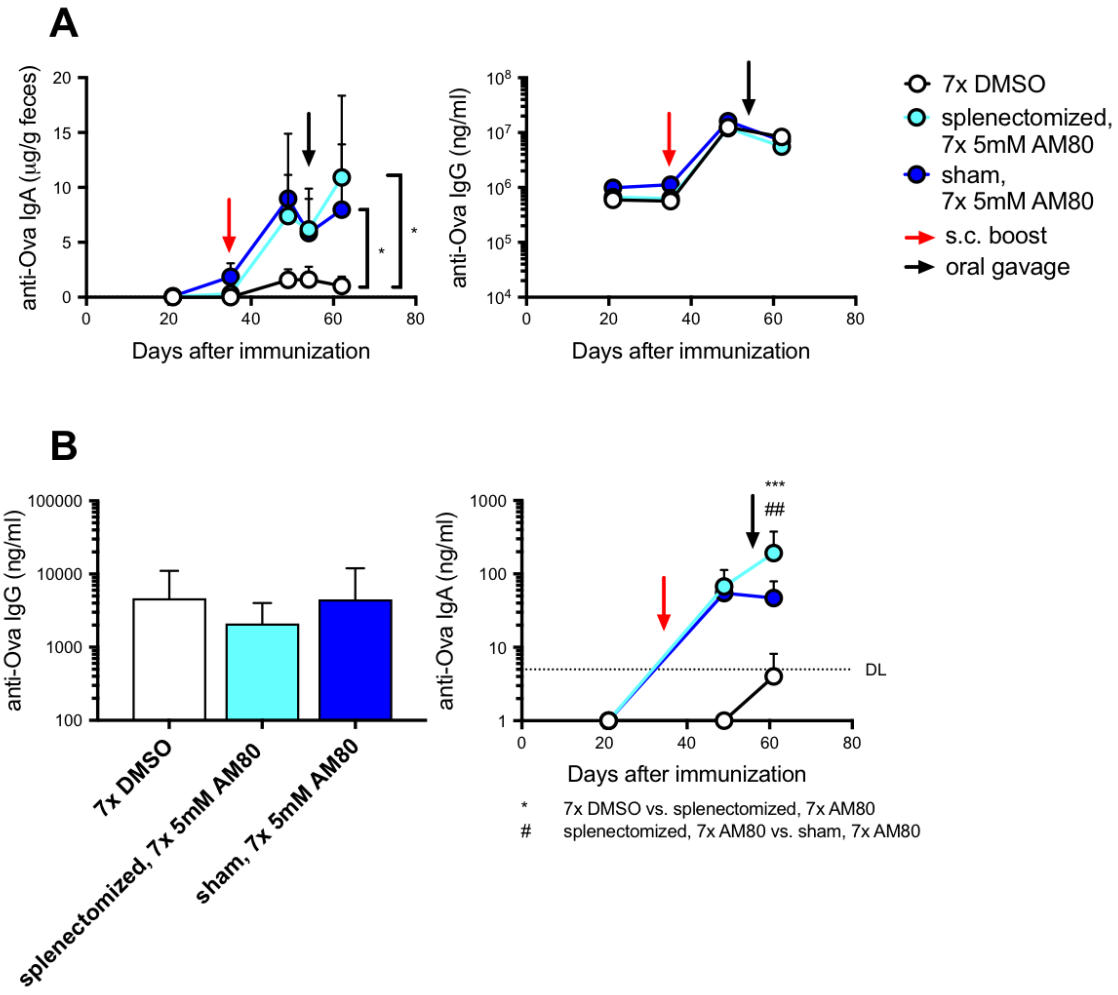

Figure 8: Generation of antigen-specific IgA by parenteral AM80 exposure does not depend on splenic IgA class-switching events. Fecal anti-Ova IgA (A, left panel), serum anti-Ova IgG (A, right panel) vaginal lavage anti-Ova IgG 65 days after vaccination (B, left panel) and vaginal lavage anti-Ova IgA (B, right panel) was measured by ELISA. Previously, animals either received splenectomy, sham surgery or no surgery. All animals were vaccinated with Ova and CpG and animals received regular AM80 or DMSO injections. Results are shown as mean + SD with 4-5 animals per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, DL = detection limit.

All groups had similar vaginal lavage anti-Ova IgG titers on day 65 after vaccination (Figure 8 B, left panel; DMSO vs. splenectomized p = 0.47, DMSO vs. sham p = 0.98, splenectomized vs sham p = 0.56). At day 49, vaginal anti-Ova IgA could only be detected in animals that received AM80. However, when they received Ova by oral gavage on day 54, repetition of the vaginal anti-Ova IgA measurement showed

significantly higher anti-Ova IgA titers in splenectomized animals that received AM80 than in non-splenectomized animals that received either AM80 ($p = 0.006$) or DMSO ($p < 0.001$) (Figure 8 B, right panel).

3.5. Kinetics of B cell class-switching in the draining lymph node

After characterizing the antibody response elicited by AM80- or ATRA-containing vaccine formulations in serum and at different mucosal surfaces, we next wanted to know what happens at the level of the draining lymph node. All immunizations were carried out s.c. in the scruff of the mice. In this setting, brachial lymph nodes (bLN) constitute the main draining lymph nodes of the injection site, whereas inguinal lymph nodes (iLN) are considered distal and therefore collected as non-draining lymph nodes.

Animals received an immunization containing Ova and CpG on day 0 and were subsequently exposed to ATRA, AM80 or ethanol on days 0, 1, 2, 3 and 6. For this experiment, ethanol was used as solvent for ATRA and AM80 preparations, and thus control groups were exposed to ethanol rather than DMSO. Animals were sacrificed on day 7 and cell populations in draining- and non-draining lymph nodes were investigated using flow-cytometry. As described before, a gating strategy was applied that identified B cells by absence of CD43 expression ($CD45^+CD43^-$ cells).

In the draining brachial lymph nodes (bLN), AM80 exposure increased the frequency of IgA^+IgD^- B cells at day 7 after vaccination when compared to naïve animals ($p = 0.007$). In the non-draining inguinal lymph nodes (iLN), this could not be observed (Figure 9 A; $p = 0.94$).

Interestingly, in the draining bLN, a high percentage (up to 50 %) of IgA^+IgD^- B cells were $\alpha_4\beta_7^+$ and might therefore have the ability to home to the intestinal mucosa (Figure 9 C). However, there was no difference between animals that had received ethanol, ATRA or AM80 in either frequency ($p = 0.69$) of $\alpha_4\beta_7^+$ cells within IgA^+IgD^- B cells or GeoMFI ($p = 0.50$) of $\alpha_4\beta_7$-fluorescence within the population of IgA^+IgD^- B cells (Figure 9 C, D). As absolute numbers of IgA^+IgD^- B cells in the inguinal lymph nodes were very low, this subpopulation was not analyzed for their frequency or GeoMFI of $\alpha_4\beta_7$.

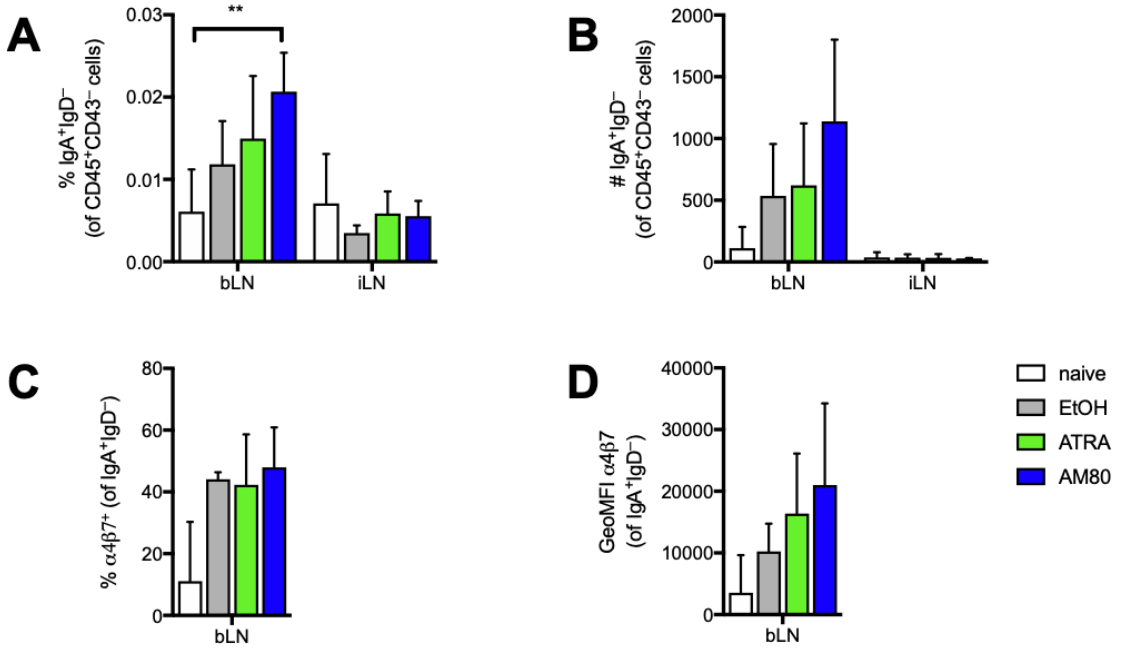

Figure 9*:* AM80 exposure during parenteral immunization increases the frequency of IgA$^+$IgD$^-$ cells in draining lymph nodes. Percentage (A) and absolute numbers (B) of IgA$^+$IgD$^-$ cells in bLN and iLN on day 7 after vaccination, analyzed by flow-cytometry. Percentage of α4β7$^+$ (C) and GeoMFI of α4β7 (D) of IgA$^+$IgD$^-$ cells in bLN and iLN on day 7 after vaccination. Results are shown as mean + SD with 3 animals per group. ** $p < 0.01$.

Apart from increasing the frequency of IgA$^+$ cells in draining bLN, AM80 exposure was also able to induce a higher frequency of IgG$^+$IgD$^-$ cells when compared to ATRA exposed mice ($p = 0.047$) or naïve animals (Figure 10 A; $p = 0.004$). Absolute cell numbers were consistent with these relative changes between the AM80 group and naïve control animals (Figure 10 B; $p = 0.007$). No difference was found between both frequency ($p > 0.99$) and absolute numbers ($p > 0.99$) of IgG$^+$IgD$^-$ B cells in the non-draining iLN (Figure 10 A, B).

When comparing the IgG$^+$IgD$^-$ with the IgA$^+$IgD$^-$ subset in the bLN on day 7 after vaccination containing AM80, the percentage of IgG$^+$IgD$^-$ cells among all B cells was up to 50 times higher than that of IgA$^+$IgD$^-$ B cells (Figure 9 A, Figure 10 A). Similarly, absolute numbers of IgG$^+$IgD$^-$ B cells were higher than that of IgA$^+$IgD$^-$ B cells (Figure 9 B, Figure 10 B).

Interestingly, whereas up to 50 % of IgA^+IgD^- B cells were $\alpha_4\beta_7^+$ (Figure 9 C), only less than 1 % of IgG^+IgD^- B cells were found to be $\alpha_4\beta_7^+$ in the bLN 7 days after vaccination with AM80 (Figure 10 C). Vaccination with AM80 could not increase the percentage of $\alpha_4\beta_7^+$ cells within IgG^+IgD^- B cells when compared to vaccination with ethanol ($p > 0.99$) and naïve ($p = 0.99$) animals (Figure 10 C). Similarly, vaccination with AM80 could not increase the GeoMFI of $\alpha_4\beta_7$ of this population when compared to ethanol injections ($p > 0.99$) or naïve animals ($p = 0.98$).

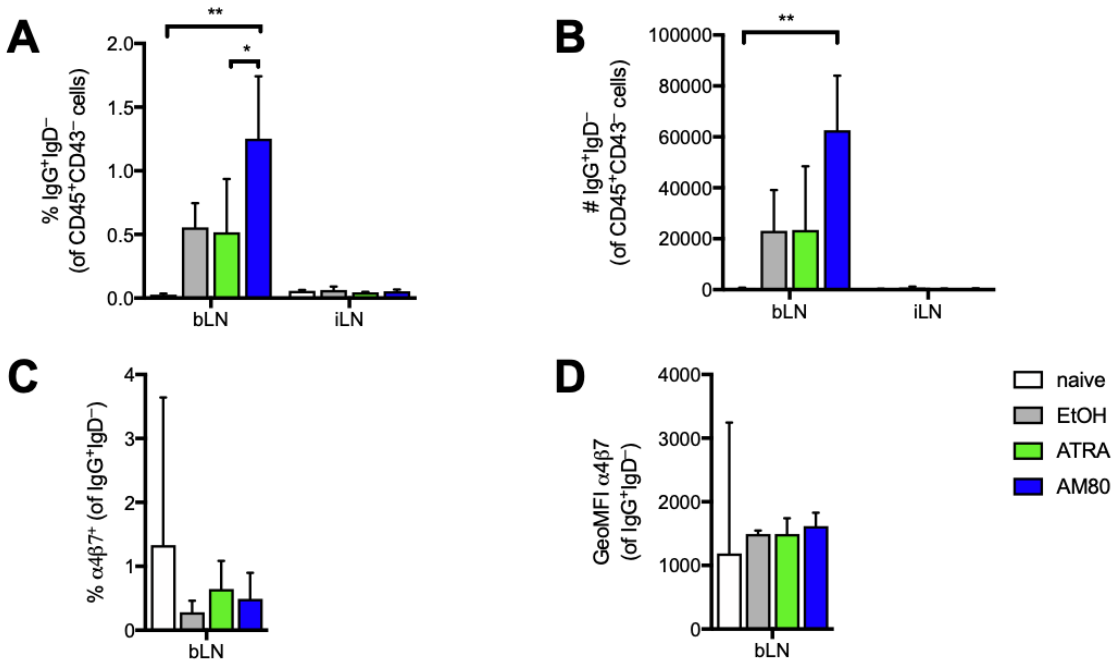

Figure 10: AM80 exposure during parenteral immunization increases the frequency of IgG^+IgD^- cells in draining lymph nodes. Percentage (A) and absolute numbers (B) of IgG^+IgD^- cells in brachial lymph nodes (bLN) and inguinal lymph nodes (iLN) on day 7 after vaccination, analyzed by flow-cytometry. Percentage of $\alpha4\beta7^+$ (C) and GeoMFI of $\alpha4\beta7$ (D) of IgG^+IgD^- cells in bLN and iLN on day 7 after vaccination. Results are shown as mean + SD with 3 animals per group. * $p < 0.05$, ** $p < 0.01$.

As some of the trends observed were not statistically significant, it was next to be determined whether there indeed is an increase in one subpopulation of B cells after vaccination with AM80 or ATRA in the draining lymph nodes and if so, how kinetics behaves. In order to better appreciate the kinetics of IgA^+ cells in draining LN after AM80 exposure, we analyzed bLN and iLN at 5 different time points within the first two weeks following immunization. Here, animals were immunized with Ova and CpG on day 0 and

received injections of ATRA, AM80 or ethanol on days 0, 1, 2, 3, 6, 9 and 13. Organs were collected at days 1, 3, 7, 10 and 14 after vaccination. Frequency of IgA^+IgD^- and IgG^+IgD^- cells among all B cells was assessed at all time points.

In draining bLN, the frequency of IgA^+IgD^- cells among all B cells was increased between day 3 and day 10 after vaccination in mice exposed to AM80 when compared to naïve animals ($p = 0.047$ for day 3, $p = 0.04$ for day 7, $p < 0.001$ for day 10). On day 10, AM80 exposure led to an increased frequency of IgA^+IgD^- cells among all B cells also when compared to animals that were immunized and exposed to ethanol (Figure 11, left panel; $p = 0.007$).

When the frequency of IgG^+IgD^- cells among all B cells was looked at in the draining lymph node after vaccination, the disparity between the groups was even more apparent. Immunization and ethanol exposure increased the frequency of IgG^+IgD^- cells among all B cells significantly when compared to naïve animals ($p = 0.03$). Whereas ATRA exposure did not show a significantly different pattern than ethanol in that regard ($p = 0.20$), AM80 exposure increased the frequency of IgG^+IgD^- cells among all B cells in the draining lymph node (Figure 11, right panel) relatively to all other experimental groups ($p < 0.0001$). A peak in frequency of IgG^+IgD^- cells could be observed around day 10 in mice exposed to AM80 or ATRA.

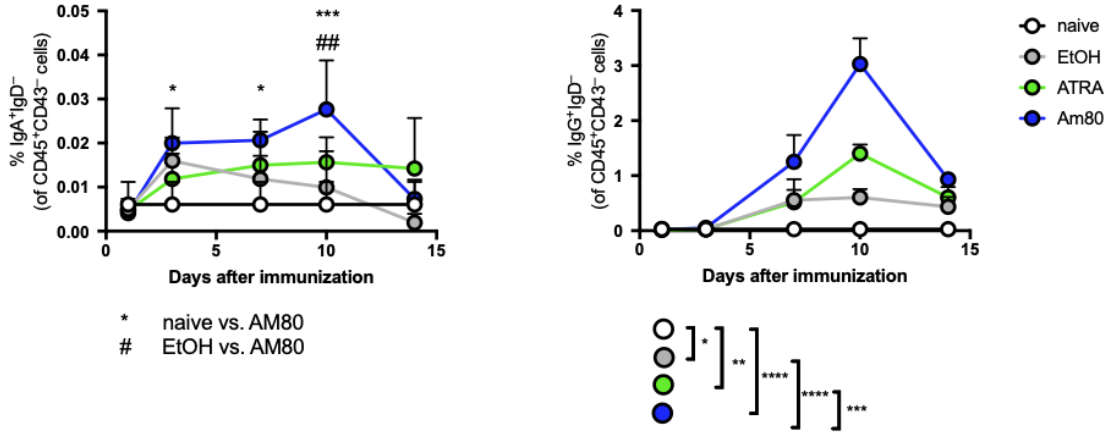

Figure 11: AM80 exposure increases the frequency of IgA^+IgD^- and IgG^+IgD^- B cell populations in the draining lymph nodes over time after vaccination. Time-course of the percentage of IgA^+IgD^- (left panel) and IgG^+IgD^- (right panel) subset within B cells in draining brachial lymph node after vaccination. Results are shown as mean + SD with 3 animals per group and time point. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Whereas it was not possible in this setting to determine the antigen-specificity of these IgA^+IgD^- and IgG^+IgD^- populations, their location in the lymph node could give valuable information on whether affinity maturation and therefore relevant interactions with the antigen could be possible.

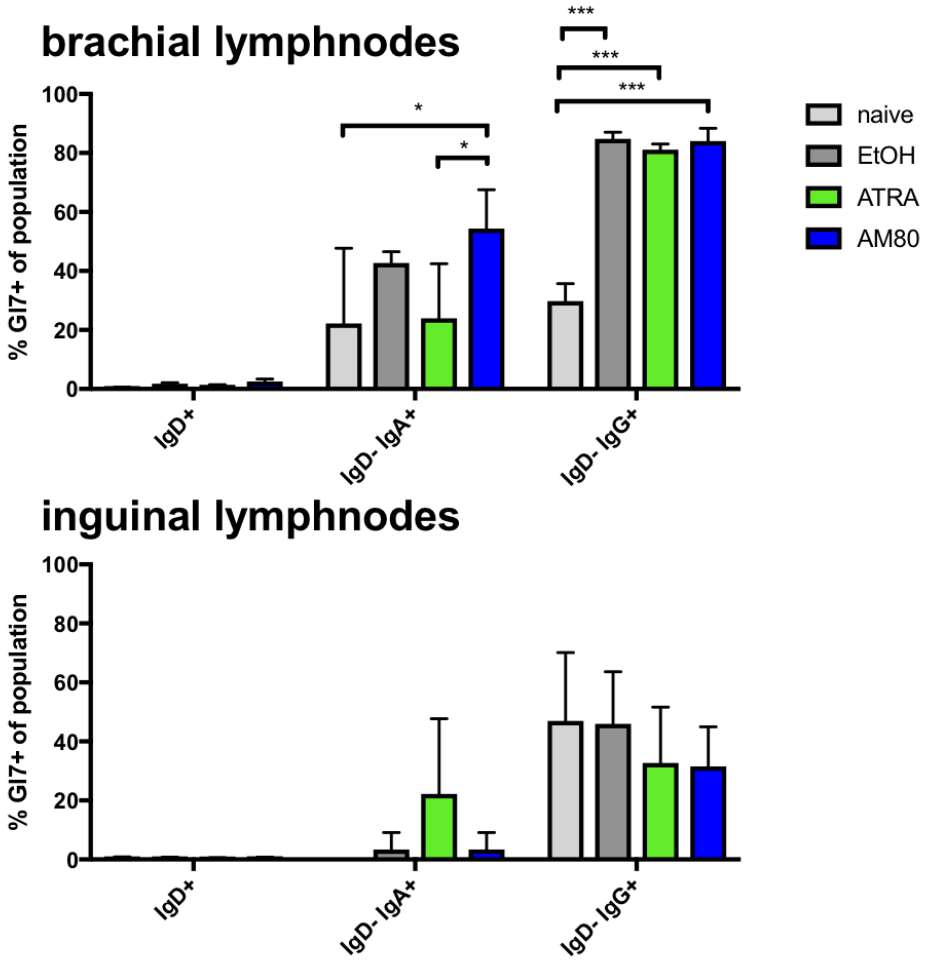

Figure 12: Effect of immunization, ethanol, ATRA and AM80 exposure on GL7 expression on different B cell subsets. Percentage of $GL7^+$ cells among IgD^+, IgD^- IgA^+ and IgD^-IgG^+ B cell populations 7 days after immunization and exposure to ethanol, ATRA or AM80 in draining bLN (upper panel) and non-draining iLN (lower panel). Results are shown as mean + SD with 3 animals per group. * $p < 0.05$, *** $p < 0.001$.

Therefore, the IgA^+IgD^- and IgG^+IgD^- populations and a presumably naïve IgD^+ B cell population were investigated upon their expression of GL7, a marker expressed by germinal center B cells, on day 7 after vaccination.

As expected, in the naïve IgD^+ B cell population in all immunization settings almost no

$GL7^+$ cells were found in the draining bLN (Figure 12, upper panel). The same was observed in the non-draining iLN (Figure 12, lower panel). Additionally, no difference in GL7 expression was found in the IgA^+IgD^- and IgG^+IgD^- populations from inguinal lymph node origin after vaccination in the scruff (Figure 12, lower panel; $p = 0.77$). However, in the draining bLN, AM80 exposure increased the percentage of $GL7^+$ cells among IgA^+IgD^- B cells compared to ATRA exposed mice ($p = 0.001$) and naïve animals ($p < 0.001$; Figure 12 upper panel). In the IgG^+IgD^- population, immunization itself was able to increase the percentage of $GL7^+$ cells among IgG^+IgD^- B cells. Interestingly, whether animals received subsequent injections of ethanol, ATRA or AM80 did not matter as all groups showed similar percentages of IgG^+IgD^- B cells that were positive for GL7 (Figure 12, upper panel).

3.6. Cytokine levels in draining lymph nodes

So far, the generated data clearly shows that parenteral immunization with ATRA/AM80 exposure results in elevated and sustained fecal antigen-specific IgA titers, when compared to standard parenteral immunizations without ATRA/AM80 exposure. This phenotype was observed alongside an increased frequency of IgG and IgA class-switch in draining LN, which occurred independently of the presence of the spleen. We then raised the question whether different cytokines, or different levels of the same cytokine, are produced within the draining lymph nodes, and that could help explaining the observed results. Since we had previously observed that three consecutive exposures to AM80 following immunization resulted in a similar intestinal IgA phenotype as that of seven exposures during the first two weeks post-immunization, we were especially interested in measuring the LN cytokine milieu during the first 48 hours after immunization.

Hence, animals were immunized with Ova and CpG and received either ATRA or the solvent DMSO as a control. Animals that did not receive any treatment served as a naïve control group. Animals were then sacrificed 24h or 48h after immunization and the draining bLN was collected. The total production of cytokines was measured after 20h incubation of the resulting bLN-derived cell suspension.

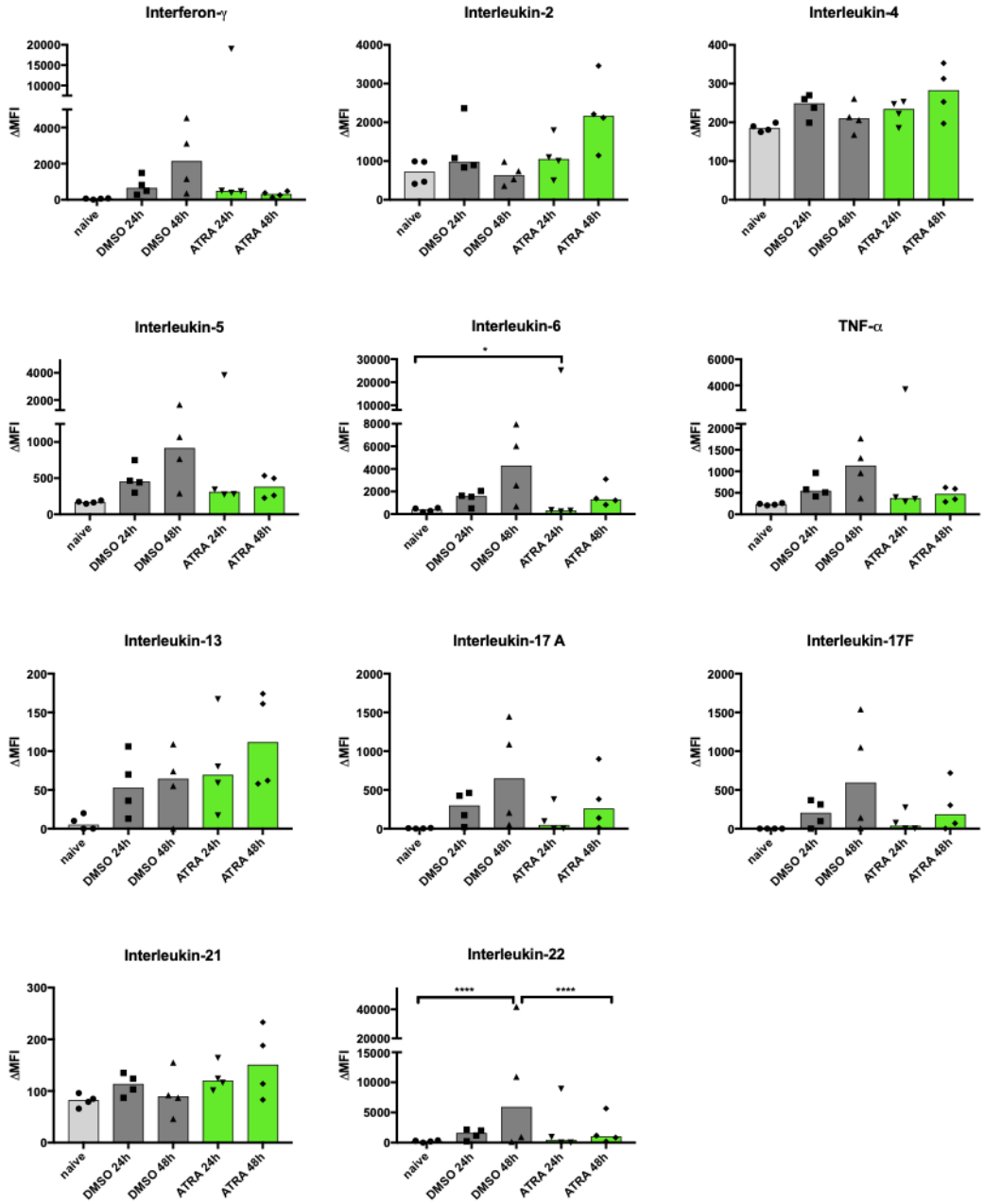

Figure 13: Cytokines produced ex vivo from draining lymph nodes 48h post-immunization with and without ATRA exposure. Cytokines were measured from draining lymph nodes *ex vivo* after immunization and exposure to DMSO or ATRA using a multiplex flow cytometry cytokine detection kit at two different time points. Results are shown as median with four animals per group. * $p < 0.05$, **** $p < 0.0001$.

No differences were observed for the cytokines interferon-γ (IFN- γ), IL-2, IL4, IL-5, IL-6, IL-13, IL-17A, IL17-F and IL-21 as well as tumor necrosis factor alpha (TNF-α) between ATRA and DMSO exposed mice at each of the collected time points ($p > 0.05$). Only IL-22 was significantly lower 48 hours after immunization in animals exposed to

ATRA comparatively to DMSO exposed ones ($p < 0.0001$). When comparing these two time points with a Mann-Whitney rank test, no statistical significance was found. However, we were not able to experimentally reproduce this observation.

3.7. Microscopy of the small intestine

At this stage, we had found titers of fecal anti-Ova IgA to increase after vaccination with exposure to ATRA or AM80 when compared to standard vaccination formulations with exposure to the solvent (DMSO or ethanol). However, we wanted to know whether a histological correlate could be found in the small intestine of vaccinated mice. Thus, animals were vaccinated with Ova and CpG on days 0 and 35 (priming and boosting, respectively) and received either DMSO or AM80 on days 0, 1, 2, 3, 5, 7, 9, 35, 36, 37, 38, 43 and 44. On day 70, animals received an oral challenge with Ova and were later sacrificed on day 84 of the experiment. The small intestine was cut into pieces and proximal, medial and distal pieces relative to the stomach were stained separately. Each piece of intestine was further cut longitudinally in order to expose the lumen of the gut. Cellular nucleus was stained with DAPI, IgA^+ B cells were stained with anti-IgA-biotin and streptavidin-PE and Alexa Fluor 488 (AF488)-coupled ovalbumin was included, in order to identify Ova-specific Ig-producing cells. Mounting the tissue for microscopic imaging was done in a manner in order for the laser to initially penetrate the adventitia-covered outside of the small intestine. Therefore, the circular structures in the microscopical images (Figure 14, Figure 15) could be identified as crypts with connective tissue surrounding them.

In animals immunized and exposed to DMSO, no structures positive for AF488-coupled Ova were found, suggesting that very few or no cells that produce Ova-specific antibodies are present (Figure 14). Similar observations were taken in all sections of the small intestine (proximal, medial and distal).

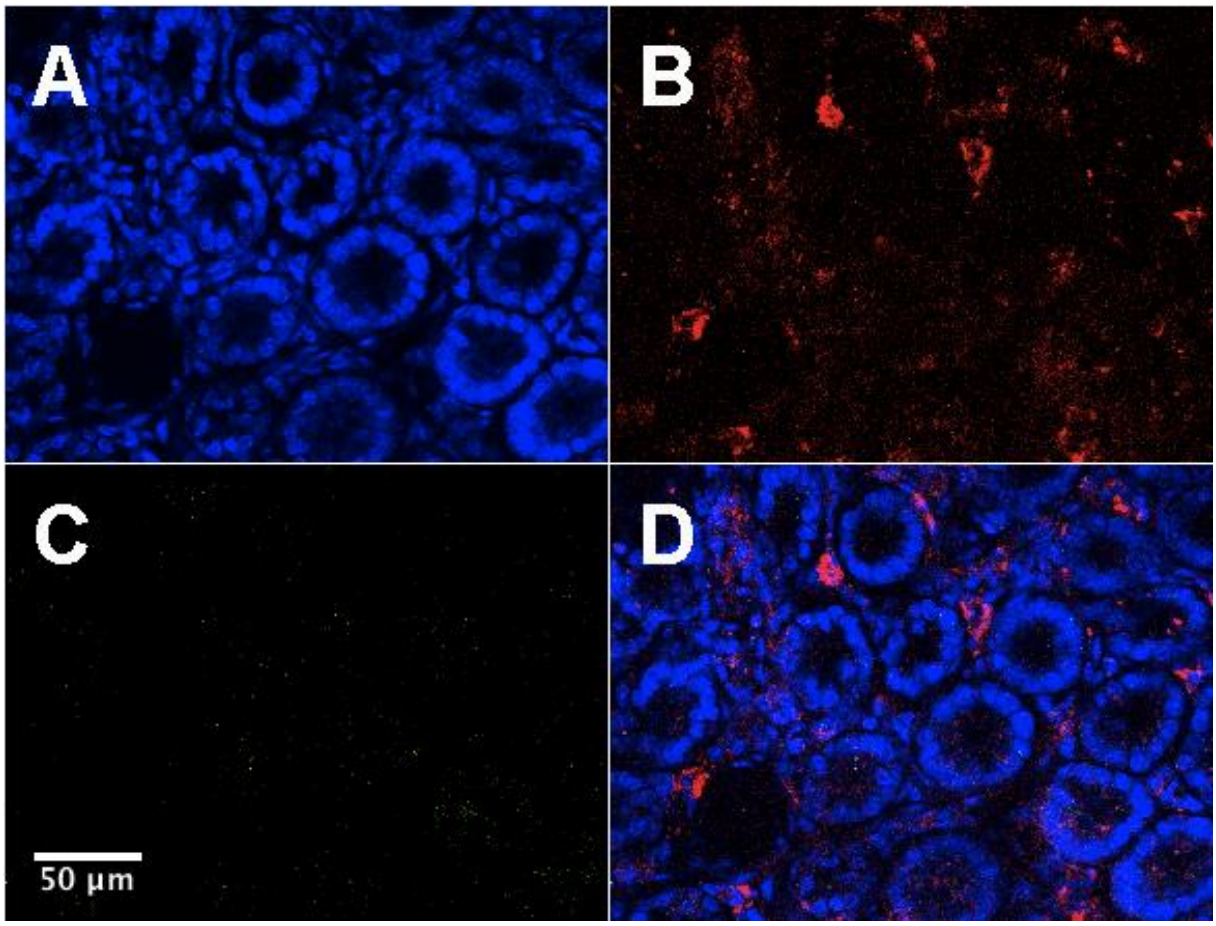

Figure 14: No anti-Ova antibodies producing cells can be found in the small intestine after immunization and exposure with DMSO. Distal small intestine was visualized by two-photon-microscopy. A: blue channel (DAPI), B: red channel (anti-IgA), C: green channel (ovalbumin), D: merge of all three channels. The microscopy image is representative of three biological replicates from more than three animals.

However, in animals immunized and subsequently exposed to AM80, structures with reactivity to fluorescent Ova could be found at least in every other field of view (Figure 15). Small structures with high signal in the green channel co-localize with nuclei that are stained with DAPI, we interpreted these structures as cells that produce anti-Ova antibodies. Interestingly, most of these cells also co-localized with signal in the red channel (i.e. Figure 15, signal in the lower right quadrant) indicating that these are anti-Ova IgA producing cells. Even though the amount of such cells was not quantified, no obvious difference was observed between the proximal, medial and distal part of the small intestine.

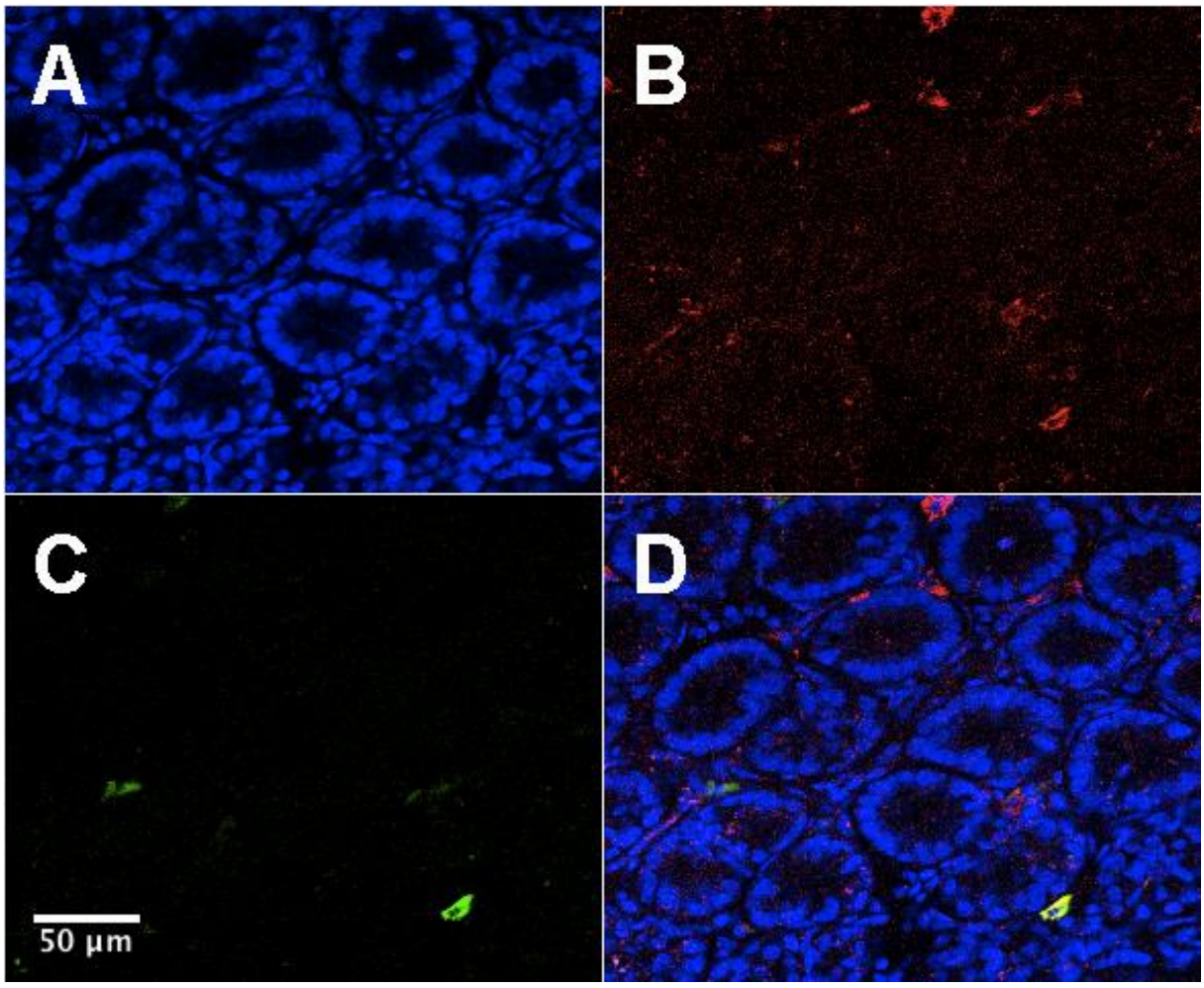

Figure 15: Anti-Ova IgA antibody producing cells can be found in the small intestine after immunization and injections with DMSO. Distal small intestine was visualized by two-photon-microscopy. A: blue channel (DAPI), B: red channel (anti-IgA), C: green channel (ovalbumin), D: merge of all three channels. The microscopy image is representative of three biological replicates from more than three animals.

4. Discussion

4.1. Summary of the main results

In this study, B cell responses were characterized after parenteral immunizations consisting of a priming and boosting schedule. The responses were primarily compared between animals that were vaccinated and subsequently exposed to all-trans retinoic acid (ATRA) or a retinoic receptor agonist (AM80), and animals vaccinated and hence exposed solely to the appropriate solvents, DMSO or ethanol.

Whereas no statistical difference was found in terms of serum and fecal antigen-specific IgG titers, titers of antigen-specific IgA in both feces and serum were elevated in animals exposed to ATRA or AM80. The elevated antigen-specific IgA titers in the feces were stably present for more than 4 months after initial immunization and can thus be seen as a long-term immune response. The first three days after vaccination were found to be crucial for inducing this particular response, as exposure to AM80 at early stage was sufficient to induce mucosal antigen-specific IgA production. Importantly, a single dose of AM80 at the time point of immunization was not enough to induce a significant increase in fecal antigen-specific IgA titers.

Not only in the feces, but also in saliva and vaginal lavage elevated antigen-specific IgA titers could be detected after vaccination and exposure to AM80. For vaginal lavage, no difference in antigen-specific IgG was found between animals that received AM80 or DMSO. For vaginal antigen-specific IgA, a difference between AM80 and DMSO injections was found after boosting injection with higher titers in animals that were exposed to AM80. However, injections with ATRA did not show titers of antigen-specific IgA titers above detection limit in vaginal lavage.

Splenectomy did not compromise the fecal antigen-specific IgA or serum antigen-specific IgG response in the vaccination setting, indicating that these B cell responses are not primarily developed in the spleen.

In the draining lymph nodes, a higher fraction of IgA^+ as well as IgG^+ B cells was found after AM80 exposure when compared to DMSO exposed animals. These frequencies peaked at around 10 days after vaccination. In the draining lymph node, whereas over

40 % of IgA^+IgD^- B cells were positive for $\alpha_4\beta_7$, only around 1 % of IgG^+IgD^- B cells were positive for $\alpha_4\beta_7$, independent of vaccination setting. However, no difference was found in MFI of $\alpha_4\beta_7$ or percentage of $\alpha_4\beta_7^+$ cells within these two populations when comparing mice exposed to ethanol, ATRA or AM80.
The expression of GL7 could be mostly observed on IgG^+IgD^- B cells in the draining lymph nodes and to a lesser extent on IgA^+IgD^- B cells, although similar between experimental groups.

Cytokines produced ex vivo in culture from cells obtained from draining lymph nodes did not differ significantly between animals exposed to ATRA or DMSO during the first 48 hours.

Using two-photon microscopy, antigen-specific IgA-producing cells could be identified in the small intestinal lamina propria of mice exposed to AM80, but not in tissues from mice exposed to DMSO.

4.2. Discussion of selected methods

4.2.1. Immunizations

All antigen immunizations and further injections of DMSO, ethanol, ATRA and AM80 were performed subcutaneously in the scruff. This is a common method used for parenteral vaccination [109] and has been used to answer various questions [110-113].
In the clinic in Germany, most parenteral vaccines are administered via the intramuscular route, with one exception being the STAMARIL® vaccination against yellow fever, that is usually administered subcutaneously. However most of the vaccines that are administered intramuscularly can also be administered via the subcutaneous route. For example, the most commonly used vaccine against Hepatitis B, Engerix®-B, can also be administered subcutaneously in patients with risk for hemorrhage [114]. Several publications have reported no difference between s.c. and i.m. immunizations in the arm in humans [115, 116] whereas others describe a superiority of i.m. over s.c. immunizations [117, 118]. These contrasting results likely depend on several factors

including the composition of the vaccine, the adjuvant used, the exact site of injection and the immunogenicity of the antigen among others.
Subcutaneous immunization is therefore an appropriate administration route for this study. However, in the further course of the project, different immunization routes have to be evaluated to ensure transfer into clinic.

4.2.2. Sample collection

Fecal and blood samples were usually taken in one session. In some experiments, blood samples were taken first, then animals were separated into cardboard boxes and feces was collected. However, some animals still had residual drops of blood on their bodies, therefore contamination of fecal samples with blood could not be excluded. The results from these experiments were therefore disregarded, since minimal contamination could have a dramatic effect on resulting fecal antibody titers, particularly those of IgG isotype. For all data presented here, fecal samples were obtained prior to blood sampling.
When sampling feces, blood and saliva at the same day was necessary, fecal and blood samples were collected before injection of pilocarpine, as this compound has side effects on enterocytes [119] and possibly also on other cells. Blood contamination of saliva cannot be excluded as mice were not anesthetized and drools of saliva had to be collected from the oral region with a pipette, which could possibly have resulted in injury of the mucosa in the oral cavity. In a previous study, the percentage of serum protein has been quantified in a range of 0.02 % to 0.2 % of total protein in the saliva [120]. In our vaccination setting, serum anti-Ova IgA antibodies were approximately 10 times higher in the serum than in saliva. To fully explain anti-Ova IgA titers measured in the saliva through blood contamination, a contamination of 10 - 20 % would therefore have to be assumed. This is unlikely as saliva was clear and not red. However, in further studies, blood contamination should be assessed by counting red blood cells in the saliva.
Importantly, because pilocarpine induces high salivation in rodents [121] it is possible that the antibody titers quantified were diluted down and therefore results from the pilocarpine-induced salivation approach underestimated the abundance of antibodies in normal unstimulated saliva.

4.2.3. ELISA

ELISA was used to quantify antibody titers in feces, serum, vaginal wash and saliva. ELISA plates were coated with ovalbumin and blocked with a 2 % bovine serum albumin solution. Samples were diluted in PBS and applied to the plate as well as a monoclonal anti-Ova IgA or IgG antibody as standard in a wide range of dilutions (1:3 to 1:729). Subsequently, the plate was washed to remove antibodies that did not specifically bind Ova. Next, IgA or IgG antibodies were detected. The concentration of anti-Ova antibodies in the sample was interpolated from the standard curve.

ELISA is a robust, cheap and widely used method for the detection of very low antibody concentrations. Through a dilution series of each sample, also higher concentrations of antibody could be measured, so that groups with high and low anti-Ova antibody titers could be compared.

In this study, monoclonal anti-Ova antibodies were used to define the standard curve. However, the response created through vaccination in mice is likely to be of polyclonal characteristics. From that fact arise several concerns. Firstly, using a monoclonal antibody for the standard curve does not appropriately control for unspecific binding that occurs in a polyclonal setting. Also, differences in affinity and avidity are likely. Most importantly, we cannot exclude the possibility that higher titers measured do not arise from more antibodies, but also partially or fully from the recognition of more epitopes with time in the polyclonal setting. Therefore, antibody quantifications from ELISA data are to be interpreted as equivalent of the corresponding monoclonal antigen-specific antibody. For future investigations, polyclonal antigen-specific antibodies should be generated and used to define the standard curve.

Nevertheless, mice exposed to DMSO/Ethanol are expected to mount an antigen-specific B cell response in a similar manner as mice exposed to ATRA or AM80, as mice have the same genetic background and are exposed to the same amount of antigen and adjuvant during the vaccination process. Therefore, differences between the experimental groups are unlikely to result from any of the concerns mentioned above but are likely to be maintained if measured at a polyclonal level.

4.2.4. Microscopy of whole mount sample of small intestine

Small intestinal samples of mice vaccinated and exposed to DMSO or AM80 were analyzed by two-photon microscopy for the presence of Ova-specific IgA producing cells. Two-photon microscopy is advantageous for the purpose of imaging whole tissues without the need of sectioning, thus providing an unprocessed 3D view of the tissue. Here, the small intestine was imaged from the adventitia towards the lumen. Compared with standard confocal microscopy, this technique can reach deeper into tissues. However, as this technique does not use a pinhole, fluorescence from other z-planes could give a signal and impede co-localization analysis. Therefore, a z-stack was recorded, and adjacent z-planes considered in the evaluation.

4.2.5. Flow-cytometry

Cell populations in the bLN were investigated using flow-cytometry. For that purpose, animals were sacrificed at several time points after vaccination and draining bLN and non-draining iLN were manually dissociated and stained with immunofluorescent antibodies.

After defining live cells, B cells were either defined as the $CD45^+CD19^+$ or the $CD45^+CD43^-$ subpopulation. CD45 is only expressed on cells with a hematopoietic origin [122] and is therefore commonly used to identify this subset of cells. As expected in the lymph node, the great majority of cells was found to be positive for CD45. On the other hand, CD43 is not expressed on mature B cells, but on most other cell types of hematopoietic origin [123]. This marker can therefore be used for a negative gating of mature B cells, a concept also used in a commercially available B cell purification kit based on magnetic microbeads (Militenyi Biotec, Order No. 130-049-801). More commonly used in flow cytometry is a positive gating of B cells through CD19 [124, 125]. However, not all plasma cells can be captured through that selection as in mice they often do not express CD19 [126]. Regardless of the strategy employed, both approaches showed similar frequencies of B cells within live cells in our experiments.

Subsequently, IgA^+, IgD^+ and IgG^+ cells were identified using appropriate antibodies. Surprisingly, when we investigated the changes in frequency of the total IgA^+ and IgG^+

subpopulations over time in the draining lymph nodes, we found no difference. However, when we sub-divided the IgA^+ and IgG^+ populations given their expression of IgD, we saw a significant amount of cell co-expressing IgA and IgD or IgG and IgD on their surface respectively.

This was a surprise to us, because naïve B cells stop expressing IgD when they class-switch to IgA or IgG [127]. Double expression of IgG (or IgA) and IgD is not possible in genetic terms, since the genetic information coding for the constant region of IgD is excised from the genome once switching to IgG or IgA occurs. This means that the expression of either of these isotypes automatically excludes the expression of the other. Hence, there has most likely been a cross-reactivity of the anti-IgD antibody, even though this antibody has been used in several studies before [128, 129]. It was therefore only after gating on the IgD^- IgA^+ or IgD^- IgG^+subpopulation, that changes in the time course after immunization became apparent.

Whereas cells from lymph nodes do not need to be permeabilized before staining for IgG, as it is expressed on the surface [130], cells are usually permeabilized to identify IgA^+ B cells [131]. However, in the intestinal lamina propria plasma cells have been described that express surface IgA [132]. As no permeabilization was performed in this experiment, it is unclear, whether the frequency of IgD^-IgA^+ cells is underestimated.

Of note, the results discussed here origin from animals after primary vaccination, not after boost. Whereas some results in antibody titers could be obtained only after boost, no booster shot setup was chosen here to be able to specifically look at the primary immune response.

4.2.6. Measurement of cytokine production from lymph nodes

Cytokines from lymph node cells were measured after immunization comparing subsequent ATRA to DMSO injections. Draining lymph nodes were dissociated into a single cell suspension, incubated and cytokines produced were measured in the supernatant using a multiplex kit for flow cytometric evaluation.

Analyzing the supernatant with the multiplex kit has the advantage of getting an overview over the production of several cytokines. However, no conclusions can be made about the exact cellular origin of the cytokines measured.

As signal strength detected by flow cytometry was very low, for some samples even below 2 pg/ml, MFI was not converted to concentration via a standard curve, but rather compared with control wells.

Analyzing *ex vivo* cytokine release is a commonly used technique [133, 134]. In order to determine, which subset of cells is producing which cytokines, another method such as intracellular cytokine staining or fluorospot should be used.

4.3. Discussion of results

4.3.1. Antibody titers in feces and serum

Mice were immunized and boosted with Ova and CpG and were further exposed to DMSO, ATRA or AM80 after priming and boosting immunizations. Animals were also orally challenged with Ova 85 days after primary immunization.

We have robustly characterized fecal and serum IgG and IgA responses that arise from these vaccinations (Figure 4) and found no significant differences in fecal anti-Ova IgG between groups that received ATRA or AM80 and control groups that received DMSO. Differences found in serum anti-Ova IgG do not seem to have biological relevance for long-term protection as most differences were observed shortly after booster immunization. Long term titers between all groups were very comparable. Most importantly, exposure to ATRA or AM80 after immunization did not lead to a systematic decline in antigen-specific IgG in feces or serum. These results therefore do not speak for a decreased long-term protection from serum antigen-specific IgG antibodies. Our observations confirm findings from Hammerschmidt et al. [40], where 14 days after immunization and ATRA exposure, no difference in serum anti-Ova IgG was found, as well as long-term responses.

Interestingly, kinetics of anti-Ova IgG titers in feces and serum seemed to differ. Whereas in the serum, 8 days after boost, a clear increase in anti-Ova IgG can be seen (Figure 4), this was not observed in the feces at that time point, but only later. Until today, it is unclear in the literature whether fecal IgG antibodies are predominantly derived from serum IgG or produced locally [135]. In this experiment, the different kinetics of serum and fecal anti-Ova IgG suggests that there might co-exist a separate subpopulation of anti-Ova IgG producing cells, likely in the intestines, that contributes to the total fecal anti-

Ova IgG titers. This has not been confirmed by microscopy as fecal anti-Ova IgG titers are 10 – 100 times lower than fecal anti-Ova IgA titers and anti-Ova IgG producing cells in the intestines are therefore very few and difficult to find. The difference in kinetics could also be explained differently, for example by up-regulation of FcRn receptors in the intestines. As mentioned earlier, the FcRn receptor has been shown to be crucial for the transport of antibodies of the IgG isotype to the small intestinal lumen in rodents [86]. However, it is unclear, to which percentage of fecal IgG in adult rodents it contributes. Potentially, ATRA or AM80 could up-regulate the expression of FcRn receptors in the small intestine thereby increasing also the titers of serum-derived anti-Ova IgG in the feces.

ATRA and AM80 exposures were found to increase anti-Ova IgA in feces and serum compared to DMSO injections. These exposures resulted in long-term B cell responses that were present for at least 4.5 months after immunization. Immunization with the same amount of AM80 lead to an even higher long-term anti-Ova IgA response in feces and serum than that observed with ATRA. Our data confirms in principle the findings from Hammerschmidt et al. [40] where antigen-specific IgA was measured 14 days after vaccination and ATRA exposure, at the same time it extends these observations to longer periods of time. More recently, the use of a nanoparticle-based i.m. vaccination approach in mice using ATRA and CpG has been found to even increase intestinal antigen-specific IgA titers when compared to delivery without nanoparticles [136]. AM80 has been chosen by us as it has been shown previously to induce IgA class switch *in vitro* [137] and has to our knowledge been used in this vaccination approach for the first time.

The biological relevance regarding a protective function of fecal IgA is subject of discussion. Traditionally, the functional concept of fecal IgA is that it coats [138] commensal bacteria thereby regulating the gut flora [139]. However, animal experiments have demonstrated a crucial role for IgA in the protection from gastrointestinal infections [140, 141]. On the other hand, human selective IgA deficiency does not lead to severe signs of disease and some patients are even asymptomatic [142]. Protective relevance of IgA will likely also depend on the pathogen and its virulence factors. The vaccination approach presented here can be used to answer some of the questions arising in that field as antigen-specific IgA response in the intestine can be induced. The evaluation of the

protective function of the vaccine will be crucial before translation into clinical application can be possible.

Vaccination and subsequent ATRA deliveries have led to relative protection in a salmonella infection model [40]. However, it is unclear which sub-compartment of the immune system is responsible for this protective phenotype. This early study presents certain limitations, particularly because animals were only subjected to a priming immunization and ATRA was used as the mucosal tropism inducer. Since our approach includes a boosting immunization and employs AM80 (shown to be more potent in inducing mucosal tropism) rather than ATRA, it will be interesting to evaluate the protection capacity of our formulation in an infection model of diarrheal disease.

The original injection scheme with ATRA/AM80 was adapted from Hammerschmidt et al. [40] where the compound was injected 7 times within 14 days after immunization. When we challenged that protocol with a limited number of AM80 injections on days 0, 1 and 2 after immunization, or with a single exposure to AM80, we found that three AM80 exposures even outperformed the original protocol, whereas a single exposure to AM80 was not capable of inducing elevated titers of fecal anti-Ova IgA (Figure 5).

Our data therefore indicates that the first three days after immunization or boost are crucial for the generation of antigen-specific IgA-producing cells with intestinal homing properties. One single injection of AM80 co-delivered with immunization and boosting time point does not suffice to generate an antigen-specific IgA phenotype at the intestinal mucosa that is significantly different than that from control immunized animals.

The final goal of the overall project is to encapsulate ATRA/AM80 in nanoparticles and deliver it to humans via i.m. or s.c. immunization. For that purpose, nanoparticles have to be designed in a way so that they release the AM80 not only at the adequate anatomical location, but also at the correct time point when cells are susceptible to acquire mucosal tropism. The better this time point is defined, the lower the concentration of AM80 that has to be administered. Similar experiments with other time points of AM80 injections could help to even better define susceptible time points.

4.3.2. Antibody titers on other mucosal surfaces

We found increased anti-Ova IgA titers not only in the feces and serum, but also in the saliva after immunization with AM80. In the female genital tract, vaginal lavage revealed an increased anti-Ova IgA in animals that were immunized and exposed to AM80, but not in animals that were immunized and exposed to ATRA or DMSO. Anti-Ova IgG in the female genital tract did not differ between the groups, again mirroring the similar serologic titers between the experimental groups (Figure 6).

The observation that other mucosal tissues than the intestinal mucosa were being targeted by our vaccination approach came as a surprise, particularly because B cells incubated with ATRA *in vitro* have previously been shown to very specifically home to the small intestine [39]. However, the female genital tract and salivary glands were not included in that previous study. A common or generalized mucosal immune system has been proposed earlier [143] and this has been supported by the observation of high expression of the chemokine receptor CCR10 on IgA^+ plasmablasts from several mucosal surfaces such as the intestines, salivary glands and the tonsils [144]. However, given the different pattern of mucosal surfaces targeted by different mucosal immunization routes (Table 1) for reasons not yet understood, this common mucosal immune system cannot be seen as one single entity.

Whereas disseminated IgA responses after mucosal vaccination have been well studied even in humans [145], parenteral vaccinations have been thought to be incapable of inducing mucosal IgA responses for sustained periods of time [146]. However, there is growing evidence that in some situations, also parenteral vaccination approaches are able to target mucosal surfaces [147]. We have been able to show in a parenteral vaccination approach that the delivery of AM80 after vaccination leads to a disseminated mucosal IgA response. Further identification of the homing mechanisms induced in our study could lead to a better understanding of physiological responses after mucosal antigen uptake and the role of RAR-agonists.

Interestingly, we have not found antigen-specific IgA in vaginal washes in animals that were immunized and have received ATRA injections (Figure 7). Even intra-uterine challenge with Ova did not result in a robust measurable anti-Ova IgA response. As we have seen lower anti-Oval IgA in the feces after ATRA than after AM80 injections, possibly ATRA elicits also lower, if any, titers of antigen-specific IgA in the female

genital tract compared to AM80 exposures. Titers could therefore have been mostly below detection limit. However, it is possible that there is a different mechanism of action between ATRA and AM80, where ATRA leads to less dissemination of IgA^+ plasmablasts. More sensitive detection methods can help answer that question. Moreover, one cannot exclude the possibility that the observed vaginal anti-Ova IgA titers may mostly derive from systemic, rather than local, IgA-producing cells. The observation that AM80 induces significantly higher IgA titers in the serum than ATRA is in line with this possibility (Figure 4).

Similar to what is discussed above regarding the intestinal tract, whether antigen-specific IgA has a protective function at other mucosal surfaces it is presently unclear. Nevertheless, it has been implied that IgA in saliva can have a protective function on caries development [148], whereas IgA in the uterus has been associated with shedding of *Chlamydia trachomatis* [149].

4.3.3. Role of the spleen in the vaccination approach

Splenectomy had no impact on fecal IgA and serum IgG titers after vaccination. A difference in vaginal IgG titers was not observed, whereas differences in vaginal IgA titers were observed only at a late time point and after oral challenge with the antigen.

It has been suggested that the immune response to vaccines is reduced after splenectomy in humans [150]. In the clinic, antibody titers are therefore controlled after some vaccinations in asplenic patients in Germany and possibly patients have to be vaccinated multiple times. The vaccination approach presented here has not been found to result in impaired antibody response in splenectomized mice in both serum and feces (Figure 8). Therefore, the spleen does not seem to be crucial in the induction of both a systemic and mucosal antibody response upon s.c. immunization. This observation suggests that the draining lymph nodes are the anatomical location where the intestinal mucosa antigen-specific IgA phenotype is induced, indicating that both antigen and ATRA/AM80 have to be present at the time of lymphocyte activation. However, in order to prove that the draining lymph nodes are essential for the antibody response, antigen-specific antibody producing cells should be identified in the draining lymph nodes by simultaneously blocking the egress, e.g. using Fingolimod (FTY720) and subsequently evaluating fecal antibody titers.

4.3.4. Kinetics of B cell class-switching in draining lymph node

Seven days after vaccination, the IgA^+IgD^- subpopulation was found to be more frequent in the draining lymph nodes of AM80 exposed animals when compared to naïve animals. However, no difference in the percentage of IgA^+IgD^- B cells that were $\alpha_4\beta_7^+$ or in GeoMFI of $\alpha_4\beta_7$ was observed after AM80 injections (Figure 9).

Similarly, an IgG^+IgD^- subpopulation was more frequent in relative and absolute numbers when comparing animals that have received AM80 injections to naïve animals. The proportion of IgG^+IgD^- B cells that were $\alpha_4\beta_7^+$ and GeoMFI of $\alpha_4\beta_7$ were found similar between the experimental groups, although a much lower percentage of IgG^+IgD^- B cells were $\alpha_4\beta_7^+$ when compared to IgA^+IgD^- B cells. These observations were not very surprising, since IgA producing cells are known to preferentially occur at mucosal sites and therefore the expression of $\alpha_4\beta_7$ is more likely to be observed on these cells, in comparison to IgG producing cells (Figure 10).

In order to confirm the tendencies found in relative numbers of IgG^+IgD^- and IgA^+IgD^- B cells, this quantification was repeated for different time points after vaccination showing an increased frequency of IgG^+IgD^- and IgA^+IgD^- B cells at several time points after AM80 injections when compared to naïve animals and ethanol injections (Figure 11).

As the reaction in the draining lymph nodes has been induced through vaccination with one antigen, it is likely that a very significant part of the generated IgG^+ and IgA^+ B cells is antigen-specific. However, we have not been able to prove antigen-specificity by flow cytometry. Another concern with the interpretation of the results is the fact that kinetics of lymphocyte egress or influx could be altered through injections of compounds such as ATRA or AM80 and the creation of IgG^+IgD^- and IgA^+IgD^- B cells could be overestimated. This is unlikely as serum IgG titer kinetics do not differ between the experimental groups, however the effect on ATRA or AM80 on lymphocyte egress from lymph nodes has not been studied previously.

ATRA [39] and AM80 [137] have been shown to induce IgA class switch *in vitro*. In our *in vivo* vaccination model, we have been able to show the induction of IgA class switch through AM80 injections *in vivo*. The fact that in the draining lymph nodes, but not in the non-draining lymph nodes, changes in cell populations were found indicates that the

process induced by vaccination and ethanol, ATRA or AM80 exposure is a local process restricted to the draining lymph node.

There has, however, another mechanism been suggested that is not controlled for in this study. Potentially, DCs at the injection site and the draining lymph node could develop a gut-homing phenotype through exposure with ATRA/AM80. Subsequent homing to the mucosal lymph system such as mesenteric lymph nodes could lead to lymphocyte maturation distant from the draining lymph nodes [151].

Apart from inducing TGF-β, ATRA has been found not to induce IgG class switch *in vitro* [137]. It is therefore surprising to find a clear increase in IgG^+IgD^- B cells after vaccination with AM80 when compared to ethanol. As this increase in cellular proportion does not correlate with measured serum or fecal anti-Ova antibody titers, the biological relevance of the increase in IgG^+IgD^- B cells in the draining lymph node is unclear. Nevertheless, one needs to keep in mind that the nature of the generated antibody titers is of polyclonal properties, and therefore potentially not properly estimated when using monoclonal antibodies in the detection assay. It has previously been implied that class switch towards IgA occurs via an intermediate switch to IgG [152]. As ATRA is naturally present in the small intestine, a possible mechanism would be the homing of IgG^+IgD^- B cells to the small intestine and class switch to IgA in the intestines. Further research will be required to determine the exact mechanism, however that knowledge might not be relevant for the development of a vaccine.

The induction of gut-homing properties through ATRA have been described *in vitro* [39]. Hence, it came as a surprise to us to find high percentage of $\alpha_4\beta_7^+$ cells among IgA^+IgD^- B cells not only in animals that received AM80 or ATRA, but also in control animals that received ethanol. A tendency towards a higher expression level of $\alpha_4\beta_7$ was observed in ATRA or AM80 exposed animals, although not statistically significant when compared to control mice. This result should be re-evaluated with a bigger cohort of animals, however given the small absolute changes in GeoMFI between the groups, the question of biological relevance is unclear.

As IgG^+IgD^- B cells had only very few $\alpha_4\beta_7$ Integrin on their surface in all vaccination settings, we do not expect extended homing of these cells to the small intestine. However, lymphocyte homing is a multi-step process. Homing receptors to the small intestine

include others than $\alpha_4\beta_7$, such as the chemokine receptor CCR9 [39]. In order to get a complete idea of homing properties, a more detailed characterization of surface receptors should be strived for and homing properties should be confirmed in competitive homing assays.

In the draining lymph nodes 7 days post vaccination, the IgG^+IgD^- subpopulation exhibits high expression of germinal center markers in all vaccination settings, whereas the IgA^+IgD^- subpopulation shows a low expression of the same markers (Figure 12). IgA class switch has been described to occur in a T cell dependent or T cell independent manner [100]. We have reported T cell dependency for serum anti Ova-IgG and also fecal anti-Ova IgA production in this vaccination setting (unpublished observations; complementary of this study). However, in order to effectively bind the antigen, B cells generally have to go through somatic hypermutation of their variable regions. This has also been implied for IgA class switched cells in Peyer's patches, and to occur in the germinal center [153]. Our finding that 20 – 50 % of IgA^+ cells express GL7 (Figure 12) indicates that at least a portion of these cells is present in the germinal center and could potentially go through somatic hypermutation. Importantly, injections of ATRA or AM80 do not impair the frequency of IgG^+IgD^- cells present in germinal centers and therefore does not seem to interfere with somatic hypermutation of IgG^+IgD^- cells. It is interesting to note, however, that at least 50 % of IgA^+ cells are not observed in germinal center structures, although their existence requires T cell help (data not included in this project, but complementary of this study). Even though it has recently been shown that class switch occurs mainly outside of germinal centers and before germinal center formation [154], these observations raise the question whether IgA^+ cells originated in peripheral lymph nodes contain somatically mutated or germline encoded sequences. To validate both results, the avidity and affinity of the resulting antibodies after immunization and ethanol or AM80 injections should be compared.

4.3.5. Cytokine levels in draining lymph nodes

No difference was found in cytokine production profiles of the draining lymph nodes after immunization and exposures to DMSO or ATRA (Figure 13).

Several cytokines have been described to synergize with ATRA in IgA class switch, such as IL-2, IL-4, IL-5, IL-6 and IL-10 [100]. ATRA has been shown to enhance IL-10 and to decrease TNF-α production by macrophages after co-culture with lipopolysaccharide *in vitro* [155], and to reduce the expression of IL-2 gene transcripts in mice *in vivo* [156]. The lack of differences in our vaccination model could be methodological. Nevertheless, it is interesting to note that IL-2, IL-4, IL-13 and IL-21 show a relative increase in some animals exposed to ATRA, compared to control mice. Given the roles of these cytokines in induction of IgA (IL-4) or IgE (IL-13) class-switch, as well as their production by follicular T helper cells (IL-21), it is of importance to further explore the obtained results. Interestingly, IL-6 and TNF-α levels show a relative increase in control animals that have received DMSO when compared to animals that have received ATRA. It is possible that there has been an activation of innate immune cells through the administered compounds which could also possibly mask potential effects of ATRA in the context of other cytokines. In order to approach both concerns, intracellular staining of cytokines in cells from the draining lymph nodes should be assayed after each of the vaccination settings here described.

4.3.6. Microscopy of the small intestine

Anti-Ova IgA producing cells in the small intestine were identified by two-photon microscopy. These cells were only found in animals that have received AM80, but not in animals that have received the solvent DMSO.

This finding confirms the local production of anti-Ova IgA in the small intestine, and which was suggested by the different kinetics in fecal and serum anti-Ova IgA titers observed earlier (Figure 4, Figure 5). Another conclusion that can be drawn from this observation is that AM80 has mediated the generation of B cells with gut-homing capacity, and which reside and produce local antibodies of the IgA-isotype, 70 days post-immunization. However, these findings still do not answer the mechanism of the vaccination as it is possible that class-switch occurred in the small intestine.

4.4. Future directions

We have shown that AM80 exposure after vaccination leads to increased antigen-specific IgA titers in the small intestine and on other mucosal surfaces. The biological meaning of this result should be tested in a gastro-intestinal infection model to see whether protection arises from the vaccination approach. Not only antibody producing cells, but also T cells and other immune cellular subsets play a role in protection from infections in the small intestine, these populations should be regarded in greater detail.

The ultimate goal of the overall project is to generate a nanoparticle-based vaccine that can be used in humans. By encapsulating AM80, potential toxicity could be reduced by exact delivery to the target structures and reduction of amount of the compound needed. By optimizing encapsulated formulations of AM80 regarding their release kinetics, concentration and size, the outcome of the vaccine should be guaranteed by two injections to ensure transferability to humans.

The biological relevance of anti-Ova IgA on other mucosal surfaces following immunizations with AM80 can be tested in multiple fashions such as infection models or caries protection models.

The exact mechanism of anti-Ova IgA induction at the small intestine and other mucosal surfaces has not been unraveled by this study. Whereas for the transfer into humans the results rather than the mechanism seem more relevant, further investigations looking into the mechanism of induction of fecal antibodies could lead to more fundamental information on IgA class-switch and lymphocyte homing.

5. References

1. UNICEF/WHO, *Diarrhoea: Why children are still dying and what can be done.* WHO Library Cataloging-in-Publication Data, 2009.
2. Kotloff, K.L., et al., *Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study.* The Lancet, 2013. **382**(9888): p. 209-222.
3. Troeger, C., et al., *Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015.* The Lancet Infectious Diseases, 2017. **17**(9): p. 909-948.
4. Hosangadi, D., P.G. Smith, and B.K. Giersing, *Considerations for using ETEC and Shigella disease burden estimates to guide vaccine development strategy.* Vaccine, 2017.
5. Vos, T., et al., *Causes of child death: comparison of MCEE and GBD 2013 estimates – Authors' reply.* The Lancet, 2015. **385**(9986): p. 2462-2464.
6. Liu, L., et al., *Causes of child death: comparison of MCEE and GBD 2013 estimates.* The Lancet, 2015. **385**(9986): p. 2461-2462.
7. Kirk, M.D., et al., *World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral Diseases, 2010: A Data Synthesis.* PLoS Medicine, 2015. **12**(12): p. e1001921.
8. Fischer Walker, C.L., D. Sack, and R.E. Black, *Etiology of Diarrhea in Older Children, Adolescents and Adults: A Systematic Review.* PLoS Neglected Tropical Diseases, 2010. **4**(8): p. e768.
9. Nations, U. *Millenium Development Goals after 2015; GOAL 4: REDUCE CHILD MORTALITY*. [cited 2018 06.09.2018]; Available from: http://www.un.org/milleniumgoals/childhealth.shtml
10. Troeger, C.E., et al., *Quantifying risks and interventions that have affected the burden of diarrhoea among children younger than 5 years: an analysis of the Global Burden of Disease Study 2017.* The Lancet Infectious Diseases, 2020. **20**(1): p. 37-59.
11. Qazi, S., et al., *Ending preventable child deaths from pneumonia and diarrhoea by 2025. Development of the integrated Global Action Plan for the Prevention and Control of Pneumonia and Diarrhoea.* Arch Dis Child, 2015. **100 Suppl 1**: p. S23-8.
12. Haroon, S., et al., *Breastfeeding promotion interventions and breastfeeding practices: a systematic review.* BMC Public Health, 2013. **13 Suppl 3**: p. S20.
13. Lamberti, L.M., et al., *Breastfeeding and the risk for diarrhea morbidity and mortality.* BMC Public Health, 2011. **11 Suppl 3**: p. S15.
14. Dreibelbis, R., et al., *The impact of school water, sanitation, and hygiene interventions on the health of younger siblings of pupils: a cluster-randomized trial in Kenya.* Am J Public Health, 2014. **104**(1): p. e91-7.

15. Nicholson, J.A., et al., *An investigation of the effects of a hand washing intervention on health outcomes and school absence using a randomised trial in Indian urban communities.* Trop Med Int Health, 2014. **19**(3): p. 284-292.
16. Munos, M.K., C.L. Walker, and R.E. Black, *The effect of oral rehydration solution and recommended home fluids on diarrhoea mortality.* Int J Epidemiol, 2010. **39 Suppl 1**: p. i75-87.
17. Bhandari, N., et al., *Effectiveness of zinc supplementation plus oral rehydration salts compared with oral rehydration salts alone as a treatment for acute diarrhea in a primary care setting: a cluster randomized trial.* Pediatrics, 2008. **121**(5): p. e1279-85.
18. Walker, C.L. and R.E. Black, *Zinc for the treatment of diarrhoea: effect on diarrhoea morbidity, mortality and incidence of future episodes.* Int J Epidemiol, 2010. **39 Suppl 1**: p. i63-9.
19. Harrell, J.E. and S.X. Cheng, *Inability to reduce morbidity of diarrhea by ORS: can we design a better therapy?* Pediatric Research, 2018. **83**: p. 559.
20. Porter, C.K., B. Kowalcyk, and M.S. Riddle, *Chronic Health Consequences of Acute Enteric Infections in the Developed World.* The American Journal Of Gastroenterology Supplements, 2016. **3**: p. 12.
21. Guerrant, R.L., et al., *The impoverished gut--a triple burden of diarrhoea, stunting and chronic disease.* Nat Rev Gastroenterol Hepatol, 2013. **10**(4): p. 220-9.
22. Rheingans, R., et al., *Exploring Household Economic Impacts of Childhood Diarrheal Illnesses in 3 African Settings.* Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 2012. **55**(Suppl 4): p. S317-S326.
23. Pinkerton, R., et al., *Early Childhood Diarrhea Predicts Cognitive Delays in Later Childhood Independently of Malnutrition.* Am J Trop Med Hyg, 2016. **95**(5): p. 1004-1010.
24. Niehaus, M.D., et al., *Early childhood diarrhea is associated with diminished cognitive function 4 to 7 years later in children in a northeast Brazilian shantytown.* Am J Trop Med Hyg, 2002. **66**(5): p. 590-3.
25. Lycke, N., *Recent progress in mucosal vaccine development: potential and limitations.* Nat Rev Immunol, 2012. **12**(8): p. 592-605.
26. McGhee, J.R. and K. Fujihashi, *Inside the Mucosal Immune System.* PLOS Biology, 2012. **10**(9): p. e1001397.
27. Ahluwalia, B., M.K. Magnusson, and L. Ohman, *Mucosal immune system of the gastrointestinal tract: maintaining balance between the good and the bad.* Scand J Gastroenterol, 2017. **52**(11): p. 1185-1193.
28. Brandtzaeg, P., *History of oral tolerance and mucosal immunity.* Ann N Y Acad Sci, 1996. **778**: p. 1-27.
29. Wiedle, G., D. Dunon, and B.A. Imhof, *Current concepts in lymphocyte homing and recirculation.* Crit Rev Clin Lab Sci, 2001. **38**(1): p. 1-31.
30. J. L. Gowans, E.J.K., *The route of re-circulation of lymphocytes in the rat.* Proceedings of the Royal Society of London. Series B. Biological Sciences, 1964. **159**(975): p. 257.
31. von Andrian, U.H. and T.R. Mempel, *Homing and cellular traffic in lymph nodes.* Nat Rev Immunol, 2003. **3**(11): p. 867-78.

32. Butcher, E.C. and L.J. Picker, *Lymphocyte homing and homeostasis.* Science, 1996. **272**(5258): p. 60-6.
33. Mora, J.R., et al., *Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells.* Nature, 2003. **424**(6944): p. 88-93.
34. Nakache, M., et al., *The mucosal vascular addressin is a tissue-specific endothelial cell adhesion molecule for circulating lymphocytes.* Nature, 1989. **337**(6203): p. 179-81.
35. Svensson, M., et al., *CCL25 mediates the localization of recently activated CD8alphabeta(+) lymphocytes to the small-intestinal mucosa.* J Clin Invest, 2002. **110**(8): p. 1113-21.
36. Stenstad, H., et al., *Differential homing mechanisms regulate regionalized effector CD8αβ⁺ T cell accumulation within the small intestine.* Proceedings of the National Academy of Sciences, 2007. **104**(24): p. 10122.
37. Kunkel, E.J., et al., *Lymphocyte Cc Chemokine Receptor 9 and Epithelial Thymus-Expressed Chemokine (Teck) Expression Distinguish the Small Intestinal Immune Compartment.* The Journal of Experimental Medicine, 2000. **192**(5): p. 761.
38. Iwata, M., et al., *Retinoic acid imprints gut-homing specificity on T cells.* Immunity, 2004. **21**(4): p. 527-38.
39. Mora, J.R., et al., *Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells.* Science, 2006. **314**(5802): p. 1157-60.
40. Hammerschmidt, S.I., et al., *Retinoic acid induces homing of protective T and B cells to the gut after subcutaneous immunization in mice.* J Clin Invest, 2011. **121**(8): p. 3051-61.
41. Shannon, B., et al., *Impact of Asymptomatic Herpes Simplex Virus Type 2 Infection on Mucosal Homing and Immune Cell Subsets in the Blood and Female Genital Tract.* The Journal of Immunology, 2014. **192**(11): p. 5074.
42. Goode, D., et al., *HSV-2-driven increase in the expression of alpha4beta7 correlates with increased susceptibility to vaginal SHIV(SF162P3) infection.* PLoS Pathog, 2014. **10**(12): p. e1004567.
43. Kelly, K.A., et al., *Two different homing pathways involving integrin beta7 and E-selectin significantly influence trafficking of CD4 cells to the genital tract following Chlamydia muridarum infection.* Am J Reprod Immunol, 2009. **61**(6): p. 438-45.
44. Arthos, J., et al., *The Role of Integrin alpha4beta7 in HIV Pathogenesis and Treatment.* Curr HIV/AIDS Rep, 2018. **15**(2): p. 127-135.
45. Davila, S.J., A.J. Olive, and M.N. Starnbach, *Integrin alpha4beta1 is necessary for CD4+ T cell-mediated protection against genital Chlamydia trachomatis infection.* J Immunol, 2014. **192**(9): p. 4284-93.
46. Perry, L.L., et al., *Distinct Homing Pathways Direct T Lymphocytes to the Genital and Intestinal Mucosae in Chlamydia-Infected Mice.* The Journal of Immunology, 1998. **160**(6): p. 2905.
47. Johansson, E.L., et al., *Distribution of lymphocytes and adhesion molecules in human cervix and vagina.* Immunology, 1999. **96**(2): p. 272-7.
48. Qualai, J., et al., *Adhesion Molecules Associated with Female Genital Tract Infection.* PLoS One, 2016. **11**(6): p. e0156605.

49. Caldeira-Dantas, S., et al., *The Chemokine Receptor CXCR3 Promotes CD8(+) T Cell Accumulation in Uninfected Salivary Glands but Is Not Necessary after Murine Cytomegalovirus Infection.* J Immunol, 2018. **200**(3): p. 1133-1145.
50. Woyciechowski, S., M. Hofmann, and H. Pircher, *α4β1 integrin promotes accumulation of tissue-resident memory CD8+ T cells in salivary glands.* European Journal of Immunology, 2016. **47**(2): p. 244-250.
51. Czerkinsky, C. and J. Holmgren, *Mucosal delivery routes for optimal immunization: targeting immunity to the right tissues.* Curr Top Microbiol Immunol, 2012. **354**: p. 1-18.
52. Czerkinsky, C. and J. Holmgren, *Vaccines against enteric infections for the developing world.* Philos Trans R Soc Lond B Biol Sci, 2015. **370**(1671).
53. Johansson, E.-L., et al., *Comparison of different routes of vaccination for eliciting antibody responses in the human stomach.* Vaccine, 2004. **22**(8): p. 984-990.
54. Polak, D., et al., *Mucosal Vaccination Shapes the Expression of Salivary Antibodies and Establishment of CD8+ T-Cells.* Journal of Periodontology, 2014. **85**(7): p. 991-997.
55. Amuguni, H., et al., *Sublingual immunization with an engineered Bacillus subtilis strain expressing tetanus toxin fragment C induces systemic and mucosal immune responses in piglets.* Microbes Infect, 2012. **14**(5): p. 447-56.
56. Czerkinsky, C., et al., *Antibody-producing cells in peripheral blood and salivary glands after oral cholera vaccination of humans.* Infect Immun, 1991. **59**(3): p. 996-1001.
57. Kleanthous, H., et al., *Rectal and Intranasal Immunizations with Recombinant Urease Induce Distinct Local and Serum Immune Responses in Mice and Protect against Helicobacter pyloriInfection.* Infection and Immunity, 1998. **66**(6): p. 2879.
58. Johansson, E.-L., et al., *Nasal and Vaginal Vaccinations Have Differential Effects on Antibody Responses in Vaginal and Cervical Secretions in Humans.* Infection and Immunity, 2001. **69**(12): p. 7481.
59. Stary, G., et al., *VACCINES. A mucosal vaccine against Chlamydia trachomatis generates two waves of protective memory T cells.* Science, 2015. **348**(6241): p. aaa8205.
60. Mestecky, J., et al., *Selective induction of an immune response in human external secretions by ingestion of bacterial antigen.* Journal of Clinical Investigation, 1978. **61**(3): p. 731-737.
61. Jackson, D.E., et al., *Migration of IgA-bearing lymphocytes into salivary glands.* Cellular Immunology, 1981. **63**(1): p. 203-209.
62. Fukuizumi, T., et al., *Tonsillar application of formalin-killed cells of Streptococcus sobrinus reduces experimental dental caries in rabbits.* Infect Immun, 1999. **67**(1): p. 426-8.
63. Sun, Y., et al., *Flagellin-PAc Fusion Protein is a High-efficacy Anti-caries Mucosal Vaccine.* Journal of Dental Research, 2012. **91**(10): p. 941-947.
64. Holmgren, J. and A.-M. Svennerholm, *Vaccines against mucosal infections.* Current Opinion in Immunology, 2012. **24**(3): p. 343-353.
65. *Cholera vaccines: WHO position paper.* Wkly Epidemiol Rec, 2010. **85**(13): p. 117-28.
66. Harris, J.B., et al., *Cholera.* The Lancet, 2012. **379**(9835): p. 2466-2476.

67. Svennerholm, A.M., et al., *Local and systemic antibody responses and immunological memory in humans after immunization with cholera B subunit by different routes.* Bulletin of the World Health Organization, 1984. **62**(6): p. 909-918.
68. Arifuzzaman, M., et al., *Antigen-Specific Memory T Cell Responses after Vaccination with an Oral Killed Cholera Vaccine in Bangladeshi Children and Comparison to Responses in Patients with Naturally Acquired Cholera.* Clinical and Vaccine Immunology, 2012. **19**(8): p. 1304.
69. van Loon, F.P., et al., *Field trial of inactivated oral cholera vaccines in Bangladesh: results from 5 years of follow-up.* Vaccine, 1996. **14**(2): p. 162-6.
70. Sur, D., et al., *Efficacy and safety of a modified killed-whole-cell oral cholera vaccine in India: an interim analysis of a cluster-randomised, double-blind, placebo-controlled trial.* Lancet, 2009. **374**(9702): p. 1694-702.
71. Hallander, H.O., et al., *Calibrated serological techniques demonstrate significant different serum response rates to an oral killed cholera vaccine between Swedish and Nicaraguan children.* Vaccine, 2002. **21**(1): p. 138-145.
72. Pasetti, M.F., et al., *Immunology of gut mucosal vaccines.* Immunol Rev, 2011. **239**(1): p. 125-48.
73. Chong, A., et al., *The Role of Typhoid Toxin in Salmonella Typhi Virulence* . The Yale journal of biology and medicine, 2017. **90**(2): p. 283-290.
74. Dougan, G. and S. Baker, *Salmonella enterica serovar Typhi and the pathogenesis of typhoid fever.* Annu Rev Microbiol, 2014. **68**: p. 317-36.
75. Parry, C.M., et al., *Typhoid fever.* N Engl J Med, 2002. **347**(22): p. 1770-82.
76. Anwar, E., et al., *Vaccines for preventing typhoid fever.* Cochrane Database Syst Rev, 2014(1): p. Cd001261.
77. Szu, S.C., K.P. Klugman, and S. Hunt, *Re-examination of immune response and estimation of anti-Vi IgG protective threshold against typhoid fever-based on the efficacy trial of Vi conjugate in young children.* Vaccine, 2014. **32**(20): p. 2359-2363.
78. Sanderson, C., et al., *Global review of rotavirus morbidity and mortality data by age and region.* Report to WHO/IVB, 2011.
79. Desselberger, U., *Rotaviruses.* Virus Res, 2014. **190**: p. 75-96.
80. *Rotavirus vaccines. WHO position paper - January 2013.* Wkly Epidemiol Rec, 2013. **88**(5): p. 49-64.
81. Angel, J., M.A. Franco, and H.B. Greenberg, *Rotavirus immune responses and correlates of protection.* Current opinion in virology, 2012. **2**(4): p. 419-425.
82. Vesikari, T., et al., *Efficacy of human rotavirus vaccine against rotavirus gastroenteritis during the first 2 years of life in European infants: randomised, double-blind controlled study.* Lancet, 2007. **370**(9601): p. 1757-63.
83. Madhi, S.A., et al., *Effect of human rotavirus vaccine on severe diarrhea in African infants.* N Engl J Med, 2010. **362**(4): p. 289-98.
84. Parker, E.P., et al., *Causes of impaired oral vaccine efficacy in developing countries.* Future Microbiol, 2018. **13**: p. 97-118.
85. Woof, J.M. and J. Mestecky, *Mucosal immunoglobulins.* Immunol Rev, 2005. **206**: p. 64-82.
86. Horton, R.E. and G. Vidarsson, *Antibodies and their receptors: different potential roles in mucosal defense.* Frontiers in immunology, 2013. **4**: p. 200-200.

87. Jones, E.A. and T.A. Waldmann, *The mechanism of intestinal uptake and transcellular transport of IgG in the neonatal rat.* J Clin Invest, 1972. **51**(11): p. 2916-27.
88. Leach, J.L., et al., *Isolation from human placenta of the IgG transporter, FcRn, and localization to the syncytiotrophoblast: implications for maternal-fetal antibody transport.* J Immunol, 1996. **157**(8): p. 3317-22.
89. Baintner, K., *Transmission of antibodies from mother to young: Evolutionary strategies in a proteolytic environment.* Vet Immunol Immunopathol, 2007. **117**(3-4): p. 153-61.
90. Gill, R.K., et al., *IgG binding and expression of its receptor in rat intestine during postnatal development.* Indian J Biochem Biophys, 1999. **36**(4): p. 252-7.
91. Israel, E.J., et al., *Expression of the neonatal Fc receptor, FcRn, on human intestinal epithelial cells.* Immunology, 1997. **92**(1): p. 69-74.
92. Latvala, S., et al., *Distribution of FcRn Across Species and Tissues.* J Histochem Cytochem, 2017. **65**(6): p. 321-333.
93. Ye, L., et al., *Efficient mucosal vaccination mediated by the neonatal Fc receptor.* Nature biotechnology, 2011. **29**(2): p. 158-163.
94. Carvalho, J.J., et al., *Non-invasive monitoring of immunization progress in mice via IgG from feces.* In Vivo, 2012. **26**(1): p. 63-9.
95. Hansen, G.H., et al., *Antibodies in the small intestine: mucosal synthesis and deposition of anti-glycosyl IgA, IgM, and IgG in the enterocyte brush border.* Am J Physiol Gastrointest Liver Physiol, 2006. **291**(1): p. G82-90.
96. Besser, T.E., et al., *Transfer of functional immunoglobulin G (IgG) antibody into the gastrointestinal tract accounts for IgG clearance in calves.* Journal of virology, 1988. **62**(7): p. 2234-2237.
97. Kutteh, W.H., S.J. Prince, and J. Mestecky, *Tissue origins of human polymeric and monomeric IgA.* J Immunol, 1982. **128**(2): p. 990-5.
98. Brandtzaeg, P.E.R., *Presence of J chain in human immunocytes containing various immunoglobulin classes.* Nature, 1974. **252**: p. 418.
99. Johansen, F.E., et al., *Absence of epithelial immunoglobulin A transport, with increased mucosal leakiness, in polymeric immunoglobulin receptor/secretory component-deficient mice.* J Exp Med, 1999. **190**(7): p. 915-22.
100. Cerutti, A., *The regulation of IgA class switching.* Nat Rev Immunol, 2008. **8**(6): p. 421-34.
101. Sonoda, E., et al., *Differential regulation of IgA production by TGF-beta and IL-5: TGF-beta induces surface IgA-positive cells bearing IL-5 receptor, whereas IL-5 promotes their survival and maturation into IgA-secreting cells.* Cell Immunol, 1992. **140**(1): p. 158-72.
102. Macpherson, A.J., et al., *IgA responses in the intestinal mucosa against pathogenic and non-pathogenic microorganisms.* Microbes Infect, 2001. **3**(12): p. 1021-35.
103. Cazac, B.B. and J. Roes, *TGF-beta receptor controls B cell responsiveness and induction of IgA in vivo.* Immunity, 2000. **13**(4): p. 443-51.
104. Castigli, E., et al., *TACI and BAFF-R mediate isotype switching in B cells.* J Exp Med, 2005. **201**(1): p. 35-9.
105. Ng, L.G., C.R. Mackay, and F. Mackay, *The BAFF/APRIL system: life beyond B lymphocytes.* Mol Immunol, 2005. **42**(7): p. 763-72.

106. Weiner, H.L., *Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells.* Immunol Rev, 2001. **182**: p. 207-14.
107. Macpherson, A.J., et al., *The immune geography of IgA induction and function.* Mucosal Immunol, 2008. **1**(1): p. 11-22.
108. Naeim, F., et al., *2 - Principles of Immunophenotyping*, in *Atlas of Hematopathology*, F. Naeim, et al., Editors. 2013, Academic Press. p. 25-46.
109. Machholz, E., et al., *Manual restraint and common compound administration routes in mice and rats.* J Vis Exp, 2012(67).
110. Hirunpetcharat, C., et al., *Complete protective immunity induced in mice by immunization with the 19-kilodalton carboxyl-terminal fragment of the merozoite surface protein-1 (MSP1[19]) of Plasmodium yoelii expressed in Saccharomyces cerevisiae: correlation of protection with antigen-specific antibody titer, but not with effector CD4+ T cells.* J Immunol, 1997. **159**(7): p. 3400-11.
111. Li, W.M., M.B. Bally, and M.P. Schutze-Redelmeier, *Enhanced immune response to T-independent antigen by using CpG oligodeoxynucleotides encapsulated in liposomes.* Vaccine, 2001. **20**(1-2): p. 148-57.
112. Remy, J.J., et al., *Immunization against exon 1 decapeptides from the lutropin/choriogonadotropin receptor or the follitropin receptor as potential male contraceptive.* J Reprod Immunol, 1996. **32**(1): p. 37-54.
113. Wang, J., et al., *Eliciting T cell immunity against poorly immunogenic tumors by immunization with dendritic cell-tumor fusion vaccines.* J Immunol, 1998. **161**(10): p. 5516-24.
114. gsk. *Fachinformation Engerix(R)-B Erwachsene*. 2015 [cited 2018 11/25].
115. de Lalla, F., et al., *Immune response to hepatitis B vaccine given at different injection sites and by different routes: a controlled randomized study.* Eur J Epidemiol, 1988. **4**(2): p. 256-8.
116. Knuf, M., et al., *Safety, immunogenicity and immediate pain of intramuscular versus subcutaneous administration of a measles-mumps-rubella-varicella vaccine to children aged 11-21 months.* Eur J Pediatr, 2010. **169**(8): p. 925-33.
117. Ikeno, D., et al., *Immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1, NIBRG-14) vaccine administered by intramuscular or subcutaneous injection.* Microbiol Immunol, 2010. **54**(2): p. 81-8.
118. Cook, I.F., et al., *Reactogenicity and immunogenicity of an inactivated influenza vaccine administered by intramuscular or subcutaneous injection in elderly adults.* Vaccine, 2006. **24**(13): p. 2395-402.
119. von Zglinicki, T. and G.M. Roomans, *Changes of ion and water content of mouse intestinal cells after pilocarpine and isoproterenol stimulation.* Scanning Microsc Suppl, 1994. **8**: p. 25-34; discussion 34-5.
120. Crawford, P.C. and W.B. Clark, *Fimbria-specific antibodies in serum and saliva of mice immunized with Actinomyces viscosus T14V fimbriae.* Infect Immun, 1986. **54**(2): p. 507-15.
121. Damas, J., *Pilocarpine-induced salivary secretion, kinin system and nitric oxide in rats.* Archives Internationales de Physiologie, de Biochimie et de Biophysique, 1994. **102**(2): p. 103-105.
122. Hermiston, M.L., Z. Xu, and A. Weiss, *CD45: a critical regulator of signaling thresholds in immune cells.* Annu Rev Immunol, 2003. **21**: p. 107-37.

123. Tsimbouri, P., M.A. O'Donnell, and J.B. Wilson, *Selection and Enrichment of B Cells from Lymphoid Tissues*, in *Epstein-Barr Virus Protocols*, J.B. Wilson and G.H.W. May, Editors. 2001, Humana Press: Totowa, NJ. p. 411-421.
124. van Baarsen, L.G., et al., *The cellular composition of lymph nodes in the earliest phase of inflammatory arthritis.* Ann Rheum Dis, 2013. **72**(8): p. 1420-4.
125. Llop-Guevara, A., et al., *A GM-CSF/IL-33 pathway facilitates allergic airway responses to sub-threshold house dust mite exposure.* PLoS One, 2014. **9**(2): p. e88714.
126. Wang, K., G. Wei, and D. Liu, *CD19: a biomarker for B cell development, lymphoma diagnosis and therapy.* Exp Hematol Oncol, 2012. **1**(1): p. 36.
127. Stavnezer, J., J.E.J. Guikema, and C.E. Schrader, *Mechanism and regulation of class switch recombination.* Annual review of immunology, 2008. **26**: p. 261-292.
128. Park, S.Y., et al., *Focal adhesion kinase regulates the localization and retention of pro-B cells in bone marrow microenvironments.* J Immunol, 2013. **190**(3): p. 1094-102.
129. Rouaud, P., et al., *Elucidation of the enigmatic IgD class-switch recombination via germline deletion of the IgH 3' regulatory region.* J Exp Med, 2014. **211**(5): p. 975-85.
130. Davis, T.A., D.K. Czerwinski, and R. Levy, *Therapy of B-Cell Lymphoma with Anti-CD20 Antibodies Can Result in the Loss of CD20 Antigen Expression.* Clinical Cancer Research, 1999. **5**(3): p. 611.
131. Jin, J., et al., *The kinase TBK1 controls IgA class switching by negatively regulating noncanonical NF-kappaB signaling.* Nat Immunol, 2012. **13**(11): p. 1101-9.
132. Kamata, T., et al., *Increased Frequency of Surface IgA-Positive Plasma Cells in the Intestinal Lamina Propria and Decreased IgA Excretion in Hyper IgA (HIGA) Mice, a Murine Model of IgA Nephropathy with Hyperserum IgA.* The Journal of Immunology, 2000. **165**(3): p. 1387-1394.
133. Ampel, N.M., et al., *Ex Vivo Cytokine Release, Determined by a Multiplex Cytokine Assay, in Response to Coccidioidal Antigen Stimulation of Whole Blood among Subjects with Recently Diagnosed Primary Pulmonary Coccidioidomycosis.* mSphere, 2018. **3**(3).
134. Spierenburg, E., et al., *Stability of individual LPS-induced ex vivo cytokine release in a whole blood assay over a five-year interval*. Vol. 460. 2018.
135. Brandtzaeg, P., *Induction of secretory immunity and memory at mucosal surfaces.* Vaccine, 2007. **25**(30): p. 5467-5484.
136. Du, Y., et al., *Exploiting the Lymph-Node-Amplifying Effect for Potent Systemic and Gastrointestinal Immune Responses via Polymer/Lipid Nanoparticles.* ACS Nano, 2019. **13**(12): p. 13809-13817.
137. Seo, G.Y., et al., *Retinoic acid, acting as a highly specific IgA isotype switch factor, cooperates with TGF-beta1 to enhance the overall IgA response.* J Leukoc Biol, 2013. **94**(2): p. 325-35.
138. Macpherson, A.J. and T. Uhr, *Induction of Protective IgA by Intestinal Dendritic Cells Carrying Commensal Bacteria.* Science, 2004. **303**(5664): p. 1662.
139. Fagarasan, S., et al., *Critical Roles of Activation-Induced Cytidine Deaminase in the Homeostasis of Gut Flora.* Science, 2002. **298**(5597): p. 1424.

140. Blutt, S.E., et al., *IgA is important for clearance and critical for protection from rotavirus infection.* Mucosal immunology, 2012. **5**(6): p. 712-719.
141. Silvey, K.J., et al., *Role of immunoglobulin A in protection against reovirus entry into Murine Peyer's patches.* J Virol, 2001. **75**(22): p. 10870-9.
142. Yel, L., *Selective IgA deficiency.* Journal of clinical immunology, 2010. **30**(1): p. 10-16.
143. Weisz-Carrington, P., et al., *Organ and isotype distribution of plasma cells producing specific antibody after oral immunization: evidence for a generalized secretory immune system.* J Immunol, 1979. **123**(4): p. 1705-8.
144. Kunkel, E.J., et al., *CCR10 expression is a common feature of circulating and mucosal epithelial tissue IgA Ab-secreting cells.* J Clin Invest, 2003. **111**(7): p. 1001-10.
145. Kozlowski, P.A., et al., *Comparison of the oral, rectal, and vaginal immunization routes for induction of antibodies in rectal and genital tract secretions of women.* Infection and immunity, 1997. **65**(4): p. 1387-1394.
146. Boyaka, P.N., *Inducing Mucosal IgA: A Challenge for Vaccine Adjuvants and Delivery Systems.* The Journal of Immunology, 2017. **199**(1): p. 9.
147. Su, F., et al., *Induction of mucosal immunity through systemic immunization: Phantom or reality?* Human vaccines & immunotherapeutics, 2016. **12**(4): p. 1070-1079.
148. Mandal, P.K., et al., *Salivary IgA versus HIV and Dental Caries.* Journal of clinical and diagnostic research : JCDR, 2016. **10**(9): p. ZC61-ZC64.
149. Brunham, R.C., et al., *Correlation of host immune response with quantitative recovery of Chlamydia trachomatis from the human endocervix.* Infection and immunity, 1983. **39**(3): p. 1491-1494.
150. Balmer, P., et al., *Immune Response to Meningococcal Serogroup C Conjugate Vaccine in Asplenic Individuals.* Infection and Immunity, 2004. **72**(1): p. 332.
151. Xia, Y., et al., *Bridging Systemic Immunity with Gastrointestinal Immune Responses via Oil-in-Polymer Capsules.* Advanced Materials, 2018. **30**(31): p. 1801067.
152. Brinkmann, V., S. Muller, and C.H. Heusser, *T cell dependent differentiation of human B cells: direct switch from IgM to IgE, and sequential switch from IgM via IgG to IgA production.* Mol Immunol, 1992. **29**(10): p. 1159-64.
153. Bergqvist, P., et al., *T Cell-Independent IgA Class Switch Recombination Is Restricted to the GALT and Occurs Prior to Manifest Germinal Center Formation.* The Journal of Immunology, 2010. **184**(7): p. 3545.
154. Roco, J.A., et al., *Class-Switch Recombination Occurs Infrequently in Germinal Centers.* Immunity, 2019. **51**(2): p. 337-350.e7.
155. Wang, X., C. Allen, and M. Ballow, *Retinoic Acid Enhances the Production of IL-10 While Reducing the Synthesis of IL-12 and TNF-α from LPS-Stimulated Monocytes/Macrophages.* Journal of Clinical Immunology, 2007. **27**(2): p. 193-200.
156. Ertesvag, A., et al., *Retinoic acid inhibits in vivo interleukin-2 gene expression and T-cell activation in mice.* Immunology, 2009. **126**(4): p. 514-522.

6.Appendix

6.1. Abbreviations

ATRA	All-*trans* retinoic acid	**HEPES**	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
ANOVA	Analysis of Variance	**HPV**	Human papillomavirus
APRIL	A Proliferation-Inducing Ligand	**HRP**	Horseradish peroxidase
BAFF	B-cell activating factor	**HSV**	Herpes simplex virus
bLN	Brachial lymph node	**i.m.**	Intramuscular
BSA	Bovine serum albumin	**i.n.**	Intranasal
cAMP	Cyclic adenosine monophosphate	**Ig**	Immunoglobulin
Cat. No.	Catalog number	**iLN**	Inguinal lymph node
CCL	CC chemokine ligand	**LN**	Lymph node
CCR	CC chemokine recepor	**mAb**	Monoclonal antibody
CD	Cluster of Differentiation	**MAdCAM**	Mucosal addressin cell adhesion molecule
Clone no	Clone Number	**MFI**	Mean fluorescence intensity
CT	Cholera toxin	**Ova**	Ovalbumin
CXCR	CXC chemokine receptor	**PBS**	Phosphate-buffered saline
DAPI	4′,6-diamidino-2-phenylindole	**PEG**	polyethylene glycol
DC	Dendritic cell	**PS**	Penicillin:streptomycin solution
ddH_2O	double-distilled water	**RAR**	Retinoic acid receptor
DL	Detection limit	**s.c.**	subcutaneous
DMSO	Dimethyl sulfoxide	**SD**	Standard deviation
ELISA	Enzyme-linked Immunosorbent Assay	**SHIV**	Simian-human immunodeficiency virus
ETEC	Enterotoxigenic *Escherichia coli*	**SIV**	Simian immunodeficiency virus
FACS	fluorescence-activated cell scanning	**spp.**	several species
FBS	Fetal bovine serum	**STIKO**	Ständige Impfkommission
FcRn	Neonatal Fc receptor	**TGF-β**	Transforming growth factor-β

GeoMFI	Geometric mean fluorescence intensity	**VCAM**	Vascular cell adhesion molecule
HBSS	Hank's Balanced Salt Solution	**WHO**	World Health Organization

6.2. Acknowledgements

The thesis presented here was accomplished in the von Andrian Laboratory, Department of Immunology at Harvard Medical School between August 2017 and March 2018.

First of all, I would like to thank Prof. Ulrich von Andrian for giving me the unique opportunity to pursue this medical doctoral thesis in his laboratory and for constant valuable and constructive feedback.
My thanks also go to Dr. Bruno Raposo who patiently taught me all the methodology necessary, was always available for many questions and whose enthusiasm for research made my time in the lab exciting and my interest in immunology grow.
Thank you also to all other members of the von Andrian lab for support, discussions and teaching in many occasions.

I also want to give many thanks to Prof. Manfred Lutz and PD Dr. Götz Ulrich Grigoleit for agreeing to act as Supervisor and even Primary Supervisor in a project abroad and for the helpful advice in the several thesis meetings.

Thank you to the Graduate School of Life Sciences (GSLS) for the scholarship including the financial funding that made the participation in this project possible for me and for the personal support with the organization of this thesis project.
Thank you also to the Studienstiftung des deutschen Volkes for financially and logistically supporting me in this research project.
Thank you to the Bill & Melinda Gates Foundation who funded the overall project mentioned in the thesis.

I would also like to thank the Studentische Statistische Beratung, Lehrstuhl für klinische Epidemiologie und Biometrie at Universität Würzburg for helpful advice with the statistics in this thesis.

And last but not least, thank you to my family and friends.

6.3. Affidavit

I hereby confirm that my thesis entitled “Enhancing mucosal B cell responses with all-*trans* retinoic acid” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis
Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg,

Michael Reinhart

Hiermit erkläre ich an Eides statt, die Dissertation “Enhancing mucosal B cell responses with all-trans retinoic acid“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.
Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat

Würzburg,

Michael Reinhart

6.4. Curriculum Vitae