Doctoral Thesis

Mucosal and systemic antibody responses induced by virus-like particles

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MUCOSAL AND SYSTEMIC ANTIBODY RESPONSES INDUCED
BY VIRUS-LIKE PARTICLES

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1 SUMMARY

Vaccination represents the most successful and cost-effective preventive measure against infectious diseases. Most vaccines currently in use are administered through intramuscular or subcutaneous injection. These routes of immunization are efficient at inducing strong systemic IgG responses which can easily be monitored in blood samples; however they elicit poor mucosal immunity. As most infectious pathogens enter the body through mucosal surfaces an ideal prophylactic vaccine should, in addition to inducing systemic humoral and T cell immunity, also induce mucosal immunity which is mainly mediated by secretory IgA (SIgA).

The aim of this work was to evaluate the feasibility of using virus-like particles (VLPs) as antigen carriers to develop intranasal (i.n.) vaccines for the induction of antibody responses which are the principal mediator of protection induced by vaccination. In particular, we studied the mechanisms of induction of IgA responses which are the hallmark of mucosal immunity.

The first part of this study aimed to characterize the effect of the immunization route on the ability of VLPs to induce mucosal and systemic IgA and IgG responses. We compared the ability of i.n. and subcutaneous (s.c.) routes of immunization to induce VLP-specific antibody responses. We found that both routes efficiently induced VLP-specific IgG and IgA responses in serum, and germinal centers and memory B cells were induced with similar efficiency by both routes of immunization. In a marked contrast, the induction of mucosal IgA responses was an intrinsic feature of the i.n. route.

As we found that the i.n. administration of VLPs efficiently induces mucosal IgA responses, whilst serum IgA can be induced by the i.n. as well as the s.c. routes, we further studied the mechanisms controlling the regulation of mucosal and systemic IgA responses. We surprisingly found that distinct mechanisms regulated IgA responses dependent on the site of antigen exposure. Mucosal IgA responses elicited by the i.n. route of immunization required T helper (Th) cells as well as BAFF, APRIL and TGFβ. In addition, TLR signaling to lung dendritic cells and alveolar macrophages was required for efficient mucosal IgA. In contrast, the systemic IgA response elicited by the s.c. route was independent of Th cells and simply required direct TLR signaling to B cells in combination with the stimulus given by the multivalent antigen.
Finally, the mechanism underlying the generation of the systemic IgG response upon i.n. immunization was assessed. We found that B cells take up VLPs in the lung and transport it through the bloodstream to the splenic follicular compartment where the systemic B cell response is initiated.

Taken together, this thesis has confirmed and extended the notion that VLPs can efficiently be applied as a vaccine platform for the development of mucosal antibody-based vaccines. Furthermore it has brought new insights into the mechanisms controlling the regulation of IgA as well as IgG responses induced by the i.n. route of immunization.
2 ZUSAMMENFASSUNG

Impfen ist die wirksamste und kosteneffektivste Schutzmassnahme gegen infektiöse Krankheiten. Die meisten Impfstoffe werden zurzeit intramuskulär oder subkutan injiziert. Diese Administrationsrouten induzieren starke systemische IgG Antworten, welche einfach in Blutproben nachgewiesen werden können; allerdings lösen sie schwache Immunantworten im Schleimhautsystem aus. Weil die meisten infektiösen Pathogene via die Schleimhäute in den Körper eintreten, sollte eine ideale Schutzimpfung, zusätzlich zur systemischen humoralen und T-Zell vermittelten Immunität, auch das Immunsystem der Schleimhäute anregen, in welchem der Antikörperisotyp IgA (SIgA) dominiert.

Ziel dieser Arbeit war die Entwicklung einer intranasalen (i.n.) Impfung mit virus-ähnlichen Partikeln als Antigenträger, so genannte „virus like particles (VLPs)“, mit deren Hilfe Antikörperreaktionen hervorgerufen werden, welche den Impfschutz gewährleisten. Ins Besonderem studierten wir die Induktionsmechanismen der IgA Antwort, die speziell für das Immunsystems in der Schleimhaut sind.

Im ersten Teil dieser Studie charakterisierten wir den Einfluss der Immunisierungsroute; wir untersuchten, ob VLPs in der Lage sind eine IgA und IgG Antwort in der Schleimhaut und systemisch im Körper auszulösen. Daher verglichen wir, ob i.n. und subkutane (s.c.) Immunisierungsrouten VLP-spezifische Antikörperantworten induzieren können. Wir stellten fest, dass beide Routen gleich effizient VLP-spezifische IgG und IgA Antworten im Serum induzierten und dass sie auch die gleiche Anzahl an B-Zellen in den Keimzentren und B-Gedächtniszellen generierten. Im Gegensatz dazu wurde nur mit der i.n Route auch in den Schleimhäuten eine IgA Anwort hervorgerufen.

Weil wir herausfanden, dass die i.n. Administration von VLPs eine effiziente IgA Antwort in der Schleimhaut auslöste, während IgA im Serum mit Hilfe der i.n. wie auch s.c. Route induziert werden konnte, befassten wir uns mit dem Mechanismus, welcher die Schleimhaut und die systemische IgA Antwort reguliert. Überraschenderweise entdeckten wir, dass verschiedene Mechanismen die IgA Antwort regulierten und sie abhängig vom Ort der Antigenexposition waren. Eine IgA Antwort in den Schleimhäuten, die durch eine i.n. Immunisierung ausgelöst wurde, benötigte T-Helferzellen sowie auch BAFF, APRIL und TGFβ. Zusätzlich wurden auch TLR Signale zu dendritischen Zellen in der Lunge und alveolaren Makrophagen für eine effiziente Produktion von IgA in der Schleimhaut gebraucht. Im Gegensatz dazu benötigte eine systemische IgA Antwort, die durch die
...subkutane Route hervorgerufen wurde, keine T-Helferzellen, sondern brauchte nur ein direktes TLR Signal zu den B-Zellen und Stimulation durch ein multivalentes Antigen.

Schlussendlich wurde auch noch der Mechanismus untersucht, welcher systemische IgG Antworten generiert, die durch i.n. Immunisierung hervorgerufen wurden. Wir beobachteten, dass B-Zellen VLPs in der Lunge aufnehmen und sie via den Blutstrom zum Follikel bringen, wo eine systemische B-Zell Antwort initiiert wird.

Diese Studie hat bestätigt und weiter abgesichert, dass VLPs als effiziente Schutzimpfung für die Entwicklung von Antikörper-basierten Impfungen in der Schleimhäuten eingesetzt werden können. Darüber hinaus haben wir neue Erkenntnisse über den Mechanismus gewonnen, der IgA sowie auch IgG Antworten, welche mit der i.n. Route hervorgerufen wurden, reguliert.
3 GENERAL INTRODUCTION

3.1 B cell activation

Protection from a great diversity of pathogens is accomplished by the combination of the innate and adaptive arms of the immune system. While the innate branch of the immune system contributes to the rapid and nonspecific response to pathogens, the adaptive arm is responsible for mediating a more elaborated immune response which is able to confer specificity, diversity, self-nonself discrimination and memory.

The adaptive immune system relies on the appropriate activation of B and T lymphocytes upon antigenic stimulation. The activation of B cells is initiated in response to specific antigens which bind to and cross-link B cell receptors (BCRs) leading to tyrosine phosphorylation of the intracellular Igαβ immunoreceptor tyrosine activation motifs (ITAMs) by Src-family kinases, such as Lyn. This leads in turn to the activation of Syk (1-4). This activation events triggers the assembly of the B cell signalosome which is composed of a great range of intracellular signaling molecules, such as Vav, Bruton’s tyrosine kinase (Btk), phosphoinositide 3-kinase (PI3K), and phospholipase C-γ2 (PLCγ2) (4, 5), as well as adaptor proteins, such as B cell linker (Blnk) (6). The B cell signalosome occurs in the site where BCR microclusters are formed and they might be responsible for the initiation of signaling (7, 8). As a consequence of their activation, B cells up regulate MHC class II (major histocompatibility complex II) molecules, co stimulatory molecules such as B7-2 and adhesion molecules such as ICAM1(9). Furthermore, the antigens are internalized via the BCR and processed within endosomal compartments. After proteolyses the resulting peptides are subsequently presented in the context of MHC class II molecules in order to activate and/or receive cognate T cell help (10). Thus, B cell activation requires the coordination of both intracellular signaling pathways and intercellular communication (7).
3.2 Activation by TI antigens

Many antigens are multivalent and therefore have mitogenic properties. By themselves, they can provide a level of BCR crosslinking optimal for B cell activation. In this case the presence of Th cells is dispensable and such antigens are classified as T cell-independent (TI) antigens. Bacterial polysaccharides and repeating surface molecules on viruses are typical examples of such antigens. Activation of B cells by TI antigens leads to a strong and rapid antibody response (11, 12). Pioneer study into the initiation of B cell activation in response to multivalent antigen showed that a cluster of 10 to 20 mIg receptors was necessary to induce BCR crosslinking and activate B cells. Such structures were defined as “immunons” (13). It has also been shown that the first signal for B cell activation in response to a TI antigen is provided by a small number of highly cross-linked clusters of mIg (14).

TI antigens can be further subdivided into two categories: TI type 1 (TI-1) and TI type 2 (TI-2). TI-2 antigens are polysaccharide antigens which usually have high molecular weight and highly repetitive antigenic epitopes (15). Prototype TI-2 antigens are TNP-Ficoll, bacterial polysaccharides and TNP-dextran. Because TI-2 are not protein antigens, they can not be degraded into peptides and therefore can not activate T cells through the recognition of peptide-MHC class II complex by T cell receptors (TCRs). TI-2 antigens were defined based on their inability to induce antibody responses in CBA/N (xid/xid mice) in contrast to TI-1 and TD antigens (16-18). The CBA/N mouse strain has a well-characterized mutation in Bruton’s tyrosine kinase (Btk) gene which is localized on the X-chromosome (19). Btk is an enzyme expressed at all stages of B cell development (except the terminally differentiated plasma cell stage) (20-22) and is associated with BCR signaling. Therefore, BCR signaling is decreased in Btk-deficient mice. Furthermore this syndrome affects B cell development because pre B cells are unable to make genetic rearrangement and consequently they can not differentiate into mature B cells (23). Although the current paradigm is that the highly repetitive epitopes (characteristic of TI-2 antigens) are sufficient to induce BCR cross-linking which in turn efficiently induce plasma cell differentiation (11) (Fig.1.1), there is an emerging model in which antigen-presenting cells (APCs) such as dendritic cells (DCs) can directly induce class switch recombination (CSR) in B cells (24, 25). This DC-dependent, but T cell and CD40L-independent CSR, is accomplished by the secretion of B lymphocyte stimulator protein (BLyS/BAFF) and proliferation-inducing ligand (APRIL) by DCs which can directly
stimulate B cells upon interaction with their receptors such as TACI (transmembrane activator and calcium modulator and cyclophylin ligand interactor) and BAFF receptor (BAFFR) which are expressed on B cells (26) (Fig. 1.2).

TI-1 antigens differ from TI-2 antigens as they can induce antibody response in CBA/N xid mice and moreover they usually do not require a second signals (i.e. cytokines) for B cell activation as TI-2 antigens usually do (27). The most known TI-1 antigen is lipopolysaccharide (LPS), which has mitogenic activity and induces polyclonal antibodies when used at high concentrations while at low concentrations it induces LPS-specific antibodies. LPS can also activate B cells through Toll-like receptor 4 (TLR4) (28).

Also the glycoprotein of vesicular stomatitis virus (VSV-G) is considered TI-1 antigen, but in contrast to LPS (at high concentrations) it does not induce a polyclonal B cell activation (27). VSV-G behaves as TI-1 antigen when it is found in a highly organized conformation in the viral envelope. In contrast, VSV-G when expressed on infected cells in the absence of VSV nucleoprotein or matrix protein is poorly organized and therefore is unable to induce antibody responses in CBA/N xid mice as the highly organized form does. Additionally, the soluble form of VSV-G, which is completely non-organized, was found to be a TD antigen. Therefore, the same antigen can behave as a TI-1, TI-2 or even a TD antigen depending on the degree of organization (29).

Antigens which are highly organized and have a proteinic nature can be TI and TD antigens at the same time. Most viruses and virus-like particles (VLPs) are such antigens. Similarly to VSV-G in the viral particle, VLPs are highly organized and can strongly crosslink BCRs, behaving as TI-1 antigen and inducing high IgM responses in the absence of Th cells (30). But, as VLPs are essentially protein particles, they can be processed by APCs and the derived peptide can be presented to T helper cells in association with MHC class II molecules, which in turn elicits a strong IgG response. Actually, most antigens found in nature contain both TI and TD antigens.
Figure 1.1: Activation of B cell by TI antigen. Binding of multivalent antigens to B cells is sufficient to induce massive BCR cross linking which is sufficient to activate B cells and induce plasma cell differentiation.

Figure 1.2: Emerging model of B cell activation by TI antigen. DCs take up antigens, become activated and secrete BAFF and APRIL which interacts with TACI and BAFFR on B cells. Additionally, the unprocessed antigen is transferred in an intact form to B cells causing BCR cross linking. Both mechanisms lead to intracellular signaling transduction events and plasma cells differentiation.
3.3 Activation by TD antigens

Pioneering studies of B cell activation were performed with haptens. Haptens usually are small chemical molecules (2,4-dinitrophenyl, DNP is a prototype hapten) which need to be coupled to protein carriers (such as chicken gamma globulin, CGG) in order to elicit an antibody response.

It was observed that such antigens alone were unable to activate B cells by themselves and therefore required additional signals provided by T cell help to cause B cell division and differentiation. For this reason their designation as thymus dependent (TD) antigens. Any antigen containing protein falls into this category of antigen. As mentioned before, most natural pathogens contain both TI and TD antigens (Figure 1.3) and most surfaces of pathogens have TI (repetitive) and TD (protein) characteristics.

The most important outcome of B cell activation is the generation of humoral memory which is the maintenance of specific antibody levels for long period of time post infection or vaccination. Generally, it is accomplished by follicular (FO) B2 B cells after an antigen-specific germinal center (GC) reaction. Only TD antigens can induce germinal center responses. Germinal centers are specialized sites in the B cell follicle of secondary lymphoid organs (31) where B cells undergo massive rounds of proliferation. Most importantly, the GC is the site where B cells undergo somatic hypermutation (SHM) and class-switch recombination (CSR). Class-switch recombination is a DNA rearrangement event in which deletion replaces one Ig heavy chain (IgH) constant region gene segment (usually μ) with a more 3’ gene segment (γ, ε or α) (32). The mouse genome encodes 4 Cγ (γ1, γ2a, γ2b and γ3), one Cε and one Cα region gene. After CSR, B cells can switch the IgM responses to IgG1, IgG2a, IgG2b, IgG3, IgA and IgE isotypes (33). IgM antibodies are mainly found in serum and are responsible for controlling the early stages of infection (34). IgG is the predominant immunoglobulin found in serum and their different isotypes differ in their capacity to opsonize bacteria and lyse infected target cells. Particularly, IgG2a has been shown to be potent in controlling viral infection (35) due to its capacity to activate the complement system (36), bind to Fc receptors expressed on phagocytes (37) and induce antibody dependent cell-mediated cytotoxicity (ADCC) (38). In humans, IgG1 and IgG4 correspond to IgG2a and IgG1 in the mouse system (39). Yet IgA is the most prominent antibody found at mucosal secretions. There, IgA is the primary mediator of immunity due to its capacity to prevent the initial adherence and infection of incoming pathogens (40). IgA is also found at lower levels
in serum. Systemic IgA have the function to bind pathogens that have invaded the circulation and via binding to FcαRI expressed on Kupfer cells it mediates phagocytosis (40). Last, the IgE antibodies are normally found at low concentrations in serum and their levels are highly elevated in atopic diseases such as asthma, allergic rhinitis and atopic dermatitis as well as in patients with parasitic infections. In contrast to other Ig isotypes, most IgE is found bound by its high-affinity receptor, FcεRI, expressed on mast cells and basophils. The binding of specific antigen promotes IgE crosslinking and subsequent release of inflammatory mediators (i.e. histamine and leukotrienes) which in turn mediate the clinical manifestations of allergy (41).

The switch process requires a variety of transcription factors and enzymatic activities mediated by several cell type-specific and general DNA repair enzymes. Activation-induced cytidine deaminase (AID) plays an essential role in the CSR process (42, 43), as Aicda-knockout mice or patients with AICDA mutations develop hyper-IgM type 2 syndrome and fail to generate class-switched antibodies (44-46).

During the germinal center reaction, after encountering the cognate antigen, FO B cells receive help provided by germinal center T cells (47). Antigen-specific T helper cells and follicular dendritic cells (FDCs) are essential for the generation of GC reactions and both cells are enriched in the light zone of GCs (48). The main role of FDCs is to retain antigens in an intact form for long periods of time via binding of antigen-antibody immune complexes to the complement (CD21 and CD35) and Fc receptors expressed on the surface of FDCs (49-51). T helper cells provide help to B cells via interaction of CD40 ligand (CD40L)-CD40 and inducible T-cell co-stimulator (ICOS)-ICOS ligand, which are expressed on T and B cells respectively (52-54). Additionally, B cell activation can be amplified by T cells derived cytokines such as IL-4, IL-5, IL-21, TGFβ and IFNγ (55).

Germinal centers are also the site of affinity maturation. It is thought that centrocytes (GC B cells of the light zone) are selected by antigen on FDCs and only B cells with high affinity receptors are able to survive. Such high affinity B cells emerging in germinal centers give rise to long-lived plasma cells and memory B cells which are ascribed to provide the humoral memory (56). The Figure 1.4 schematically illustrates a germinal center reaction.
Figure 1.3: Activation of B cell by TD antigen. Naturally occurring pathogens are taken up by APCs and their protein antigens are processed and presented to cognate CD4+ T cell help in association with MHC class II molecules. Consequently, activated T cells up regulate CD40L which interacts with CD40 on B cells. Additionally, pathogens also contain repetitive surface structures and therefore can induce BCR cross linking. Both, CD40-CD40L interaction and BCR cross linking induce signaling for B cell activation and plasma cell differentiation.
3.4 Mucosal immunity and secretory IgA

The great majority (~90%) of human mucosal IgA plasma cells (PCs) generally synthesize the J (“joining”) chain (57, 58), an essential peptide responsible for correct polymerization of polymeric IgA, pIgA (IgA + J) and pentameric IgM (59). This leads to the formation of IgA dimers which may bind to the polymeric Ig receptor (pIgR). The resulting ligand-receptor complexes are endocytosed, transcytosed through the epithelial cell and after apical pIgR cleavage, dimeric IgA is released into the mucosal secretion. The secretory component (SC) remains bound to the SIgA and confers stability to secretory antibodies (SIgA is the most stable antibody due to the covalent bonding between SC and α-chain of pIgA) (59, 60).

The translocation of pIgA to the human intestine lumen by the pIgR mechanism of transport (~40mg/kg) exceeds the daily amount of IgG production (~30mg/kg). For this reason, the intestinal mucosa is probably quantitatively the most important effector organ of antibody-mediated immunity and certainly the most important site of antibody production.

In collaboration with a variety of innate mucosal defence mechanisms (61), the two most important functions of SIgA is to perform immune exclusion (a mechanism in which SIgA
can promote the entrapment of antigens in the mucus, preventing direct contact of pathogens with the mucosal surface) of exogenous antigens and mediate intracellular neutralization of incoming pathogens within epithelial-cell vesicular compartment during pIgR-mediated transport (62, 63) (Figure 1.5). Furthermore, due to its high stability, SIgA can perform its activity for prolonged period of time in hostile environments rich in proteases such as mucosal cavities as the gut lumen, respiratory tract and oral cavities (61).

SIgA can alternatively mediate defence at mucosal surfaces by blocking or hindering the molecules of pathogens which are responsible for epithelial adherence (64). Furthermore, dimeric IgA (secreted by local IgA plasma cells) underlying the epithelial barrier might prevent mucosal-cell infection by transporting the pathogens (which eventually breached the epithelial barrier) back to the lumen via pIgR(65) or even by mediating antibody-dependent cell-mediated cytotoxicity (ADCC) which in turn leads to the lyses of infected cells (40, 66). For these reasons, IgA antibodies are important effectors of the immune system and understanding the mechanisms that control IgA class switching are therefore of interest.

**Figure 1.5: Major mechanisms of defence mediated by IgA.** 1. Immune exclusion; 2. Intracellular neutralization; 3. In case the antigen escapes from the two previous mechanisms, it can still be trapped in the lamina propria and further transported back to the lumen.

### 3.5 Regulation of IgA class switching and IgA responses

IgA class switching varies depending on the tissue where IgA plasma cells are differentiating. It can occur via both T-cell-dependent (TD) and T-cell-independent (TI) pathways, and the resulting antibodies can act against both pathogenic and commensal microorganisms (157).
3.6 T-cell-independent (TI) IgA class switching

TD antibody responses usually take from 5 to 7 days to be mounted, which are far too late to combat infectious pathogens that replicate quickly and commensal bacteria (157). For this reason some B cell subsets can rapidly switch to IgA in the absence of CD40L and T cell help (67).

In the human immune system B1 B cells are absent, but nevertheless TI IgA responses might occur because HIV infected patients with CD4⁺ T cell-deficiency as well as patients lacking CD40 still are able to induce intestinal IgA class switching (68). A B cell subset which might contribute to this TI IgA response in humans is the transitional B cells. Transitional B cells are immature new bone marrow emigrants B cells found in the periphery, which express polyreactive antibodies encoded by unmutated V(D)J genes (69-72).

In mice, intestinal TI IgA responses are mediated mainly by peritoneal B1 B cells which differ from conventional B2 (follicular) B cells. Indeed, B1 B cells express unmutated IgA which have not undergone SHM and therefore they can recognize multiple specificities with low affinities (73, 74). Moreover, the repertoire of B1 B cells is usually restricted and its reactivity is skewed towards bacterial carbohydrate antigens and autoantigens (75). Not only in the gut, but also systemically, TI IgA responses can occur. For the systemic TI IgA responses, splenic marginal zone (MZ) B cells seem to be the major antibody producer (73). Indeed, *in vitro* studies have shown that upon BAFF, LPS and TGFβ stimulation, MZ and B1 B cells present enhanced capacity for IgA class switching when compared to B2 B cells (76). Similar to B1 B cells, splenic MZ B cells also express polyreactive IgA (in addition to IgM) which may confer protection against bacteria that escaped the epithelial-cell barrier (67, 73).

Although the role of TLR ligands as IgG switching factor has been previously described *in vitro* (77) as well as *in vivo* (78-82), the role of TLR signaling in regulating IgA responses has not been well studied so far. Actually, there is one study showing that LPS together with TGFβ1 can initiate germline Cα gene transcription and CSR from Cµ to Cα in mouse B cells (83); however the mechanisms by which TLR ligands trigger IgA CSR remains unclear and whether there is an *in vivo* relevance for these *in vitro* findings is unclear.

Microbes can stimulate B cells not only by delivering TLR signals. They additionally stimulate DCs which in turn secrete BAFF and APRIL. These two molecules are soluble B-cell stimulating factors belonging to the tumour necrosis factor (TNF) family which resemble the CD40L (24, 68, 84). *In vivo*, DCs sample TI antigens and thereafter transfer them to a
non-degradative endocytic pathway (85). A fraction of the endocytosed antigen is subsequently recycled to the plasma membrane and consequently presented to B cells (85-88). Thus, the same DC presents native antigens to B cells and stimulates them with BAFF and APRIL.

The effect of BAFF and APRIL in controlling TI IgA CSR seems to depend on the expression of TACI on B cells, as TACI-deficient B cells do not express AID and are unable to undergo CSR in response to BAFF or APRIL (26) and IgA levels specific to TI antigens are reduced in serum of TACI\(^{-/}\) mice (89). Interestingly, B1 B cells express higher levels of TACI which further support the pivotal role of TACI in TI IgA CSR (90). In addition to induce TI IgA CSR, it has been recently shown that TACI also enhances CD40-dependent plasma cell differentiation (91). APRIL and BAFF can also bind to B cell maturation antigen (BCMA) and BAFF has an additional, specific receptor, the BAFF receptor (BAFFR). Binding of BAFF to BAFFR promotes B cell survival signals and to a lower extent it can also induce CSR in peripheral B cells (26, 92). In contrast, the binding of BAFF and APRIL to BCMA does provide survival signals to plasma cells (93, 94), but has no effect on CSR. In contrast to the mouse system, where BAFF and APRIL are sufficient to induce IgA secretion (26), human B cells seem to require additional signals via BCR or TLR. Although there is accumulating evidence for a key role of TACI in regulating IgA CSR, its clear mechanism of action remains unknown.

Furthermore, it was recently shown that nitric oxide (NO) plays a crucial role in IgA class switching and secretion (95). In this report, the authors showed that mice deficient in the inducible isoform of nitric oxide synthase (iNOS) presented a strong reduction in serum as well as in intestinal IgA. Importantly, iNOS controlled TI and TD IgA responses (95). The TI IgA responses probably were affected because the expression of APRIL and BAFF in iNOS\(^{-/}\) DCs from the GALT (gut associated lymphoid tissue) was strongly decreased compared to wild type DCs. This in turn might explain the abrogation of TI IgA responses in iNOS\(^{-/}\) mice. Interestingly, iNOS expression showed to be dependent on TLR stimulation, as DCs from germ-free or MyD88\(^{-/}\) mice express reduced levels of iNOS. The direct role of iNOS in regulating IgA CSR and secretion was demonstrated by the transfer of WT GALT-DCs into iNOS\(^{-/}\) mice which could restore the serum IgA levels in the recipient mice.

All these studied mentioned above were trying to elucidate the mechanisms controlling IgA responses in the gut. However, much less is known about the mechanisms controlling IgA responses induced in the respiratory tract. One of the few examples studied is influenza virus
infection, where virus-specific IgA responses generated in the respiratory tract were independent of cognate T-B interaction but needed bystander CD4⁺ T cell help; in contrast to IgM and IgG responses which were more dependent on cognate interactions (96).

3.7 T-cell-dependent (TD) IgA class switching

As mentioned above, most antigens occurring in nature, initiate antibody responses in germinal centers which generally are dependent on cognate interactions between antigen-specific B cells and CD4⁺ T cell help expressing CD40L, a tumour-necrosis factor (TNF) family member, which binds to CD40 expressed on B cells (97).

In the intestine, the richest site for IgA production, TD antibody responses are strongly biased towards IgA and the inductive sites of B cell responses are mainly the organized lymphoid tissue of Peyer’s patches, mesenteric lymph nodes and isolated lymphoid follicles (73, 74, 98).

In addition to CD40L, TGFβ1 (a cytokine secreted by many cell-types, including B cells and various CD4⁺ T cells subsets) is also essential for TD IgA class switching because the promoters upstream of Cα genes, more specifically the Iα promoters, become activated in response to TGFβ1 (99-101).

In mice and humans, CD40L can also induce IgA class switching in combination with other cytokines than TGFβ1, (especially with Th2 cytokines), including IL-2, IL-4, IL-5, IL-6, IL-10 and vasoactive intestinal peptide (VIP) (58, 102-107). The mechanism by which these cytokines affect IgA responses seems to be due to the enhancement of TGFβ1 secretion by B cells exposed to CD40L. Additionally, these cytokines may promote B cell proliferation and plasma-cell differentiation of IgA B cells which previously switched in response to CD40L and TGFβ1(58, 108).

As mentioned above, it has been shown that iNOS regulates IgA responses via both, the TD and TI mechanisms (95). The TD IgA responses were impaired because B cells from iNOS⁻/⁻ mice expressed lower levels of TGFβRII, the ligand binding chain of the TGFβR (109, 110) and Smad proteins (which are downstream molecules of the TGFβR induced upon TGFβ1 and CD40 stimulation) (95). The role of iNOS in controlling IgA responses by non-GALT DCs (i.e. systemic DCs and mucosal DCs present in other sites such as respiratory tract) remains elusive.
3.8 Virus-like particles as antigen carrier for intranasal vaccination

Prevention of infectious disease by vaccination remains a compelling goal in an effort to improve public health in industrialized and developing communities.

The vast majority of infectious involve the mucosae with regard to initial microbial colonization and/or entry into the body. For these reasons mucosal routes of vaccine delivery is of a great interest. Among the possible mucosal routes (including oral, intranasal, rectal, conjunctial and vaginal) for immunization of humans, oral and intranasal (i.n.) are practical for all ages and both genders (111). Specialized microfold (M) cells overlying mucosa-associated lymphoid tissues (MALT) in the intestine and nose constitute effective portals by which vaccines reach underlying inductive sites for immune responses (112). Properly formulated mucosally administered vaccines can stimulate any relevant type of immune response: secretory IgA (SIgA), neutralizing systemic IgG (against toxins and viruses) and cell-mediated responses. Because they elicit SIgA, mucosal vaccines are attractive for application against pathogens that cause mucosal infection or invade through the mucosa.

Regarding the best immunization route for mucosal vaccines, i.n. vaccination has shown particular potential because in addition to induce humoral immunity, it can also elicit CTL immune responses, not only at the site of delivery, but also at distal tissues (61). Specifically, it has been shown that in mice, monkeys and humans, i.n. vaccination has been able to induce specific mucosal IgA responses in the salivary glands, upper and lower respiratory tract, male and female genital tracts and at lower extend in the small and large intestine (113-116). Despite these data, it has also been shown that IgA AFCs generated in the airways lack the expression of α4β7 integrin (the gut homing receptor) and therefore they might fail to migrate to the intestine (117). Thus, the capacity of i.n. vaccination in inducing IgA antibodies in the gut is somewhat contradictory. The i.n. route is also able to induce cytotoxic T cell responses (CTLs) in distant mucosal tissues including the female genital tract (118). Additionally, it has been shown that i.n. vaccination induced higher systemic antibody responses when compared to other mucosal immunization routes (113, 114).

Although a great number of studies have been performed in the past years evaluating strategies to develop efficient and safe mucosal vaccines, it has to be mentioned that the development of mucosal vaccines still remains a challenge. This is because similarly to pathogens, mucosal vaccines delivered onto mucosal surfaces usually can be diluted in mucosal secretions, trapped in the mucus, degraded by proteases and even excluded by
epithelial barriers (61). In order to overcome these problems, mucosal vaccine formulations should mimic mucosal pathogens in the sense that they might be particulate, stimulate the innate immune system and target the mucosa and underlying DCs. In this respect vaccines based on virus-like particles (VLPs) are promising candidates for development of effective mucosal vaccines for use in humans. In practice, however large amounts of VLPs are needed for mucosal immunization because, as mentioned above, small particles tend to be trapped in the mucus and moreover only a small proportion of the administered dose will enter the mucosal inductive sites. Since this subject is of special interest for the present work, it will be better discussed in the chapter 2.1 “Efficient induction of mucosal and systemic immune responses by virus-like particles administered intranasally: implications for vaccine design”.
4 RESULTS

4.1 Part I: Efficient induction of mucosal and systemic immune responses by virus-like particles administered intranasally: implications for vaccine design

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Abbreviations used in this paper: AFC, antibody-forming cell; VLP, virus-like particle; i.n., intranasal; s.c., subcutaneous; LN, lymph node; BAL, bronchus-alveolar lavage; BM, bone-marrow; MLN; mediastinal lymph node; CLN, cervical lymph node; DC, dendritic cells; BCR, B cell receptor; GC, germinal center; FcRn, neonatal Fc receptor; pIgR, polymeric immunoglobulin receptor; Ab, antibody; PNA, peanut agglutinin; SC, secretory component; rVV-GP, recombinant vaccinia virus expressing LCMV glycoprotein p33.
4.1.1 Abstract

Intranasal (i.n.) immunization aims to induce local as well as systemic immune responses. In the present study, we assessed a vaccine platform based on virus-like particles (VLP) derived from the RNA phage Qβ for i.n. immunization. We found that both i.n. and subcutaneous (s.c.) administration of Qβ-VLP elicited strong and comparable specific IgG responses in serum and lung. Surprisingly, both routes also induced high levels of specific IgA in serum. In contrast, only i.n. administration of Qβ-VLP resulted in local IgA production in the lung. Efficient induction of B cell responses by i.n. administration of VLP was further supported by the presence of large numbers of germinal centers (GC) as well as memory B cells in the spleen and plasma cells in the bone marrow. Results obtained for the VLP itself could be extended to a peptide antigen covalently attached to it. Specifically, i.n. immunization of mice with VLP displaying the influenza virus derived ectodomain of the M2 protein resulted in strong M2-specific antibody (Ab) responses as well as anti-viral protection. In contrast, i.n. immunization with VLP displaying p33 peptide, the major CTL epitope of lymphocytic choriomeningitis virus (LCMV), induced relatively inefficient cytotoxic T cell (CTL) responses, resulting in low numbers of specific T cells and poor effector cell differentiation. Taken together, these results suggest that effective antibody-based vaccines are achievable by i.n. administration of Qβ-VLP displaying specific antigens.
4.1.2 Introduction

Most current vaccines are administered parenterally through intramuscular or s.c. injection (61). While these routes of immunization are efficient at inducing strong systemic IgG responses, they poorly elicit mucosal immunity. However, as most infectious pathogens enter the body through mucosal surfaces, an ideal vaccine should not only induce systemic humoral and CTL immunity, but additionally induce mucosal immunity. Mucosal immunity is mainly mediated by secretory IgA (SIgA) (74) and has been shown to be effectively induced by i.n. immunization (113, 115). However, most antigens fail to efficiently trigger SIgA responses upon i.n. immunization, even when administered together with mucosal adjuvants. Indeed, the success of an immune response induced at mucosal sites depends on the nature of the antigen. For instance, harmless microbes and nutrients as well as soluble proteins which are captured by the mucosal immune system and may even induce immune tolerance (61, 119).

Mucosal immunity is best induced by vaccines which mimic mucosal pathogens. Such vaccines usually have at least one of the following features: i) they are particulate, ii) they stimulate innate immune responses, and, iii) they target the mucosa and the underlying dendritic cells (DC) (61).

Virus-like particles (VLP) are antigen carriers fulfilling these requirements. We thus set out to investigate the potential of VLP derived from the bacteriophage Qβ (120) to induce immune responses upon i.n. immunization. Qβ-VLP can easily be produced and purified from E.coli by over-expression of the phage coat protein which spontaneously assembles into VLP in the absence of phage RNA. The resulting 30 nm VLP are similar in size to many icosahedral RNA viruses and this may facilitate their interaction with antigen-sampling mucosal DC and M cells. The latter are generally found in organized lymphoid tissues such as Peyer’s patches in the intestine, nasopharynx-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT) in the airways. Furthermore, Qβ-VLP spontaneously package bacterial RNA during assembly and therefore carry ligands for TLR7 and TLR3. Finally, Qβ-VLP are highly ordered and exhibit a repetitive array of capsid proteins which strongly cross-link B cell receptors (BCR) and induce potent B cell responses. In fact, it has been shown that antigens coupled to the surface of Qβ-VLP induce high IgG responses in mice (121-123) and humans (124-126) when administered s.c. or intramuscularly.

It has previously been shown that i.n. immunization with VLP induces systemic and mucosal IgG and SIgA responses, while parenteral immunization only induces systemic and mucosal
IgG responses but fails to induce SIgA (127, 128). In the current study, we compared systemic and mucosal antibody (Ab) responses induced by Qβ-VLP upon i.n. and s.c. immunization. Furthermore, we assessed the efficiency of these routes to induce VLP-specific germinal centers (GC), long-lived plasma cells and circulating memory B cells and determined whether viral peptide antigens displayed by the VLP were able to induce protective B cells and CTL responses. The results demonstrate that i.n. administration of VLP induces strong systemic B cell responses including high levels of IgG and IgA Ab, GC formation, memory B and plasma cell formation. Additionally, i.n. immunization of Qβ-VLP-M2 resulted in protective B cell responses. In contrast, i.n. immunization with Qβ-VLP-p33 was less efficient at inducing cytotoxic T cells (CTL) responses, resulting in low numbers of poorly differentiated CTL.

Taken together, the findings presented here suggest that Qβ-VLP represent an efficient platform for the generation of antibody-based vaccines that can be administered mucosally in a needle free manner.
### 4.1.3 Results

**Intranasal and subcutaneous immunization with Qβ-VLP induces strong systemic antibody responses**

We have previously shown that a single intravenous or s.c. injection of Qβ-VLP in the absence of adjuvant is sufficient to induce a strong anti-Qβ-VLP IgG response which peaks 3-4 weeks after immunization (129, 130). Here, we determined the potential of Qβ-VLP to induce local and systemic immune responses upon i.n. immunization and further investigated its protective capacity in an influenza infection model. Considering that Nardelli-Haefliger *et al.* have suggested that VLP induce more efficient Ab responses when they reach the lung of vaccinated individuals (131) we first wanted to see whether Qβ-VLP reach the lung after i.n. immunization. To this end 100 µg of Alexa488 labelled-Qβ-VLP were administered i.n. and visualized 24 hours later using a UV-lamp. As shown in Fig 2.1 A, Qβ-VLP could readily be detected in lungs of vaccinated animals, indicating that i.n. administered VLP efficiently reach this organ.

Next, we measured the systemic Qβ-VLP Ab response in mice after a single i.n. or s.c. immunization with Qβ-VLP. A strong Qβ-VLP specific IgG and IgA response was detected in serum of both groups of mice (Fig 2.1 B, C). This response was sustained for up to 4 months after immunization. Thus, i.n. immunization resulted in IgG and IgA responses of comparable magnitude to those observed after classical s.c. immunization.

**Frequencies of Qβ-VLP specific AFC parallel the antibody levels found in serum**

To further characterize the Ab response, numbers of antibody forming cells (AFC) in the spleen and bone marrow (BM) were quantified at time points from 2 weeks to 4 months after immunization. High numbers of AFC secreting Qβ-VLP-specific IgG were found in the spleen of both i.n. and s.c. immunized mice. As expected, frequencies declined over time and were roughly 10-fold lower 4 months after immunization ($p = 0.05$ for the i.n. and $p < 0.01$ for the s.c. group, Fig 2.2 A). The numbers of splenic AFC secreting Qβ-VLP- specific IgA were much lower when compared to AFC secreting Qβ-VLP-specific IgG. However, in contrast to specific IgG AFC, the number of specific IgA AFC did not decline significantly through-out the 4 month period (Fig 2.2 B).
In contrast to the spleen, comparable frequencies of IgG and IgA producing AFC were found in the BM. Moreover, the numbers of AFC remained relatively constant in this compartment over time both in s.c. and i.n. immunized mice (Fig 2.2 C, D).

Figure 2.1: Systemic antibody responses induced by intranasal immunization with QB-VLP. (A) Visualization of Alexa488 labelled QB-VLP in the lung of i.n. immunized mice. Mice were immunized i.n. with 100µg of Alexa488 QB-VLP or 100µl of PBS. 24 hours after immunization mice were killed and Alexa488-QB-VLP traced using a UV light tool (*tissue = lung, **tissue = heart). (B, C) Mice were immunized either i.n. or s.c. with 50µg of QB-VLP. (B) Comparison of anti-QB-VLP IgG titer in serum of i.n. or s.c. immunized mice. Geometric mean IgG ELISA titers +1 SD (n=6) are shown. (C) Comparison of anti-QB-IgA titer in serum of i.n. or s.c. immunized mice. Geometric mean IgA ELISA titers +1 SD are shown. The data shown is representative of two independent experiments.
RESULTS

Figure 2.2: Antibody forming cells (AFC) secreting Qβ-VLP-specific antibodies in systemic compartments (spleen and lung. Numbers of AFC secreting Qβ-VLP IgG (A) and IgA (B, C) were determined by ELISPOT in the spleen (A, B) and in the lung (C, D). Mean values +1 SD (n=3). The data shown is representative of 3 independent experiments. Difference between splenic IgG AFC numbers at day 14 and 120 are statistically significant as assessed by the Student’s t-test (*, \( p = 0.05 \) and **, \( p < 0.01 \)).

Only mice that received Qβ-VLP intranasally generate mucosal IgA
Antibodies, in particular SIgA, represent a first line of defence on mucosal surfaces. In order to assess the ability of the i.n. and s.c. routes of immunization to induce Qβ-VLP-specific Ab in mucosal secretions, the bronchial alveolar lavage (BAL) was collected. Qβ-VLP specific IgG were found in BAL of both i.n. and s.c. immunized mice. IgG levels were slightly higher in the i.n. group, reaching statistical significance on day 27 (\( p < 0.01 \), Fig 2.3 A). In marked contrast, only mice that received Qβ-VLP i.n. had specific IgA in BAL (\( p < 0.001 \), Fig 2.3 B). Thus, whereas both routes of immunization induced Qβ-VLP specific IgG and IgA as well as IgG Ab in BAL, only i.n. immunization resulted in local IgA production in the lung.
RESULTS

Figure 2.3: Mucosal antibody response. Mice were immunized i.n. with 100µg of Qβ-VLP. BAL was collected at days 18, 27 and 120 and Ab titers measured by ELISA. In A, anti-Qβ-VLP IgG titers and in B, anti-Qβ-VLP IgA titers were determined. Difference in the levels of Qβ-VLP IgG between the two groups only at day 27 is statistically significant (**, p < 0.01) and the difference in the Qβ-VLP IgA levels between the two groups is statistically significant at days 18 and 27 (**, p < 0.001). Statistical significance was assessed by the Student’s t-test. Geometric mean titers + 1 SD (n=6) are shown. n.d., not detectable. The data shown is representative of four independent experiments.

IgA and IgG responses induced by the influenza virus-derived ectodomain of the M2 protein displayed on Qβ-VLP

We have previously shown that antigens coupled to Qβ-VLP induce strong IgG responses upon s.c. immunization (123, 132). In order to address whether this was also true for the i.n. route we vaccinated mice with M2 protein covalently attached to the Qβ-VLP. Immunization with the influenza M2 protein has previously been shown to induce protection against lethal challenge with influenza A virus when fused to hepatitis B core (HBc) VLP (133, 134). When administered i.n., M2-Qβ-VLP induced both Qβ-VLP and M2-specific IgG and IgA responses in serum (Fig 2.4 A, B) as well as in BAL (Fig 2.4 C, D). S.c. immunization elicited similar Ab responses compared to the i.n. route, but failed to induce mucosal IgA in BAL. However the observed differences were not statistically significant (p > 0.05, Fig 2.4 D). Importantly, the Ab response against M2 qualitatively followed the Qβ-VLP-specific response, supporting what has been previously shown for s.c. immunization (121, 122, 124, 126).
Figure 2.4: Levels of M2-specific IgG and IgA antibodies in serum and in BAL upon immunization with M2-Qβ-VLP. Mice were immunized at day 0 with 50µg of M2-Qβ-VLP. Serum and BAL were collected after 14 and 28 days and Ab titers against M2 and Qβ-VLP were compared by ELISA. Geometric mean titers + 1 SD (n=3) are shown. n.d., not detectable. The data shown is representative of two independent experiments.

Thus, the response against M2 largely mirrored the response obtained against the VLP itself. Hence, these data demonstrate that i.n. immunization not only induces strong Ab responses against the VLP carrier but also against foreign epitopes displayed on the VLP. In order to evaluate the efficacy of the M2-specific Ab responses, vaccinated and control mice were challenged with a lethal dose of influenza virus. Mice vaccinated once or twice either i.n. or s.c. with M2-Qβ-VLP were protected from a lethal challenge with influenza virus and showed 100% survival (Fig 2.5 A, B). In contrast, control mice which were vaccinated once or twice s.c. with Qβ-VLP alone succumbed to lethal infection. As expected, i.n. immunization with Qβ-VLP alone does not protect from lethal influenza virus infection (data not shown). Interestingly, mice immunized once with M2-Qβ-VLP were not protected from weight loss, while two i.n., but not s.c. immunization almost completely abrogated morbidity (Fig 2.5 C, D). The difference observed between the two groups immunized twice was statistically significant (p = 0.01). Overall these results demonstrate that i.n. administration of foreign antigens coupled to Qβ-VLP efficiently induce a protective humoral immune response.
Figure 2.5: M2-Qβ-VLP immunization protects against lethal challenge with influenza virus. Mice were vaccinated either i.n. (open triangles) or s.c. (filled triangles) with a single dose or in a boost regimen (second dose administered 14 days after priming) with 50µg of M2-Qβ-VLP. Control mice were s.c vaccinated with Qβ-VLP alone, once or twice (filled squares). Fourteen days after the last immunization, mice were infected i.n. with a lethal dose (4xLD50) of influenza virus strain PR8 and mortality and morbidity were monitored after both immunization regimens. Mortality and morbidity of mice vaccinated once (A, C) and of mice vaccinated twice (B, D). Difference in the weight loss between the i.n. and s.c. group immunized twice was statistically significant (p = 0.01) as assessed by the ANOVA test. Mean values ± 1 SD (n=4) are shown. The data shown is representative of two independent experiments.

Efficient induction of Qβ-VLP specific germinal centers and memory B cells by intranasal immunization

A hallmark of specific B cell responses is the formation of GC leading to the generation of memory B cells and long-lived plasma cells. To compare the ability of the i.n. and s.c. routes to generate GC, mice were immunized and spleens were removed 10 days later. Spleen sections were stained for the presence of Qβ-VLP specific B cells and peanut agglutinin (PNA), a marker for GC B cells. Both routes of immunization efficiently induced GC, exhibiting comparable size and morphology (Fig 2.6).

We have previously developed a method to detect VLP-specific memory B cells by flow cytometry (129) (Fig 2.7). Using this technique, we assessed the frequency of Qβ-VLP specific memory B cells in the spleen and in the draining mediastinal lymph node (MLN). Similar frequencies of memory B cells were found in spleen (5% of total isotype-switched B
cells) (Fig 2.7 B) and in MLN (11% of total isotype-switched B cells) (Fig 2.7 C) 1 month after i.n. and s.c. immunization.

**B cell responses in draining lymphoid organs**

Mediastinal lymph nodes (MLN) drain the lung and are usually the site where mucosal immune responses are initiated against antigens reaching the lung. Indeed, we found high frequencies of Qβ-VLP-specific IgG producing AFC within these LN (Fig 2.8 A), consistent with the presence of IgG Ab in BAL (Fig 2.3 A). In contrast to the spleen and BM, numbers of AFC secreting Qβ-VLP specific IgG in MLN of i.n. immunized mice were slightly higher than in the s.c. immunized mice. This supports the notion that MLN are important sites of Ab induction upon i.n. immunization with VLP. As seen in spleen, frequencies of Qβ-VLP specific IgG producing AFC declined over time. After s.c. injection, Qβ-VLP reaches the blood circulation (unpublished) and is distributed throughout the peripheral LN either as free antigen or in association with blood-derived cells. This observation may explain the presence of Qβ-VLP IgG AFC in MLN of s.c. immunized mice.
In contrast to results obtained for IgG producing AFC, only mice which were immunized i.n. generated a substantial number of Qβ-VLP specific IgA AFC in MLN (Fig 2.8 B). Thus, the presence of IgA producing AFC in MLN paralleled the presence of IgA in BAL.

I.n. immunization may also lead to induction of B cell responses in the cervical LN (CLN), which drains the nose and upper airways. Compared to MLN, the number of Qβ-VLP-specific AFC were 5-10 folds lower in these LN (data not shown), indicating that the lower respiratory tract is the major site draining VLP for the induction of Ab responses.
Figure 2.7: Induction of Qβ-VLP specific memory B cells. Mice were immunized i.n. or s.c. with 50µg of Qβ-VLP and the frequency of Qβ-VLP specific memory B cells was assessed 30 days post immunization. (A) Analysis was performed by gating on isotype-switched B cells (CD19+, IgM, IgD, CD4, CD8, CD11b and Gr-1-). The percentage of Qβ-VLP specific B cells in spleen (B) and MLN (C) of i.n. and s.c. immunized mice are shown. Staining with Qβ-VLP in spleen and MLN of naïve control mice are also shown. Mean percentages ± 1 SD (n=3) are shown. Differences between i.n. or s.c. immunized mice and naïve mice are statistically significant as assessed by the Student’s t-test (in the MLN p < 0.01 and in the spleen p < 0.05).

**MLN are major sites of VLP drainage after i.n. immunization**

To directly assess, whether MLN or CLN were the major site of Qβ-VLP-drainage after i.n. immunization, we determined the frequency of cells that had taken up or bound Qβ-VLP within the respective LN. To this end, mice were immunized i.n. with Alexa488 labelled Qβ-VLP and leucocytes from MLN and CLN were analyzed by flow cytometry. While 1.15% of total live leucocytes (mainly DC, data not shown) of MLN were found to be associated with Qβ-VLP, the frequency of cells associated with Qβ-VLP in CLN of i.n. immunized mice was similar to the background level seen in PBS treated mice (Fig 2.8 C). The frequency of cells associated with Qβ-VLP in MLN was always higher than in CLN (p < 0.02). Thus, after i.n. immunization Qβ-VLP drained or were transported preferentially to MLN draining the lung and not to LN draining the nose and upper airways. These results are consistent with the hypothesis that MLN are the major inductive site of local Ab responses detected in the lung after i.n. administration with Qβ-VLP.
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Figure 2.8: Mediastinal lymph nodes are important sites for induction of antibody responses after intranasal immunization. Numbers of antibody forming cells (AFC) secreting Qβ-VLP-specific IgG (A) and IgA (B) were determined in MLN by ELISPOT. The values represent frequencies from pooled LN (n=3). The data shown is representative of three independent experiments. C shows the localization of Qβ-VLP in MLN 1 day after i.n. immunization. Mice were immunized i.n. with 100 µg of Qβ-VLP labelled with Alexa 488 and the presence of Qβ-VLP was assessed in MLN and CLN by flow cytometry 24 hours later. The percentage of live leukocytes in association with Qβ-VLP Alexa 488 is shown. The data shown is representative of two independent experiments. Difference between the percentage of leukocytes in association with Qβ-VLP Alexa 488 in MLN and CLN is statistically significant as assessed by the Student’s t-test (p < 0.02).

Qβ-VLP-specific IgA antibodies are locally produced in the lung

To further investigate the source of IgA found in BAL, we determined the numbers of AFC secreting anti-Qβ-VLP IgA in the lung tissue (Fig 2.9 A). High numbers of AFC secreting anti-Qβ-VLP IgA were found in the lung, suggesting that the IgA detected in BAL after i.n. immunization may indeed be locally produced.

It has been shown previously that upon virus infection, induced bronchus-associated lymphoid tissue (iBALT), which resembles GC structures, can be generated in the lung and contribute to the induction of local immune responses (135). To address the possibility of iBALT being an inductive site of the IgA response observed with Qβ-VLP, lungs of i.n. immunized animals were analysed histologically. However, no such GC-like structure could
be detected (data not shown). This indicates that Qβ-VLP-specific AFC migrate to the lung from draining lymphoid organs, most likely from MLN. To directly assess whether IgA was indeed produced locally in the lung and not derived from the blood due to leakage caused by irritation during i.n. VLP administration, the following experiment was performed. Qβ-VLP was first administered i.n., followed by a second VLP (AP205) which was administered s.c. Thereafter, Qβ and AP205-VLP-specific Ab were measured in BAL and serum. AP205-VLP are similar to Qβ-VLP in that they are phage-derived, loaded with E. coli RNA and exhibit similar size (136). Both VLP have very similar biological characteristics, including their ability to induce strong IgG and IgA Ab responses. Indeed, both VLP induced IgA Ab in serum (not shown). However in BAL, we only detected IgA specific for Qβ-VLP, which was administered i.n. (p < 0.01, Fig 2.9 B). Hence, the IgA found in the lung after i.n. immunization is locally produced.

Figure 2.9: Qβ-VLP-specific IgA antibodies detected in BAL are locally produced in the lung. In A, mice were immunized i.n. with 100µg of Qβ-VLP and numbers of AFC secreting Qβ-VLP IgA were determined by ELISPOT in lung digested tissue and MLN 18 and 27 days later. The value represents the average (n=3) of specific AFC numbers. The data shown is representative of two independent experiments. Of note, numbers of AFC in the lung are expressed per 1x10⁶ cells, however in average only 4x10⁵ cells were isolated. In B, mice were first administered with 50µg of VLP (Qβ) i.n. and then a second VLP (AP205) was administered s.c. and vice versa. BAL was collected 20 days post immunization and Qβ-VLP specific IgA was measured. Difference in the level of Qβ-VLP specific IgA between the two group of mice are statistically significant as assessed by the Student’s t-test (**, p < 0.01). Geometric mean titers + 1 SD (n=6) are shown. n.d., not detectable. The data shown is representative of 4 independent experiments.

Poor induction of cytotoxic T cells (CTL) upon intranasal immunization
To test whether the i.n. route is able to efficiently induce CTL responses, mice were immunized i.n. with Qβ-VLP displaying peptide p33, the major CTL epitope of lymphocytic choriomeningitis virus (LCMV) in H-2b mice. To increase the immunogenicity of VLP for the induction of CTL, VLP were loaded with immunostimulatory DNA rich in CG motifs (CpGs) (137). Mice were immunized i.n. or s.c. with p33-Qβ/CpG and frequencies of specific CD8+ T
phenotype (CD127+ blood generated after s.c. immunization were fully activated and showed an effector + reduced effector cell differentiation. Whereas the majority of specific CD8+ T cells in the blood generated after s.c. immunization not only induced lower frequencies of specific CD8+ T cells but also resulted in reduced effector cell differentiation. Whereas the majority of specific CD8+ T cells in the blood generated after s.c. immunization were fully activated and showed an effector phenotype (CD127+/CD62L-), most of the cells generated after i.n. immunization remained CD127+ (p = 0.001) and were therefore not fully differentiated (Fig 2.10 F).

Figure 2.10: Induction of CTL responses upon intranasal and subcutaneous immunization. Mice were immunized either i.n. or s.c. with 100 µg of p33-Qβ/CpG and frequencies of p33 specific CD8+ T cells were determined 8 days later in the blood (A), spleen (B), ovaries (C), MLN (D) and lung (E). In F, the activation status of specific CD8+ T cells in the blood elicited by the two routes of immunization is shown. Difference in the frequency of gp33-specific CD8+ T cells between the i.n. and s.c. group was statistically significant in blood (*, p < 0.02) and ovaries (***, p < 0.01). In blood, also the percentage of activated specific CD8+ T cells was statistically significant between the two groups (p = 0.001). Statistical significance was assessed by the Student’s t-test.
The reduced CTL response mounted in mice immunized i.n. was confirmed in an anti-viral protection experiment where immunized mice were challenged with recombinant vaccinia virus expressing peptide p33 (rVV-GP). Mice immunized s.c. with either a low or high dose of the vaccine had completely controlled viral titers by day 5 after challenge. In contrast, only 2 out of 3 mice immunized i.n. with the high dose of the vaccine were fully protected from viral replication and the mice immunized i.n. with the low dose of the vaccine essentially failed to control viral replication (Fig 2.11).

Figure 2.11: Poor CTL immunity induced by intranasal immunization translates into weak viral protection. To evaluate the protective capacity of the CTL induced, mice were immunized either i.n. or s.c. with 15 or 150 µg of p33-Qβ/CpG and 8 days later animals were challenged i.p. with 6x10⁵ PFU of rVV-GP. Viral titers were determined in the ovaries 5 days later. Difference between i.n. and s.c. mice vaccinated with 15 µg of p33-Qβ/CpG is statistically significant (p < 0.001).
4.1.4 Discussion

Mucosal immune responses are an early and important line of defence against pathogens. It has been shown previously that mucosal immune responses can be more efficiently achieved by administration of vaccines onto mucosal surfaces, i.e. oral, rectal, vaginal or i.n.. Ideally, an efficient vaccine should provide both humoral and cell-mediated protection, not only at the mucosal delivery site, but also throughout the body, including systemic compartments as well as distal mucosal tissues. In this regard, the administration of antigens through the nose or by inhalation appears to be a promising route of vaccination. In this study, we compared the ability of the i.n. and s.c. immunization to induce systemic and mucosal antigen-specific Ab and CTL responses using Qβ-derived VLP as a model vaccine. We found that both routes of immunization were efficient at inducing Qβ-VLP specific IgG and IgA Ab responses in serum and long-term memory. While only the i.n. route induced IgA in BAL, specific IgG Ab could be detected after both routes of immunization, although i.n. immunization was slightly more effective at inducing mucosal IgG. This observation was paralleled by the slightly higher numbers of Qβ-VLP specific IgG AFCs found in MLN of i.n. immunized mice. Although it has been shown that IgG AFC generated in the spleen express CD62L and may therefore migrate to peripheral lymph nodes (58), (117), the presence of AFC secreting anti-Qβ-VLP IgG in MLN of s.c. immunized mice is best explained by local induction due to the presence of the Qβ-VLP antigen in this LN (not shown). This indicates that MLN are the major inductive sites of the mucosal Qβ-VLP specific IgG response detected in BAL. In agreement with these findings, Balmelli et al. showed in mice that following nasal immunization with HPV16 VLP, the major inductive site of the specific mucosal immune response was the LN draining the lung (138). They have also shown that interaction of the antigen with the lower respiratory tract was necessary to induce high titers of neutralizing Ab, although interaction of the VLP with the NALT (located in the upper respiratory tract) was sufficient to induce a mucosal response in mice primed by s.c. immunization (127). Later, the same group showed in human volunteers that HPV-16 VLP administered by aerosol and reaching the lower airways were more immunogenic than those administered by the nasal route (131).

Other groups have pointed to the NALT as the major site for priming B cells following i.n. infection (112, 117, 139, 140). These differences may be explained by the nature of the antigen used. It is likely that pathogens replicating locally in the upper respiratory tract preferentially reach the NALT and induce Ab responses in this compartment while inhaled antigens, such as the VLP used in the present study, tend to bypass the NALT (because they
RESULTS

Most of the IgA found in mucosal secretions is SIgA which is composed of a complex of dimeric IgA (synthesized and assembled by IgA plasma cells) bound covalently to the secretory component (SC) derived from the epithelial polymeric immunoglobulin receptor (pIgR) (74). The covalent bond between the glycosylated SC and the \( \alpha \)-chain of pIgA confers high stability to SIgA, making SIgA protease-resistant because SC can mask proteolytic sites from proteases present in mucosal secretions (146, 147). An additional advantage of SIgA over neutralizing IgG is the greater potential to mediate cross protection (protecting against
heterologous strains) due to multiple binding sites increasing the avidity and its innate protective potential (148), (149). For instance, it has been shown in an influenza model that SIgA is more effective than monomeric IgA in inhibiting viral hemagglutination as well as viral neutralization (147), suggesting that the antiviral activity of the Ab is due to its avidity and molecular form (polymeric nature). Overall, both SIgA and plasma-derived IgG are important for controlling viral infection in the respiratory tract. Whereas IgG neutralizes viral replication in the lung, only SIgA has the ability to prevent the initial infection in the upper airways (150).

Current vaccines protect on the basis of the induction of specific Ab (151). Nevertheless, induction of CTL responses may be an additional benefit offered by a potent vaccine. We therefore assessed whether the i.n. route is suited for the induction of potent CTL responses. However, the i.n. route was clearly inferior to the s.c. route both in terms of frequencies of specific CD8+ T cells, their effector status and their protective capacity. Thus, the i.n. route does not appear to be particularly potent at inducing strong effector CTL responses. Whether the i.n. route may be more attractive for vaccines aiming to induce preferentially resting CTL remains to be evaluated.

Taken together, the present study shows that a single i.n. administration of Qβ-VLP is sufficient to elicit strong and long-lasting local and systemic IgG and IgA Ab responses specific for Qβ-VLP. Considering that an efficient protective vaccine should induce neutralizing Ab in the mucosa in order to hinder pathogen penetration and spreading, the data presented here suggest that VLP may provide an efficient carrier-technology for the development of protective inhaled vaccines in humans. In addition, immunization via the i.n. route has the advantage of being needle-free, avoiding the risk of vaccine-induced infection, a real challenge in developing countries (152).
4.1.5 Materials and Methods

**Mice and antigens**
To evaluate the Ab response induced by Qβ-VLP, female, 8 weeks old C57BL/6 mice (Harlan, Horst, The Netherlands) were immunized either intranasally (i.n.) or subcutaneously (s.c.) with 50µg of Qβ-VLP containing E.coli-derived ssRNA. For assessment of antiviral immunity, the p33 peptide (KAVYNFATM) derived from LCMV was coupled to VLP derived from the bacteriophages Qβ and packaging of CpG oligonucleotides into the p33-Qβ were performed as described previously (137). Mice were immunized i.n. or s.c. either with 15 or 150µg of p33-Qβ/CpG.

For measuring the Ab titer against a heterologous B cell epitope, the extracellular domain of M2 was coupled to the Qβ-VLP as described previously (137). All animal experiments were conducted in accordance with protocols approved by the Swiss Federal Veterinary Office.

Capsids of the RNA phage Qβ and AP205 were cloned into pQb10 vector and purified as described elsewhere (120).

**Immunization**
For the i.n. immunization with Qβ-VLP, mice were anesthetized with isoflurane and vaccine was administered using a 200µl pipette. S.c. vaccination was performed by Qβ-VLP injection into both sides of the abdomen. For both routes of immunization, Qβ-VLP was diluted in PBS to a final administration volume of 100µl (2x 50µl).

To assess the Ab response against a heterologous antigen coupled to Qβ-VLP, 50µg of M2-Qβ-VLP were administered either i.n. or s.c. at day 0 and 14 and 28 days later, BAL and serum were collected.

**Influenza infection**
In order to determine antiviral immunity mediated by Ab, female C57BL/6 mice were vaccinated either i.n. or s.c. with a single dose of 50µg of M2-Qβ-VLP or in a boost regimen (second dose administered 14 days after priming). Fourteen days after the last immunization, mice were i.n. infected with 4xLD50 of influenza virus PR8 followed by daily monitoring of mortality and assessment of morbidity (weight loss).

**Lung washes and lung lymphocytes isolation**
BAL samples were collected through the trachea by washing the lung 3 times with 300µl PBS/1% BSA. Samples were stored at -20°C until use.

For the isolation of lymphocytes from the lung, mice were perfused with 5ml of PBS in the heart ventricle to clear lungs of blood. Lungs were chopped in small pieces and incubated at 37°C in media containing collagenase. Finally, lymphocytes were harvest by using a 30% percoll gradient.

**In vivo imaging using UV light tool**
Mice were anesthetized with isoflurane and immunized i.n. with 100µg of Qβ-VLP labeled with Alexa-488 (50µl applied in each nostril). 24 hours after immunization, mice were killed and macro imaging was performed using UV-light device (LT-99D2-220 Illumatools, Lightools research). Pictures were recorded with a compact digital camera (Maxxum 5D, Conica Minolta).

**ELISA**
ELISA plates (Nunc Immuno MaxiSorp, Rochester, NY) were coated overnight with 100µl of Qβ-VLP (1µg/ml) and ELISA were performed according to standard protocols using HPRO-conjugated goat anti-mouse IgG (Fc gamma specific), Jackson ImmunoResearch; HPRO-conjugated goat anti-mouse IgA (alpha chain specific), Sigma.

For detection of anti-Qβ-VLP IgA in serum, IgG Ab were depleted using Protein G (Amersham Biosciences). ELISA controls showed that the elevated amount of anti-Qβ-VLP IgG in serum suppresses the measurement of anti-Qβ-VLP IgA. For detection of anti-Qβ-VLP IgG and Qβ-VLP IgA in BAL, undiluted samples were used. Anti-Qβ-VLP IgG titers in serum and in BAL are indicated as dilutions reaching half maximal absorbance at 450nm. Anti-Qβ-VLP IgA titers in serum and in BAL were measured by endpoint titer by setting the OD (450nm) cut-off at 0.2. Anti-Qβ-VLP and anti-M2 IgA in BAL of s.c. group is under the set OD cut-off of 0.2 and therefore was not detected (n.d.).

Measurement of anti-M2 IgA and IgG Ab was performed similarly with the difference of the coated protein, where 10µg/ml of M2 coupled to RNAse was used.
**ELISPOT Assay**

Qβ-VLP specific antibody-forming cells (AFC) frequencies were determined as described (129). Briefly, 24-well plates were coated with 10µg/ml Qβ-VLP. Spleen, BM, MLN or lung derived-cells were added in DMEM containing 2% FCS and incubated for 5 h at 37°C. Cells were washed off and plates were incubated either with goat anti-mouse IgG (EY Labs) or with goat anti-mouse IgA (alpha chain specific, SouthernBiotech), followed by alkaline phosphatase-conjugated donkey anti-goat IgG Ab (Jackson ImmunoResearch) before development of alkaline phosphatase color reactions.

**Flow Cytometry**

Detection of Qβ-VLP specific B cells expressing surface Ig was performed as described (30) by incubation with Qβ-VLP particles, followed by a polyclonal rabbit anti-Qβ-VLP sera (1/400; produced by RCC Ltd.) and Cy5-conjugated F(ab’)2 donkey anti-rabbit IgG serum (1/400; Jackson ImmunoResearch). Isotype-switched B cells were detected with a mixture of FITC-conjugated antibodies at 1/200 dilution (rat IgG2a anti-IgD, 11-26c.2a; goat anti-IgM serum, JacksonImmuno Research Laboratories; rat IgG2b anti-CD4, GK1.5; rat IgG2a anti-CD8, 53-6.7; rat IgG2b anti-CD11b, M1/70; rat IgG2b anti-Gr-1, RB6-8C5) and PerCP-Cy5.5-conjugated rat IgG2a anti-CD19 (1/400; 1D3). In all cases Fc-receptors were blocked with rat IgG2b anti-mouse CD16/32 (1/100; 2.4G2). Antibodies were purchased from BD Biosciences, unless otherwise specified.

**Immunofluorescence**

Freshly removed spleens were snap-frozen in liquid nitrogen. Tissue sections of 7µm thickness were cut in a cryostat and fixed with acetone. For detection of Qβ-VLP specific B cells, firstly unlabeled Qβ-VLP capsids were added, followed by a polyclonal rabbit anti-Qβ-VLP antiserum (1/1500; RCC) and detected with Alexa 488-cojugated goat anti-rabbit Ab (1/1000). PNA-binding B cells were detected with biotinylated PNA (1/800; Vector Laboratories), followed by Alexa 546-labeled Streptavidin (1/1000).

**Virus infection and peptide MHC class I streptamers**

In order to determine antiviral immunity, vaccinated female C57BL/6 mice were infected intraperitoneally with 6x10⁵ PFU of recombinant vaccinia virus expressing LCMV glycoprotein p33. After 5 days ovaries were collected and vaccinia titers were determined on BSC 40 cells as described (153). Specific CD8⁺ T cells were measured in the blood, spleen,
ovaries, MLN and lung by streptamer staining, using PE-labeled H-2D<sup>b</sup>-streptamers loaded with peptide p33 (IBA, Germany). Activation status of the specific T cells was determined by staining with rat IgG2a anti-CD127 FITC-conjugated (clone A7R34) and rat IgG2a anti-CD62L APC-conjugated (clone MEL-14).

**Acknowledgement**
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**Disclosures**
N. Schmitz, H.J. Hinton, K. Schwarz, A. Jegerlehner and M. F. Bachmann are employees of, and hold stocks or stock options with, Cytos Biotechnology.
4.2 Part II: Alveolar macrophages and lung dendritic cells sense RNA and drive mucosal IgA responses

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Short Title: TLR signaling and IgA responses

Abbreviations: Ab: antibody; AFCs: antibody forming cells; Ag: antigen; BCMA: B cell maturation antigen; BCR: B cell receptors; BM: bone marrow; CSR: class switching recombination; DCs: dendritic cells; i.n.: intranasal(ly); MLN: mediastinal lymph node; s.c.: subcutaneous(ly) TACI: transmembrane activator and calcium modulator and cyclophylin ligand interactor; Th: T cell help; TLR: toll like receptor; WT: wild type
4.2.1 Abstract

The mechanisms regulating systemic and mucosal IgA responses in the respiratory tract are incompletely understood. Using virus-like particles loaded with single-stranded RNA as a ligand for TLR7 we found that systemic versus mucosal IgA responses were differently regulated. Systemic IgA responses were T-cell independent and did not require TACI or TGFβ whereas mucosal IgA production in respiratory tract was dependent on Th cells, TACI and TGFβ. Strikingly, both responses required TLR7 signaling but systemic IgA depended upon TLR7 signaling directly to B cells, while mucosal IgA required TLR7 signaling to lung dendritic cells and alveolar macrophages. Collectively, our data show that IgA switching is controlled differently according to the cell type receiving TLR signals. This knowledge should facilitate the development of IgA inducing vaccines.
4.2.2 Introduction

Successful vaccines mediate protection via neutralizing antibodies (Ab) (151, 154). Parenteral routes of immunization are very efficient in inducing neutralizing IgG responses which can be maintained as humoral immunological memory over long periods of time (155, 156). However, these routes usually induce poor IgA responses, especially at mucosal surfaces. For this reason, intranasal (i.n.) administration might be an attractive route of vaccination, since in addition to inducing specific IgG responses this route also efficiently induces IgA responses at mucosal surfaces, which are the primary sites of pathogen entrance.

The regulation of IgA isotype class-switch recombination (CSR) is complex and both T-cell-dependent (TD) and T-cell-independent (TI) mechanisms have been described (74, 157). IgA responses induced by TD antigens (Ag) such as proteins require CD4+ T-cell help (Th) mediated by CD40L as well as TGFβ1 and are mainly mediated by B2 B cells. In contrast IgA responses induced by TI type 1 Ag (TI-1) such as LPS, or TI type 2 Ag (TI-2), such as polysaccharides; do not require CD40-CD40L interaction. Instead they rely on APRIL (secreted mainly by activated DCs and macrophages) binding to TACI on B cells and are mediated mainly by B1 B cells (73, 95).

Both TD and TI mechanisms of CSR have been described for intestinal IgA production. Specifically, it has been shown that IgA CSR in the gut can occur independently of CD40 signaling and germinal center formation (158). Retinoic acid (RA) has also been implicated in TI IgA responses and can stimulate the expression of gut-homing receptors on B cells. In addition, by synergizing with gut-associated lymphoid tissue (GALT)-DC-derived IL-6 and IL-5, RA can promote IgA secretion (159). Recently, nitric oxide (NO) secreted by dendritic cells has also been implicated in the regulation of IgA production in the gut (95).

In contrast to the gut, much less is known about the mechanisms controlling IgA responses at the respiratory mucosa. One of the few examples studied is influenza virus infection, where virus-specific IgA responses generated in the respiratory tract occurred independently of cognate T-B interaction (CD40 expression on B cells), but did require bystander CD4+ T cell help; in contrast to IgM and IgG responses which were dependent on cognate interactions (96).

Many reports have investigated the role of toll-like receptor (TLR) signals in B cell responses, including memory B cell responses(81, 160) as well as IgG CSR (77-80, 82). However, the role of TLR signals in the induction of IgA responses has been scarcely studied. Interestingly,
normal levels of serum IgA in MyD88⁻/⁻ mice are suggestive that TLR signaling is dispensable for IgA responses (79). Moreover, the role of TLR signaling in mucosal IgA responses has not been directly investigated.

Virus-like particles (VLPs) are highly effective immunogens which can induce strong Ab responses. VLPs are classic TI type 1 antigens which are able to induce IgM responses in the absence of T help because of multimeric interactions with cognate BCRs which induce a strong activation signal in B cells. In addition, we have previously shown that i.n. and subcutaneous (s.c.) immunization with VLP derived from the bacteriophage Qβ, Qβ-VLPs, (120) are efficient immunization routes for induction of IgA responses (161). Qβ-VLPs are packaged with RNA derived from *Escherichia coli* during its self assembly process. Thus, Qβ-VLPs typically mediate TLR3/7 signals which we considered to be important candidates for triggering IgA responses.

In the present study, we assessed the role of TLR7 signaling in driving IgA responses. We found that depending on the site of antigen sampling, TLR7 signaling was required either to lung DCs and alveolar macrophages or to B cells and resulted in TD versus TI IgA responses respectively.
4.2.3 Results

Site of antigen exposure governs the requirement for T cell cognate help during IgA production

VLPs are classified as Th cell-independent (TI) type 1 (TI-1) Ag because they can efficiently induce IgM responses without the need of T cell help. This property can be accredited to their highly organized structure which allows them to efficiently crosslink the BCR. At the same time they can behave as a Th cell-dependent (TD) Ag, since they are proteins. Indeed, we have observed that Qβ-specific IgM responses are induced in the absence of T cell help, whereas specific IgG responses required the cognate interaction between T and B cells (30, 78). In this study we assessed whether the IgA response against Qβ-VLP would require T cell help or whether, similarly to the IgM response, IgA could also be induced by a TI mechanism. To address this question, we immunized wild type (WT) and MHC II−/− mice via the i.n. or s.c. routes and compared the mucosal (BAL) and systemic (serum) IgA titers respectively between the two groups. The mucosal IgA response in MHC II−/− mice immunized i.n. was strongly reduced in MHC II−/− mice compared to WT mice. In marked contrast, the levels of systemic IgA were similar for MHC II−/− and WT mice immunized s.c. (Fig. 2.12 a). We further assessed the IgA response elicited in CD40−/− mice. In accordance with our findings in MHC II−/− mice, mucosal IgA levels in CD40−/− mice were also significantly reduced compared to the WT group whilst the systemic IgA titer elicited by CD40−/− mice was comparable to the WT group (Fig. 2.12 b). These observations show that IgA responses against Qβ-VLP are differentially regulated depending on the site of antigen exposure. Whereas mucosal IgA responses elicited upon mucosal administration of VLPs in the lung requires T cell help and the cognate interaction between T and B cells, Qβ-VLPs administered s.c. induced a TI systemic IgA response. As expected, the IgG response was strongly reduced in the absence of T cell help, independently of the route of immunization (not shown).
Figure 2.12: Requirement of CD4⁺ T cell help for induction of Qβ-VLP-specific IgA responses. Mice were immunized either s.c. or i.n. with 50µg of Qβ-VLP and 20 days later serum and BAL samples were collected from the s.c. and i.n. immunized mice respectively to measure specific IgA levels by ELISA. Qβ-VLP-IgA titers of C57BL/6 and MHC II⁻/⁻ (a) and C57BL/6 and CD40⁻/⁻ mice (b) are shown. Geometric mean IgA ELISA titers + SEM are indicated (n = 3); n.d., not detectable. The data shown are representative of three independent experiments. Statistical significance was assessed by unpaired Student’s t-test (*, p < 0.05 and **, p < 0.01).
**Differential requirements for TGFβ and TACI signaling for mucosal and systemic IgA production**

Transforming growth factor (TGFβ) is the major cytokine involved in the induction of IgA CSR. *In vitro* TGFβ has been shown to be involved in the Th dependent CSR to IgA (95, 162). Evidences for its *in vivo* role were provided by experiments showing that mice deficient for TGFβ receptor TβRII selectively on B cells (TβRII-B) were almost completely devoid of IgA, both in serum and in mucosal washes (110, 163). In an attempt to address the role of TGFβ in the IgA responses against Qβ-VLP *in vivo*, we used these TβRII-B mice. Following s.c. immunization, systemic IgA responses in TβRII-B mice were slightly reduced compared to the control group. In contrast, the mucosal IgA titer in BAL of TβRII-B mice was significantly lower when compared to that of control mice. This demonstrates that the systemic IgA response to Qβ-VLP is only partially dependent on TGFβ signaling to B cells, whereas TGFβ plays an important role for the induction of mucosal IgA response (Fig. 2.13 a).

IgA-specific CSR can be accomplished by TD or TI mechanisms. The TD mechanism requires CD40L expressed on activated T cells whereas the TI mechanism is thought to rely largely on APRIL (a proliferation-inducing ligand) and BAFF (B lymphocyte stimulator protein, also known as BLyS, TALL-1, THANK and zTNF4) produced by DCs which interact with their cognate receptors, TACI (transmembrane activator and calcium modulator and cyclophylin ligand interactor) and BCMA (B cell maturation antigen) expressed on B cells (73, 164). To address the role of these molecules in Qβ-VLP-specific IgA responses, we compared the IgA levels in serum between the WT and TACI;BCMA double deficient mice. Surprisingly, the systemic IgA levels in TACI;BCMA−/− mice were not significantly reduced compared to the WT group. In contrast, the mucosal IgA response in the BAL of TACI;BCMA−/− mice was strongly reduced. Thus, in addition to requiring T cell help, local IgA responses also needed signaling through TACI or BCMA, whilst TI systemic IgA responses occurred in a TACI/BCMA independent manner (Fig. 2.13 b). To investigate whether TACI or BCMA signaling was required for mucosal IgA responses, we also administered Qβ-VLP i.n. to TACI−/− mice. Similar to the double deficient TACI;BCMA−/− mice, TACI+ mice generated a significantly reduced IgA response in BAL (data not shown). This indicates that the TD IgA responses occurring at the airway mucosa requires TACI mediated signals.
Figure 2.13: TβRII and TACI play a partial role for production of systemic IgA but are critical for the production of mucosal IgA responses against Qβ-VLP. Mice were immunized either s.c. or i.n. with 50 µg of Qβ-VLP and 20 days later specific IgA were measured in serum and BAL respectively by ELISA. Qβ-VLP-IgA titers of control and TβRII-B (a) and C57BL/6 and TACI:BCMA−/− mice (b) are shown. Geometric mean IgA ELISA titers + SEM are indicated (n = 4). The data shown are representative of three independent experiments. Statistical significance was assessed by unpaired Student’s t-test (**, p < 0.01).
**RESULTS**

**TLR7 signaling is required for induction of optimal mucosal and systemic IgA responses against Qβ-VLP**

Our previous data demonstrated that the mucosal IgA production in response to i.n. immunization required T helper cell derived signals (CD40L) in addition to TACI mediated signals. In contrast, the systemic IgA response elicited by s.c. immunization did not require T helper cells nor TACI signaling. Thus, the IgA responses against VLPs are differentially regulated depending on the site of antigen exposure. We therefore sought to understand which additional factor, other than Th cells, TGFβ and APRIL/BAFF production could be involved in the regulation of the TI systemic IgA response observed following s.c. immunization.

As mentioned, Qβ-VLPs are loaded with *E.coli* derived ssRNA and provide efficient TLR7 stimulation. To determine whether TLR7 signaling played a role in regulating the IgA response we compared the IgA and the IgG isotypes in serum of mice immunized either i.n. or s.c. in the presence or absence of TLR7 and MyD88 signaling. To this end, we compared both the Ab response of mice immunized with Qβ-VLP with or without packaged RNA and the response of WT mice to TLR7 or MyD88 deficient mice. As shown in Fig. 2.14 a,b, IgA and IgG2a in serum were only induced with Qβ-VLP containing RNA. In contrast, the levels of IgA and IgG2a in WT mice immunized with Qβ-VLP devoid of RNA as well as in TLR7−/− and MyD88−/− mice were very low. Following s.c. immunization, IgG1 responses were induced in the absence of TLR7 signaling and suppressed by the presence of TLR7 signaling, which confirms previous findings for TLR9 signaling (78). In contrast, TLR7 signaling had no influence on specific IgG1 titers following i.n. immunization (Fig. 2.14 a). In the BAL, similarly to the systemic Ab response, the levels of IgA and IgG2a were completely abolished in the absence of TLR7 signaling (Fig. 2.14 c).

**The numbers of Qβ-VLP specific AFCs correlate with the Ab titer**

We have previously seen that mediastinal LNs (MLN) are the major inductive site of the Ab response upon i.n. immunization against Qβ-VLP (161). Next, the impact of TLR signaling on the number of AFCs secreting Qβ-VLP-specific IgA in these LNs was investigated. Similarly to the Ab titer, the number of AFCs secreting IgA was strongly reduced in mice immunized with VLP devoid of RNA as well as in TLR7−/− and MyD88−/− mice compared to control mice (Fig. 2.14 d). The same was true for the spleen of s.c. immunized mice (Fig. 2.14 e).

In conclusion, in contrast to T cell help, CD40L, TGFβ and TACI, TLR7 signaling was pivotal for the induction of systemic IgA responses after s.c. immunization.
RESULTS

IgA ELISA Titer

Intranasal (serum)

Subcutaneous (serum)

Intranasal (BAL)

Table:

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Intranasal (BAL)

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Figure 2.14: Role of TLR7 signaling in regulating IgA responses against Qβ-VLP. Mice were immunized either i.n. (a) or s.c. (b) with 50µg of Qβ-VLP and 20 days later, Qβ-VLP-specific IgA, IgG2a and IgG1 titers in serum of WT C57BL/6, TLR7+/+, TLR7−/− and MyD88−/− mice immunized with Qβ-VLP loaded with RNA and of WT mice immunized with empty Qβ-VLP were measured by ELISA. (c) anti-Qβ-VLP IgA and IgG2a in BAL of i.n. immunized mice are shown. Geometric mean IgA, IgG2a and IgG1 ELISA titers + SEM are indicated (n = 4). Number of AFCs secreting Qβ-VLP IgA was determined by ELISPOT in MLN of i.n. (d) and in spleen of s.c. (e) immunized group. ELISPOT data show the mean values and + SEM (n = 4). The data shown are representative of four independent experiments. n.d., not detectable. Statistical significance was assessed by unpaired Student’s t-test (*, p < 0.05 and **, p < 0.01).
IgA CSR upon s.c. immunization requires TLR7 signaling directly on B cells

In the next set of experiments we investigated how TLR7 signaling controls IgA responses to Qβ-VLP following i.n. and s.c. immunization. To this end, we reconstituted lethally irradiated wild-type C57BL/6 mice with MyD88−/− bone-marrow (BM) such that hematopoietic cells lacked MyD88 expression, whereas its expression was normal in radiation-resistant cells such as epithelial cells. Irradiated WT mice reconstituted with MyD88−/− BM cells failed to produce IgA upon i.n. immunization, suggesting that hematopoietic cells are responsible for regulating the IgA response to Qβ-VLP via TLR7 signaling (Fig. 2.15 a). We have previously shown that TLR9 signaling in B cells but not in non-B cells is essential for promoting IgG2a responses against VLPs loaded with CpGs (78). We therefore assessed whether a similar mechanism regulated IgA CSR. We addressed this question by generating BM chimeras exhibiting TLR7 expression in all hematopoietic cells except B cells. To this end JH−/− recipient mice were lethally irradiated and reconstituted with a mixture of BM cells isolated from JH−/− and TLR7−/− mice or from JH−/− and WT mice. TLR7 signaling directly to B cells played a role in regulating systemic IgA responses against Qβ-VLP following s.c. immunization but was less important for mucosal IgA responses following i.n. immunization (Fig. 2.15 b,c). This shows that systemic IgA CSR requires TLR7 signaling directly to B cells.

In contrast, the impact of TLR7 signaling on mucosal IgA responses was due to an involvement of another hematopoietic cell type.

In a marked contrast, the Qβ-VLP-specific IgG2a titers, both in serum and BAL were strongly reduced in the group of mice where the B cells where TLR7 deficient (Fig. 2.15 b,c). Based on this finding, we concluded that not only TLR9 and TLR4 signaling in B cells (78, 79, 81), but also TLR7 signaling controls IgG2a CSR independently of the immunization route and requires TLR7 expression in B cells. Importantly, IgG1 levels in chimeric mice exhibiting TLR7 deficient B cells were increased, demonstrating normal responsiveness of the B cells.

Taken together, we conclude that regardless of the immunization route, IgG2a CSR is governed by TLR signaling directly to B cells. In contrast, for IgA responses, this was the case only after s.c. immunization.
Figure 2.15: TLR7 expression directly to B cells is required for systemic but not mucosal IgA responses against Qβ-VLP. (a) Qβ-VLP IgA levels in BAL of WT and MyD88−/− BM chimeras immunized i.n. (b,c) JH−/− mice were lethally irradiated and subsequently reconstituted with BM cells isolated from JH−/− mice (80%) mixed with 20% of BM cells isolated either from C57BL/6 (WT B cells) or from TLR7−/− (TLR7−/− B cells) mice. After reconstitution, chimeric mice were immunized either i.n. (b) or s.c. (c) with 100 µg of Qβ-VLP and 20 days later specific IgA, IgG1 and IgG2a titers were determined in BAL and serum respectively by ELISA. Geometric mean IgA ELISA titers ± SEM are indicated (n = 3); n.d., not detectable. The data shown are representative of two independent experiments. Statistical significance was assessed by unpaired Student’s t-test (*, p < 0.05 and **, p < 0.01).
**Lung dendritic cells and alveolar macrophages are the major cell populations transporting Qβ-VLP from the lung to the draining lymph node**

TLR7 signaling directly to B cells was not required for mucosal IgA. Therefore, our next attempt was to identify the cell population requiring TLR7 signals to promote mucosal IgA responses. We have previously shown that upon i.n. administration Qβ-VLP can mainly be found in the lung and that MLNs which drain the lower airways and are the major sites to which Qβ-VLP are transported and where the Ab response is initiated (161).

In order to elucidate which cell population may be involved in TLR7-dependent IgA CSR, we analyzed in detail the cellular populations interacting with Qβ-VLP within the lung and the MLNs. It has been shown previously that in the lung CD11c⁺CD11b⁻ cells are alveolar macrophages whereas CD11c⁺CD11b⁺ are lung DCs (165). By analyzing Qβ⁺-cells in the lung, we observed that most of the Qβ⁺-cells were CD11c⁺CD11b⁻ cells and a minor proportion was CD11c⁺CD11b⁺ cells, identifying the major cell population interacting with Qβ-VLP as alveolar macrophages (Fig. 2.16 a). We also found a few Qβ-VLP in association with CD11c⁻CD11b⁺ cells and B cells in the lung (Fig. 2.16 a and not shown). In MLNs of mice which received Qβ-VLP i.n. the two major cell populations bearing Qβ-VLP were again alveolar macrophages and DCs (Fig. 2.16 b). Almost no CD11c⁻CD11b⁺ cells positive for Qβ-VLP were found in MLNs, indicating that this cell population is non-migratory and remains in the lung. Thus, it is possible that alveolar macrophages and DCs take up Qβ-VLP in the lung, sense the endogenous ssRNA and transport the Ag to MLN. To address this question, we investigated what happens in a situation where alveolar macrophages and DCs are unable to migrate. In CCR7⁻/⁻ mice, these two cell populations were completely absent in the MLN and most of Qβ-VLP was in association with CD11b⁺ cells. This finding suggests that in WT mice most Qβ-VLP are carried to MLNs by alveolar macrophages and DCs, whereas in situations where these cells were unable to migrate (CCR7⁻/⁻ mice), Qβ-VLP drained via the lymphatic system (Fig. 2.16 b).

Following s.c. administration a large fraction of Qβ-VLP enters the bloodstream and are distributed throughout the body (our unpublished data). By analyzing Qβ⁺-cell populations in MLNs after s.c. immunization we found that Qβ-VLP was in association with distinct cell populations such as macrophages, monocytes and lymphocytes. Most strikingly, the alveolar macrophage (CD11c⁺CD11b⁻) population was completely absent in the Qβ-VLP⁺ gate (Fig. 2.16 c and not shown).

By comparing the numbers of AFCs secreting Qβ-VLP-specific Ab in MLNs of i.n. and s.c. immunized mice, we observed that both groups elicited similar numbers of AFCs secreting
anti-Qβ-VLP IgG, however the numbers of AFCs secreting anti-Qβ-VLP IgA were significantly reduced in MLN of s.c. immunized mice (Fig. 2.16 d). This suggests that CD11c+ cells that take up Qβ-VLP and migrated to MLNs are the key cells for the induction of mucosal IgA responses.

Figure 2.16: Determination of cells in association with Qβ-VLP in the lung and MLNs. (a) Mice were immunized i.n. with 50ug of Qβ-VLP labeled with Alexa 488 and the lung cell populations in association with Qβ-VLP were assessed in the lung by flow cytometry 4 and 24 hours later. (b,c) Cells in association with Qβ-VLP in the MLN after 24 hours of i.n. (b) and s.c. (c) immunization are shown. Comparison of pattern of Qβ-VLP distribution between WT and CCR7-/- mice immunized i.n. is shown in (b). (d) Number of AFCs secreting Qβ-VLP IgA and IgG in MLNs of i.n. and s.c. immunized mice were determined by ELISPOT at day 20 post immunization. Mean values + SEM are indicated (n = 4). The data shown are representative of two independent experiments. Statistical significance was assessed by the unpaired Student's t-test (**, p < 0.01).
**TLR7 signaling in lung DCs and alveolar macrophages induces expression of APRIL and BAFF**

Next, we sought to understand why the lack of TLR7 signaling in lung DCs and alveolar macrophages has implications in the mucosal IgA response against Qβ-VLP. We have seen that TLR7 signaling has only a minor impact in lung DCs and alveolar macrophages migration to the MLNs (data not shown). Therefore, we anticipated that there must be another explanation for the need of TLR signaling in these cells in order to induce mucosal IgA responses. It has been shown that upon CpG and double-stranded RNA stimulation, mucosal DCs increases BAFF and APRIL expression (95, 166). We were therefore wondering whether TLR7 signaling in lung DCs and alveolar macrophages has similar effect. To address this question we sorted the Qβ⁺ cells from MLNs of WT and TLR7⁻/⁻ mice which received Qβ-VLP i.n. and compared the expression level of APRIL and BAFF. The Fig. 2.17 a shows that the levels of APRIL and BAFF were reduced 3 and 2 folds respectively in the Qβ⁺ cells isolated from the TLR7⁻/⁻ mice when compared to the WT group. This result indicates that optimal BAFF and APRIL expression by lung DCs and alveolar macrophages occurs following TLR stimulation *in vivo*.

**Alveolar macrophages and lung DCs sense ssRNA to induce optimal IgA response**

In order to directly address the hypothesis that the CD11c⁺ cells found in association with Qβ-VLP in MLN of i.n. immunized mice are the cell population which require TLR7 signals to promote IgA responses, we generated mixed BM chimeras using BM cells from CD11c-DTR mice, allowing us to specifically deplete DCs and alveolar macrophages. Specifically, WT C57BL/6 mice were lethally irradiated and reconstituted with BM cells isolated from CD11c-DTR mice mixed with BM cells isolated either from TLR7⁻/⁻ or from WT control mice. Upon diphtheria toxin (DT) administration, all the CD11c⁺ population in the chimeric mice completely lacked TLR7 signaling and therefore we could directly address the role of TLR7 signaling in these cells in inducing IgA responses. Fig 2.17 b shows that the levels of VLP-specific IgA in serum and BAL were reduced in chimeric mice in which DCs lack TLR7 expression, confirming therefore that TLR7 signaling in CD11c⁺ DCs and alveolar macrophages is pivotal in regulating mucosal IgA responses.
Figure 2.17: After intranasal immunization TLR7 signaling in CD11c+ cells is necessary for optimal Qβ-VLP IgA responses. (a) Qβ+-cells were sorted by flow cytometry from MLNs of WT or TLR7-/- mice immunized i.n. and the expression of BAFF and APRIL were measured by quantitative real-time PCR. Mean values + SEM are indicated (n = 3). (b) Wild type C57BL/6 mice were lethally irradiated and subsequently reconstituted i.v. with BM cells isolated from CD11c-DTR mice mixed with BM cells isolated either from C57BL/6 (WT DCs) or from TLR7-/- (TLR7-/- DCs) mice. After reconstitution (~6 weeks), chimeric mice were treated for 5 days with DT i.n. After the first DT administration, mice were immunized i.n. with 100 µg of AP205-VLP and 10 days later specific IgA titers were determined in serum and in BAL by ELISA. Geometric mean IgA ELISA titers + SEM are indicated (n = 3). We hypothesized that TLR7 deficiency in CD11c+ cells would impair the IgA response after i.n. immunization and therefore compared the statistical significance by one-tailed Student’s t-test (*, p < 0.05 and **, p < 0.01).
4.2.4 Discussion

Most pathogens invade the body through the mucosa and some cause local infections at mucosal sites. Secretory IgA (sIgA) plays a critical role in preventing infections at these sites. Furthermore, IgA is the most abundant Ig isotype in humans and understanding its regulation is of major importance.

In the present study we dissected the mechanisms involved in the regulation of IgA responses against Qβ-VLP administered via mucosa or systemic routes. Our data show that systemic IgA does not require Th cells or cognate interaction between T and B cells. In marked contrast, mucosal Qβ-VLP-specific IgA response in the lung was strongly dependent on T cell help. It has been shown that DCs can induce T cell and CD40-independent CSR through BAFF and APRIL (24). However, we observed in mice deficient for TACI and BCMA (TACI;BCMA^-/-) that BAFF and APRIL signaling were dispensable for the induction of systemic TI IgA titers in response to Qβ-VLP immunization. This finding contradicts with the literature. Actually, it has been shown that systemic Ab responses elicited by NP-ficoll (prototype TI-2 antigen) but not by NP-CGG (a TD Ag) are strongly regulated by TACI (89, 167). This difference can be explained either by the difference of Ag nature, i.e. whereas NP-ficoll is a TI-2 antigen, VLPs are TI-1 antigen and therefore with stronger capacity to generate B cell responses by its own. Another possibility is that the RNA present on VLPs overcomes the activation signals required via TACI. It still could be possible that BAFF-BAFF-R interaction plays a role in the regulation of TI systemic IgA responses. However a major role has been attributed to signaling via TACI since TACI^-/- mice have low serum IgA in response to TI type II antigens (89). Furthermore, APRIL^-/- mice also showed reduced systemic IgA in response to NP-LPS (a TI-1 Ag) (168). Collectively, these data points to an important role of APRIL-TACI interaction in regulating TI IgA CSR and make it unlikely that BAFF-BAFF-R interaction is important to regulate the systemic TI IgA responses against VLPs.

In contrast to the systemic TI IgA responses, the TD IgA titers in BAL were significantly reduced in TACI;BCMA^-/- mice as well as in TACI^-/- mice. A dominant role for TACI confirms previous findings showing that TACI is important for mediating isotype switching and IgA production by B cells (26, 169). Surprisingly, however, TACI was important for driving a TD IgA response rather than TI IgA responses. This is not consistent with the dogma that TACI mediates TI IgA CSR (89, 167). Importantly, the mucosal IgA levels in
BAL were completely abolished in both MHC II<sup>−/-</sup> as well as in CD40<sup>−/-</sup> mice whilst the reduction was only partial in TACI;BCMA<sup>−/-</sup> mice. This possibly indicates the synergistic role of T helper cells and TACI signaling. While, CD40-CD40L engagement is crucial for mucosal IgA CSR, TACI may be involved in IgA production and plasma cell survival. In agreement with this hypothesis, it has been recently shown that TACI enhances the differentiation of B cells into AFCs in cultures containing limiting conditions of CD40 ligation, suggesting that TACI may be important also for the Ab response to TD antigens (91).

TGFβ has been shown to be an important cytokine for IgA CSR in vitro (107, 108, 162). In vivo, it was found that the IgA response, both in serum and in mucosal washes, against TD antigens was completely abolished in mice which lack TGFβ-RII in B cells (110, 163). More recently it was found that expression of TGFβ-RII on naïve B-cells is induced by iNOS with IgA CSR being impaired in iNOS<sup>−/-</sup> mice (95). However, the in vivo role of TGFβ against viral particles had not yet been addressed. In the present study we found a significant reduction of Qβ-VLP-specific TD IgA titers in BAL of mice immunized i.n. which lack TGFβRII in B cells, whereas systemic TI IgA levels were only slightly reduced. Thus, mucosal IgA responses to viral particles seem to depend on the one hand on TGFβ and on the other hand on the presence of T helper cells in addition to BAFF or APRIL secreted by DCs. In contrast, induction of serum IgA upon systemic exposure to VLPs is largely independent of TGFβ. Furthermore, systemic IgA was independent of Th cells as well as signaling via TACI. These data indicate that there must be an additional, as yet undetermined, factor important for TI IgA CSR upon systemic exposure to viral particles.

The role of TLR ligands as a third signal for Ab class switching has been previously described in vitro (77). In vivo, several reports have indicated a role for TLR signaling in B cells as IgG switching factor (78-82). However, the role of TLR signaling in regulating IgA responses has not been well studied. By comparing Qβ-VLP IgA titers in serum and BAL between groups of mice where TLR7 signaling was present or not, we found, unexpectedly, that TLR7 signaling had a crucial role in regulating IgA responses against Qβ-VLPs, independently of the route of immunization. The same was true for IgG2a, but not for IgG1.

Our experiments with BM chimeras demonstrated that TLR7 signaling in B cells was required for systemic IgA CSR. Together with the notion that IgA responses under these conditions occur independently of Th cells, CD40, TGFβ and TACI, we propose that TLR7 signaling directly to B cells may represent the missing factor required for TI IgA CSR. This fits with the idea that following s.c. immunization, Qβ-VLP drains freely to bloodstream and reaches...
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different lymphoid organs including the spleen (our unpublished data). This might facilitates
the direct interaction of Qβ-VLP with B cells. In contrast, mucosal IgA production required
TLR7 signaling by alveolar macrophages and lung DCs, rather than direct TLR signalling to
B cells. Thus, under these conditions, B cells may have little direct contact with VLPs, and
IgA is induced indirectly, via CD40-CD40L engagement, TGFβ and APRIL and BAFF
signalling to TACI. Indeed, APRIL and BAFF, the ligands for TACI, were upregulated in
alveolar macrophages and lung DCs upon TLR7 triggering (Fig 2.18 a). This data is in
contrast to a recent report showing that human upper respiratory mucosa B cells expressing
TLR3 can initiate TI IgA response following dsRNA activation directly to B cells (166).
Perhaps soluble ssRNA not in association with viral particles also would trigger similar
mechanism because most likely it would target and be sampled by cell population other than
alveolar macrophages and lung DCs.

The observation that alveolar macrophages are a key population for induction of IgA seems
counter-intuitive since macrophages are usually non-migratory. Alveolar macrophages,
however, seem to be different since it is an intrinsic feature of CD11c+ alveolar macrophages
(165) to migrate to MLN and initiate immune responses against particulate Ag (170). Our
results have further demonstrated that alveolar macrophages migrate to the MLNs in a CCR7-
dependent fashion. It will be interesting to directly distinguish in vivo the role of lung DCs
and alveolar macrophages in regulating mucosal IgA responses against VLPs.

In summary (Fig 2.18) our data demonstrate that for TI systemic IgA responses, direct TLR7
signaling to B cells is crucial whilst for TD mucosal IgA responses, regulation occurs
indirectly through the ability of TLR7 signaling to activate alveolar macrophages and DCs
leading to increased BAFF and APRIL production, T helper cell activation resulting in TGFβ
secretion and delivery of CD40-CD40L cognate signals to B cells. It will be interesting to
further determine whether this is a “unique” feature of TLR7 or whether other TLR agonists
bear similar property. We have demonstrated for the first time that direct TLR signaling to B
cells in combination with multivalent antigen results in TI IgA CSR.
Figure 2.18: TLR7 regulates appropriate IgA responses against VLPs directly through signaling in B cells, and indirectly through activation of CD11c⁺ lung DCs and alveolar macrophages. (a) Mucosal IgA responses require lung DCs and alveolar macrophage activation via TLR7 stimulation. Activated DCs/alveolar macrophages modulate IgA responses on the one hand by activating cognate CD4⁺ T cells which in turn provide help via CD40L-CD40 interaction and cytokine secretion (i.e. TGFβ). Additionally, activated DCs/alveolar macrophages can directly modulate IgA responses by secreting APRIL and BAFF upon TLR7 stimulation. (b) Optimal systemic IgA responses simply require strong BCR cross-linking and TLR7 signaling directly in B cells which are provided by direct interaction with VLPs.
4.2.5 Materials and Methods

Mice
C57BL/6 mice were purchased from Harlan. TLR7\(^{-/-}\) (171), MyD88\(^{-/-}\) (172), MHC II\(^{-/-}\) (174), CD40\(^{-/-}\) (175), CCR7\(^{-/-}\) (176), CD11c-DTR/GFP (177) mice on a C57BL/6 background have been described earlier. T\(\beta\)RII\(^{fl}\) mice were purchased from MRC Harwell, UK and were crossed with CD19Cre mice (178) on a BALB/c background in order to delete the floxed target gene (T\(\beta\)RII) in B cells. The used mice T\(\beta\)RII-B were homozygous for T\(\beta\)RII\(^{fl}\) (T\(\beta\)RII\(^{fl/fl}\)) and heterozygous for CD19Cre (CD19\(^{cre/+}\)) and the control group were T\(\beta\)RII\(^{fl/fl}\) and CD19\(^{+/+}\). Depletion of T\(\beta\)RII\(^{fl/fl}\) was ~95% on splenic B cells purified from T\(\beta\)RII-B mice as assessed by PCR (not shown). TACI\(^{-/-}\) and TACI;BCMA\(^{-/-}\) were generated at and kindly provided by BiogenIdec (Cambridge, MA) (179). All animals were kept under specific pathogen-free conditions at BioSupport and were used at 8 to 12 weeks of age. Experiments were conducted in accordance with protocols approved by the Swiss Federal Veterinary Office.

Immunization and antigen
To evaluate the Ab response induced by Q\(\beta\)-VLP, C57BL/6, TLR7\(^{+/+}\), MyD88\(^{+/+}\), MHC II\(^{+/+}\), CD40\(^{+/+}\), T\(\beta\)RII-B, TACI\(^{-/-}\) and TACI;BCMA\(^{-/-}\) mice were immunized either i.n. or s.c. with 50 \(\mu\)g of Q\(\beta\)-VLP containing E. coli-derived ssRNA.

For the i.n. immunization with Q\(\beta\)-VLP, mice were anesthetized with isoflurane and vaccine was administered using a 200 \(\mu\)l pipette. S.c. vaccination was performed by Q\(\beta\)-VLP injection into both sides of the abdomen. For both routes of immunization, Q\(\beta\)-VLP was diluted in PBS to a final administration volume of 100 \(\mu\)l (2 \times 50 \(\mu\)l).

Capsids of the RNA phage Q\(\beta\) were cloned into pQ\(\beta\)10 vector and purified as described elsewhere (120). AP205 coat protein (180) was cloned into the pQ\(\beta\)10 vector (181) and expressed and purified similarly as Q\(\beta\).
**ELISA**

ELISA plates (Nunc Immuno MaxiSorp, Rochester, NY) were coated overnight with 100µl of Qβ-VLP (1µg/ml) and ELISA were performed according to standard protocols using horseradish peroxidase-conjugated secondary Abs: goat anti-mouse IgG (Fc gamma specific), Jackson ImmunoResearch; goat anti-mouse IgA (alpha chain specific), Sigma; rat anti-mouse IgG1, BD Pharmingen and rat anti-mouse IgG2a, BD Pharmingen.

For detection of anti-Qβ-VLP IgA in serum, IgG was depleted using Protein G (Amersham Biosciences). ELISA controls showed that the elevated amount of anti-Qβ-VLP IgG in serum suppresses the measurement of anti-Qβ-VLP IgA. For detection of anti-Qβ-VLP IgG and Qβ-VLP IgA in BAL, undiluted samples were used. Anti-Qβ-VLP IgG titers in serum and in BAL are indicated as dilutions reaching half maximal absorbance at 450nm. Anti-Qβ-VLP IgA titers in serum and in BAL were measured by endpoint titer by calculating the OD (450nm) cut-off as the average of the OD of the naïve sample multiplied by three folds the standard deviation of the OD of the naïve sample.

**ELISPOT Assay**

Qβ-VLP specific antibody-forming cells (AFC) frequencies were determined as described (129). Briefly, 24-well plates were coated with 10µg/ml Qβ-VLP. Spleen, BM, MLN or lung derived-cells were added in DMEM containing 2% FCS and incubated for 5 h at 37°C. Cells were washed off and plates were incubated either with goat anti-mouse IgG (EY Labs) or with goat anti-mouse IgA (alpha chain specific, SouthernBiotech), followed by alkaline phosphatase-conjugated donkey anti-goat IgG Ab (Jackson ImmunoResearch) before development of alkaline phosphatase color reactions.

**Radiation bone marrow chimeras**

Radiation BM chimeras in which all B cells were deficient in the expression of TLR7 were generated by i.v. injection of BM mixture containing 20 % of BM cells isolated from TLR7−/− mice and 80 % of BM cells isolated from JH−/− mice (1-5 x 10⁷ total injected cells) into JH−/− mice that had been lethally irradiated (950 rad) 1 day previously. Prior cell transfer, BM cell suspension was depleted from T cells. Control chimeras with WT B cells were made using a combination of C57BL/6 and JH−/− donor BM. After 6 weeks, BM reconstitution was assessed by staining peripheral blood lymphocytes. Thereafter, mice were immunized with 100µg of Qβ-VLP either i.n. or s.c.
The BM chimeras in which CD11c+ cells lack TLR7 expression were generated as follow: WT mice were lethally irradiated and reconstituted with a BM mixture containing 50% of BM cells isolated from TLR7-/- mice and 50% of BM cells isolated from CD11c-DTR mice. Control chimeras were generated using a mixture of BM cells isolated from C57BL/6 and CD11c-DTR mice. For CD11c+ cells ablation, diphtheria toxin was administrated i.n. for 5 days. In the first 3 administration (every day) the mice received 100ng of DT. For the 2 last administration (with 1 day interval) only 50ng was inoculated. Mice were immunized i.n. with 100µg of AP205-VLP 24 hours after the first DT administration.

**Lung washes and lung digestion**

BAL samples were collected through the trachea by washing the lung three times with 300 µl PBS/1% BSA. Samples were stored at –20°C until use.

For the isolation of leucocytes from the lung, mice were perfused with 5-10 ml of PBS in the heart ventricle to clear lungs of blood. Lungs were chopped in small pieces and incubated at 37°C in media containing collagenase (2.4mg/ml). Finally, leucocytes were harvest by using a 30% percoll gradient.

**Flow cytometry**

For detecting cells in association with Qβ-VLP, mice were immunized either i.n. or s.c. with Qβ-VLP labelled with Alexa-488. Twenty-four or four hours after immunization, mice were killed and cells isolated from lung and MLNs were stained with APC-conjugated rat anti-mouse CD11b and PE-conjugated hamster anti-mouse CD11c (BD Pharmingen). In all cases Fc-receptors were blocked with rat IgG2b anti-mouse CD16/32 (2.4G2) and dead cells were excluded by PI staining.

**Quantitative real-time PCR**

Synthesis of single-stranded cDNA (ss-cDNA) was done with total RNA using random nonamers (Microsynth) and SuperScriptTM II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Complementary RNA was digested by treating the ss-cDNA with 2 units of RNase H (New England BioLabs, Inc.) at 37°C for 20 minutes. The cDNA was then used as template for real-time quantitative PCR (iCycler instrument, Biorad) with the gene specific primers β-actin-F (5'-CCC TGA AGT ACC CCA TTG AAC-3'), β-actin-B (5'-CTT TTC ACG GTT GGC CTT AG-3'), APRIL-F (5'-GGG GAA GGA GTG TCA GAG TG-3'), APRIL-B (5'-GCA GGG AGG GTG GGA ATA C-3'), BAFF-F (5'-AGG CTG GAA GAA
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GGA GAT GAG-3’), BAFF-B (5’-CAG AGA AGA CGA GGG AAG GG-3’) using Brilliant SYBR Green QPCR Master Mix (Stratagene) according to the manufacturer's protocol.

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Author Contributions
J.B. and M.F.B. designed and conceptualized the research; J.B., A.J. and H.J.H performed the experiments; P.S. conceived experiments and contributed with reagents; and J.B. and M.F.B. analyzed the data and prepared the manuscript.
4.3 Part III: B cells transport viral particles into the splenic B cell follicles to initiate antibody responses

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Running Title: Regulation of systemic antibody response upon intranasal immunization

Abbreviations: Ab: antibody; AFCs: antibody forming cells; Ag: antigen; BCR: B cell receptors; C: complement; Cr2: complement receptor 2; FO: follicle; GC: germinal centers; i.n.: intranasal(ly); MZ: marginal zone; PNA: peanut agglutinin; RP: red pulp; s.c.: subcutaneous(ly)
### 4.3.1 Abstract

The lung is exposed continuously to large amounts of antigens and is an important entry site for pathogens. The lung mucosa is rich in secreted IgA and IgG antibodies and exposure of the lung to a variety of antigens results to systemic as well as local antibody responses. Indeed, intranasal administration of virus-like particles (VLPs) derived from the bacteriophage Qβ not only resulted in B cell responses in the lung-draining lymph nodes but also in the spleen, where a strong germinal centre reaction could be detected. However, no free VLPs could be detected in the blood of immunized animals, indicating that VLPs reached the spleen by cellular transport. Indeed, we found a population of VLP-carrying B cells in the lung as well as in the blood and spleen. Transfer of B cells that bound VLPs in the lung induced strong IgG responses in the recipient mice. We found that complement receptor 2 was not involved in VLP-binding but the interaction was mediated by B cell receptors (BCR). Collectively, our data demonstrate that B cells bind VLPs in the lung and transport it to the splenic B cell follicle via low affinity BCR. Thus, lung B cells seem to be crucial for mediating strong systemic IgG responses upon i.n. administration of particulate antigens.
4.3.2 Results

Intranasal administration of Qβ-VLPs induces efficient systemic IgG response

We have reported previously that Qβ-VLP efficiently reach the lung after intranasal immunization and induce a strong systemic specific IgG responses (161). Indeed, mice immunized either i.n. or subcutaneously (s.c.) with a single dose of 50μg of Qβ-VLPs mounted similar levels of systemic IgG titers which lasted up to 120 days post-immunization (Fig 2.19 A). Correspondingly, the number of Qβ-VLP-specific AFCs secreting IgG Abs as well as the number of GCs in the spleen was also similar (Fig 2.19 B,C). These results indicate that the spleen is actively participating in the Qβ-VLP-specific IgG response. The presence of VLP-specific GCs in the spleen strongly suggested that VLPs reach the spleen from the lung to initiate the B cell responses.

Figure 2.19: Generation of Qβ-VLP-specific systemic B cell responses following intranasal immunization. (A) Qβ-VLP-specific IgG titers in serum of C57BL/6 mice immunized with 50μg of Qβ-VLP either i.n. (circles) or s.c. (squares) were determined by ELISA. Geometric mean titers ± SEM (n = 6) are shown. (B) Numbers of AFC secreting Qβ-VLP IgG were determined by ELISPOT in the spleen of either i.n. (circles) or s.c. (squares) immunized mice. Mean values ± SEM (n = 3) are given. (C) Histological spleen sections from mice 12 days after s.c. (upper panel) or i.n. (lower panel) immunization were stained for PNA (red), to identify GCs and Qβ-VLP-specific B cells (green). Note that Qβ-VLP-specific B cells are found in either GCs or large aggregates as plasmablasts outside B cell follicles.
Detection of Qβ-VLPs in spleen and serum

We have previously demonstrated that Qβ-VLPs quickly reach the spleen and persists in follicular dendritic cells (FDCs) for at least 30 days. Since the GC-formation observed in the spleen after i.n. immunization indicates presence of VLPs, our next attempt was to localize Qβ-VLPs in the spleen by immunohistochemistry. For this purpose, 50µg of Qβ-VLPs (the immunogenic dosage) were administered either i.n. or s.c. and cryosections of the spleen were examined for the presence of VLPs after 24 hours. To our surprise, no Qβ-VLPs could be detected in the spleen of i.n. immunized mice (Fig 2.20 A). In contrast, Qβ-VLPs were readily detected in the marginal zone (MZ) as well as in the follicular (FO) area of the spleen after s.c. immunization (Fig 2.20 A). In order to assess whether amounts of Qβ-VLPs in the spleen may have been too low for detection, we repeated the experiment with a 20x higher dose of Qβ-VLP (1 mg). At this high Ag dose, Qβ-VLP were clearly detectable in the spleen of i.n. immunized mice. Surprisingly, however, VLP-localisation was confined to the FO compartment and no antigen was detected in the MZ and red pulp (Fig 2.20 B). In a marked contrast, in the spleen of mice immunized s.c. with 1mg of Qβ-VLP, the Ag was found throughout the whole spleen including T and B cell areas, MZ and mainly in the red pulp (RP). The spleen is the major organ which filters the blood (182) and blood born-antigens are usually trapped in the MZ and red pulp. Thus, Qβ-VLPs seem to reach the spleen via the blood after s.c. immunization. In marked contrast, the confined localization of Qβ-VLP within the B cell FO of mice which received Qβ-VLP i.n. indicated that Qβ-VLPs were not transported from the lung to the spleen via the blood stream, at least not in a free manner. To directly address this question, we immunized mice with 100µg of Qβ-VLPs either i.n. or s.c. As a positive control we also administered 100µg of Qβ-VLP intravenously (i.v.). Free Qβ-VLPs in serum were quantified by ELISA 2 and 7 hours later. The Fig 2.20 C shows that whereas a great amount of free Qβ-VLP could be detected in serum 2 hours after s.c. and i.v immunization (~40 and ~100% respectively was recovered from the 100µg initially administered), only a very small fraction (~2ng/ml) of free Qβ-VLP was detected in serum of i.n. immunized mice (Fig 2.20 C). Importantly, with this low Ag dosage, the VLP-specific IgG titer was barely induced upon i.v. administration (data not shown). Thus, Qβ-VLPs do not reach the spleen from the lung by free transport trough the bloodstream.
Figure 2.20: Detection of antigen in spleen and serum after immunization with Qβ-VLP. Immunohistochemistry of spleen sections stained for Qβ-VLP antigen from mice 24 hours after immunization with 50µg (A) and 1mg (B) of Qβ-VLP either s.c. (left panels) or i.n. (middle panels) or from naïve (right panels) mice. Arrows indicate Qβ-VLP stain in the marginal zone (MZ: upper left panel), red-pulp (RP: lower left panel) and follicular compartments (FO: upper left and lower middle panels). (C) Qβ-VLP concentrations in serum of mice 2 and 7 hours after i.n. (gray triangles), s.c. (black squares) or i.v. (red triangles) immunization with 100µg of Qβ-VLP. Mean concentrations of 3 mice are given with ± SEM.
**B cells are the major cell population transporting Qβ-VLPs in blood after i.n. immunization**

Since free circulation of Qβ-VLP is not the major mechanism of transporting Qβ-VLP from the lung into B cell FO, we tried next to identify a cell population in the blood associated with Qβ-VLP. To this end, mice were immunized either i.n. or s.c. with Qβ-VLPs labelled with Alexa 488 and 4 hours later blood cells were analyzed for binding of Qβ-VLPs. Whereas in the blood of s.c. immunized mice the great majority of Qβ-VLP (~90%) was in association with monocytes (Fig 2.21 A and not shown), i.n. immunization resulted in a small population of B cells associated with Qβ-VLP (Fig 2.21 A). This observation was rather unexpected but fitted well with the finding that Qβ-VLP were exclusively detected in the B cell FO of i.n. immunized mice (Fig 2.20 B).

Whether B cells trapped Qβ-VLPs directly from the lung was assessed next. Mice were immunized i.n. with Qβ-VLPs labelled with Alexa 488 and 4 hours later lungs were collected and digested with collagenase to allow the isolation of lymphocytes. As shown in Fig 2.21 B virtually all CD19+ B cells in the lung of i.n. immunized mice bound Qβ-VLPs. There was also a population of CD19- non-B cells bound Qβ-VLPs in the lung. These are mainly migrating CD11c+ alveolar macrophages and dendritic cells involved in the initiation of IgA responses in the mediastinal lymph nodes (data not shown). Thus, Qβ-VLPs in the lung are captured by alveolar macrophages, dendritic cells as well as B cells. While alveolar macrophages and DCs migrate to draining LN (not shown), B cells migrate through the blood to the spleen to enter the splenic B cell FO. Indeed, cryosection of the spleen stained for the B cell marker B220 showed that many Qβ-VLPs were on the surfaces of B cells (Fig. 2.21 C).
Lung-B cells from i.n. immunized mice induce specific Ab response

Next, we assessed whether the lung-B cells carrying Qβ-VLPs are able to induce a B cell response in the spleen. For this end, B cells were purified from the lung 4 hours post i.n. immunization and transferred i.v. into naïve recipient mice. Indeed, the group of recipient mice which received lung-B cells of i.n. immunized mice were able to induce high titer of Qβ-VLP IgG in serum (Fig 2.22 A). In contrast, mice receiving lung-B cells isolated from naïve mice failed to induce VLP-specific IgG responses.
Figure 2.22: B cell response induced by lung-B cells capturing Qβ-VLPs. (A) Lung-B cells from mice 4 hours after i.n. immunization and from naïve mice were adoptively transferred into naïve mice. Their Qβ-VLP specific IgG titers 12 days after B cells transfer were compared to that of naïve mice and mice immunized i.n. with 100µg of Qβ-VLP. Geometric mean titers ± SEM (n = 4) are given. (B) Staining of splenocytes 12 days after transfer of lung-B cells either from i.n. immunized (lower panel) or untreated (upper panel) congenic Ly5.2 mice into Ly5.1. Analysis was performed by gating on isotype-switched B cells (CD19+, IgM, IgD, CD4, CD8, CD3, CD11c, CD11b and Gr-1−). The data shown are representative of two independent experiments.
The previous experiment demonstrated that lung-B cells bind Qβ-VLPs; however, it remained unclear whether the IgG response was mediated by the transferred B cells themselves or by resident splenic B cells. To address this question we isolated lung-B cells from Ly5.2 C57BL/6 mice immunized i.n. 4 hours earlier and transferred them into congenic Ly5.1 C57BL/6 mice in order to distinguish donor versus host B cells. 12 days later, spleens from immunized mice were isolated and Qβ-VLP-specific B cells were detected by FCM as described before (30). All Qβ-VLP-specific B cells in the spleen of recipient mice expressed the congenic marker Ly5.1, confirming that it was the host B cells which mounted the Qβ-VLP-specific B cell response. Thus, lung B cells transport Qβ-VLPs to the spleen where resident FO B cells recognize the antigen and mount an antibody response.

**Binding of Qβ-VLP to B cells is not mediated by Cr2, but by low affinity Qβ-VLP BCR instead**

In the final set of experiments, we tried to identify the mechanism by which B cells bind Qβ-VLPs. It has been shown previously for lymph nodes that B cells resident in the subcapsular area have the ability to bind immuno-complexes (IC) and delivery them to FDCs resident in the B cell FO. Importantly, this process was found to be mediated by Cr2 expressed on B cells (183). In order to assess whether a similar mechanism was involved in the transport of Qβ-VLPs from the lung to the spleen, frequency of Qβ-VLP-binding B cells were compared in the blood of i.n. immunized wild type (WT) and Cr2−/− mice. Surprisingly, a population of B cells binding Qβ-VLP were observed in the blood of both WT as well as Cr2−/− mice (Fig 2.23 A). This indicates that the mechanisms by which B cells mediate transport of IC into FO in the lymph node differs from the one in which B cells transport inhaled viral particles into splenic FO.

Another possible way by which B cells bind Ag is via the B cell receptors (BCR). To investigate whether blood B cells were binding Qβ-VLPs after i.n. immunization via BCR, we took advantage of VI10YEN mice which express a BCR specific for vesicular stomatitis virus (VSV) (184). These mice have a drastically reduced B cell repertoire and are essentially unable to bind other Ags than VSV. When the frequency of Qβ-VLP-binding B cells in the blood of WT and VI10YEN mice immunized i.n. was compared, the latter were found to be unable to bind Qβ-VLP (Fig 2.23 B). This strongly suggests that lung-B cells transport Qβ-VLP administered i.n. into the splenic FO via binding to low affinity BCRs.
Figure 2.23: Determination of receptors involved in Qβ-VLPs binding to B cells after intranasal immunization. Analysis of uptake of Alexa 488-conjugated Qβ-VLP by B cells in the blood of naïve, C57BL/6 wild-type and Cr2−/− mice (A) and in C57BL/6 wild-type and VI10xYEN mice (B) 4 hours after i.n. immunization with 100µg of Qβ-VLP. The data shown are representative of two independent experiments.
4.3.3 Discussion

Intranasal immunization has been shown to be an efficient route for vaccination, eliciting both mucosal as well as a systemic IgG and IgA responses (127, 131, 161, 194). We have also shown that following i.n. immunization, VLPs reach the lung and subsequently induces efficient B cell responses in the lung-draining lymph nodes as well as in the spleen (161). Dendritic cells have been implicated in the transport of antigen from the lung-mucosa to the draining lymph nodes (165, 239, 240). Less is known, however, about the transport of antigen from the lung to the spleen. Since the lung is a highly perfused organ and the spleen in a way the “blood-draining lymphoid organ”, the most obvious way of antigen transportation may have been the blood. To our surprise, however, no free VLPs were detected in the blood. In addition, histological analysis revealed a distribution of VLPs within the spleen which was not compatible with blood-born transport of VLPs: while systemic exposure of VLPs primarily resulted in antigen deposition in the red pulp and the marginal zone (with some staining in the B cell follicles), intranasal immunization resulted in exclusive deposition of VLPs within B cell follicles.

Blood-born bacteria do not reach the spleen in free form but are bound to neutrophils and immature dendritic cells (241). In order to identify a cell population that may transport VLPs from the lung to the spleen, we performed a detailed analysis of blood and spleen cells associated with VLPs. To our surprise, VLPs in blood and spleen were neither bound to neutrophils nor monocytes but were associated with B cells. Moreover, B cells found within the lung-tissue of i.n. immunized mice were also associated with VLPs, indicating that B cells pick up VLPs in the lung. Transfer of such lung-derived B cells exhibiting bound VLPs into recipient mice resulted in potent antibody responses. Importantly, host B cells and not the transferred B cells themselves were responsible for the observed immune response, demonstrating that lung B cells served as a shuttle, transporting lung-derived antigen into the splenic follicles.

As mentioned above, blood-borne bacterial Ags reach the spleen via the MZ from where they can be transported within a few days into the FO by poorly defined myeloid cells binding the cysteine-rich domain of murine mannose receptor (242-244). We did not find an involvement of these cells in the transport of VLPs. This is most likely because VLPs do not reach the MZ after pulmonary exposure. Indeed, histological analysis revealed VLPs within the follicles but never in the MZ. An important role in follicular Ag transport has also been attributed to MZ B cells. It has been shown that Ags complexed to pentameric IgM are transported from the MZ
to FDCs located in the FO by MZ B cells. This transport was dependent upon C3 and Cr2, since in C3 and Cr2−/− mice, the IgM-Ag complexes were only detected in the MZ but fail to be transported into the FO (245, 246). A similar mechanism has also been reported to facilitate the follicular transport of Ags bound to complement and of a monoclonal anti-CR1/2 Ab (247, 248). Interestingly, it has been shown that MZ B cells are constantly shuttling between the MZ and FO, supporting a model where MZ B cells capture systemic Ag and deliver these to FDCs (249). However, other reports have demonstrated that MZ B cells are sessile cells and remain in the MZ for extended period of time in an integrin-dependent fashion (250) and may leave the MZ only upon activation. MZ B cells also do not seem to be involved in Ag transport upon i.n. immunization, since no antigen could be detected within the MZ neither early (4 hours) nor later (24 hours) after immunization. Furthermore VLPs could also be readily detected within the FO even in C3−/− mice (data not shown). Collectively, these findings render an important role of MZ B cells in transporting lung-borne Ags into the splenic FO very unlikely.

Currently we are investigating how lung-B cells bind VLPs. We have not observed impaired VLP-binding in mice deficient in Cr2 and components of the complement cascade, excluding that VLPs bind B cells exclusively through complement receptors. This is in contrast to a previous study using immune-complexes, where Cr2 expression on B cells was found to be critical for antigen binding and transport (183). The difference may be explained by the fact that VLPs are highly repetitive and therefore able to engage in high-avidity interactions with B cells whilst the relatively artificially created immune complexes cannot. Indeed, B cells expressing a BCR specific for vesicular stomatitis virus (VSV) (VI10YEN mice) clearly captured VLPs less efficiently in the lung and blood, suggesting that B cells may bind the VLPs through low affinity (but high avidity) interactions with their BCR. Whether natural antibodies, which also exhibit reduced diversity in these quasi-monoclonal mice, are involved in VLP-binding to B cells remains to be investigated. Interestingly, preliminary results showed that transfer of sera from wild-type mice into VI10YEN mice could not restore the ability of these B cells to bind Qβ-VLP. This argues against an involvement of natural antibodies in VLP-binding and supports a more important role of low affinity BCRs in binding Qβ-VLPs in the periphery following lung Ag exposure. These data are in line with a model that only B cells binding antigen with high affinity migrate to the T-B boundary to make cognate interaction with primed T cells while other B cells may deposit antigen on FDCs (251, 252).
In summary, we identify B cells as a key cell responsible for the shuttling of antigen from the lung mucosa to the spleen to initiate antibody responses.
4.3.4 Materials and Methods

Mice and antigens
To evaluate the Ab response induced by Qβ-VLP, female, 8 weeks old C57BL/6 mice (Harlan, Horst, The Netherlands) were immunized either intranasally (i.n.) or subcutaneously (s.c.) with 50µg of Qβ-VLP containing E.coli-derived ssRNA. All animal experiments were conducted in accordance with protocols approved by the Swiss Federal Veterinary Office. Capsids of the RNA phage Qβ were cloned into pQβ10 vector and purified as described elsewhere (120).

Immunization
For the i.n. immunization with Qβ-VLP, mice were anesthetized with isoflurane and vaccine was administered using a 200µl pipette. S.c. vaccination was performed by Qβ-VLP injection into both sides of the abdomen. For both routes of immunization, Qβ-VLP was diluted in PBS to a final administration volume of 100µl (2x 50µl).

Lung cells isolation
For the isolation of lymphocytes from the lung, mice were perfused with 5ml of PBS in the heart ventricle to clear lungs of blood. Lungs were chopped in small pieces and incubated at 37°C in media containing collagenase. Finally, lymphocytes were harvest by using a 30% percoll gradient.

Purification and adoptive transfer of lung-B cells
Leucocytes isolated from the lung were incubated with anti-CD19 MACS microbeads (Miltenyi Biotech) and B cells were positively selected through LS MACS separation columns according to the manufacturer’s instructions. Purity of CD19+ B cells was ~90%. Cells were extensively washed with PBS at 4°C, resuspended in 100µl of PBS and injected into the tail vein of a sex-matched recipient. After 10 days, recipient mice were bled for determination of Qβ-VLP-specific IgG titers.
ELISA
For determination of Qβ-VLP-specific IgG titers, ELISA plates (Nunc Immuno MaxiSorp, Rochester, NY) were coated overnight with 100µl of Qβ-VLP (1µg/ml) and ELISA were performed according to standard protocols using HPRO-conjugated goat anti-mouse IgG (Fc gamma specific), Jackson ImmunoResearch. Anti-Qβ-VLP IgG titers in serum are indicated as dilutions reaching half maximal absorbance at 450nm.

For determination of free Qβ-VLP in serum, ELISA plates (Nunc Immuno MaxiSorp, Rochester, NY) were coated overnight with 100µl of anti-Qβ-VLP monoclonal Ab (1.5µg/ml) and quantification of free Qβ-VLP in serum was determined using biotinylated anti-Qβ-VLP monoclonal Ab following HPRO-conjugated Streptavidin (Jackson Immuno Research). Optical densities were read in the ELISA reader at 450nm.

ELISPOT Assay
Qβ-VLP specific antibody-forming cells (AFC) frequencies were determined as described (129). Briefly, 24-well plates were coated with 10µg/ml Qβ-VLP. Spleen were added in DMEM containing 2% FCS and incubated for 5 h at 37°C. Cells were washed off and plates were incubated either with goat anti-mouse IgG (EY Labs), followed by alkaline phosphatase-conjugated donkey anti-goat IgG Ab (Jackson ImmunoResearch) before development of alkaline phosphatase color reactions.

Flow Cytometry
Detection of Qβ-VLP specific B cells expressing surface Ig was performed by incubation with Alexa 647-conjugated Qβ-VLP particles. Donor versus host cells were distinguished with a FITC-conjugated anti-CD45.1 (1/300; mouse anti-mouse). Isotype-switched B cells were detected with a mixture of PE-conjugated antibodies at 1/500 dilution (rat anti-IgD; goat anti-IgM serum, JacksonImmuno Research Laboratories; hamster anti-CD3; rat anti-CD4; rat anti-CD8; hamster anti-CD11c; rat anti-CD11b; rat anti-Gr-1 and PerCP-Cy5.5-conjugated rat IgG2a anti-CD19 (1/400; 1D3). In all cases Fc-receptors were blocked with rat IgG2b anti-mouse CD16/32 (1/100; 2.4G2). Antibodies were purchased from BD Biosciences, unless otherwise specified.

For detecting cells in association with Qβ-VLP in the blood, mice were immunized either i.n. or s.c. with 100µg of Qβ-VLP labelled with Alexa-488. Four hours after immunization, mice were killed and blood-cells were stained with APC-conjugated rat anti-mouse CD19 (BD
RESULTS

Pharmingen; 1/400). Fc-receptors were blocked with rat IgG2b anti-mouse CD16/32 (1/100; 2.4G2) and dead cells were excluded by PI staining.

**Immunofluorescence**
Freshly removed spleens were immersed in OCT and snap-frozen in liquid nitrogen. Tissue sections of 7μm thickness were cut in a cryostat and fixed with acetone. For detection of Qβ-VLP specific B cells, firstly unlabeled Qβ-VLP capsids were added, followed by a polyclonal rabbit anti-Qβ-VLP antiserum (1/1500; RCC) and detected with Alexa 488-conjugated goat anti-rabbit Ab (1/1000). PNA-binding B cells were detected with biotinylated PNA (1/800; Vector Laboratories), followed by Alexa 546-labeled Streptavidin (1/1000). Qβ antigens were detected by incubating sections with anti-Qβ antiserum (1/1500; produced by RCC Ltd., Switzerland), followed by Alexa 488-conjugated goat anti-rabbit Ab (1/1000; Molecular Probes). B cell follicles were identified with Alexa 647 rat anti-mouse B220 (1/200; BD Pharmingen).

**Immunohistochemistry**
Freshly removed spleens were immersed in OCT and snap-frozen in liquid nitrogen. Tissue sections of 7μm thickness were cut in a cryostat and fixed with acetone. For detection of Qβ-VLP antigens, sections were incubated with rabbit anti-Qβ antiserum, followed by alkaline phosphatase-labeled goat antibodies to rabbit immunoglobulins (Jackson ImmunoResearch Laboratories) and alkaline phosphatase-labeled donkey antibodies against goat immunoglobulins (Jackson ImmunoResearch Laboratories) (diluted 1/80). Alkaline phosphatase was visualized using Fast Blue substrate Kit III (Vector Laboratories), which yielded a blue precipitate. Red pulp was distinguished from white pulp using Nuclear Fast Red solution (Vector Laboratories).
Vaccination against infectious disease has been one of the greatest successes of modern medicine. Protection induced by vaccination is almost always achieved by the generation of neutralizing antibodies (151, 185). Most of the commercial vaccines available are either attenuated or inactivated forms of the infecting pathogen, such as Salk and Sabin polio vaccines, respectively (186, 187). Although these forms of vaccines are usually safe, there is a low but real risk of reversion to a virulent phenotype, at least for attenuated pathogens. For instance, an oral rotavirus vaccine (RotaShield) based on live attenuated Rhesus monkey-human recombinant rotavirus had to be withdrawn after a short time on the market because of the risk of causing intussception (a blockage of the intestine) (188, 189). Therefore it is a tempting idea to develop recombinant, non-replicating prophylactic vaccines in order to overcome these possible side effects. In this respect, virus-like particles (VLPs) have shown promising results. VLPs are pseudoviruses which spontaneously assemble into a highly ordered array upon recombinant expression of viral proteins. They may be economically and easily produced and most importantly they efficiently generate antibodies against the viral particle from which they were derived as well as against foreign B cell epitopes expressed on their surface (190).

Most currently used vaccines are administered through parenteral routes such as intramuscular and subcutaneous injection which efficiently induce systemic IgG antibodies; however, they poorly induce mucosal secretory IgA (SIgA) as well as cytotoxic T cell (CTL) immunity. Taking into account that most infectious agents enter the body via the mucosal surface, protective immunity by the mucosa becomes of major importance. The development of vaccines for administration onto mucosal surfaces which efficiently induce systemic IgG as well as SIgA and CTL immunity at the site of antigen penetration is therefore an important current goal in the industry. Moreover, mucosal administration can prevent the risk of needle associated infection caused by parenteral vaccination, still a major risk in developing countries (152).
VLPs are especially interesting from a mucosal vaccine point of view since they bear many features of mucosal pathogens (i.e. they are particulate, stimulate the innate immune system and target cells under lying the mucosa, such as mucosal DCs) (61). Furthermore, when given mucosally they are exposed to the immune system as viruses usually are (191). As a prove of this promising principle, it has been shown in humans and mice that oral (192, 193) and i.n. (127, 131, 194) administration of VLPs results in systemic IgG as well as SIgA and mucosal CTL responses and consequently in protection against mucosal pathogens. Moreover, the i.n. route has the advantage that it elicits stronger antibody responses at lower doses of VLPs when compared to the oral route (194). In agreement with these reports, we also have seen that a single dose of 50µg of Qβ-VLPs in the absence of adjuvant administered i.n. resulted in strong VLP-specific local IgA responses as well as systemic IgG and IgA (161). With respect to CTL responses, the i.n. route of administration of VLPs did induce local CTL response, but compared to the s.c. route, the systemic CD8⁺ T response was clearly inferior and the CD8⁺ T cells were not fully differentiated into effector cells (161). This suggests that i.n. administration of VLPs preferentially induce local CTL responses as well as local and systemic Ab responses and therefore might be suitable for vaccination against pathogens which infect the respiratory tract.

The inductive site of the antibody response upon i.n. immunization is an area of debate. Some groups believe that the NALT is the major site for priming B cells upon i.n. infection (112, 117, 139, 140, 195). However, Balmelli et al have shown that oral, s.c. and i.n. immunization in conscious mice with HPV16-VLPs were inferior at inducing specific-SIgA in mucosal secretions when compared to i.n. administration to anesthetized mice (127). In a attempt to identify whether the upper or lower respiratory tract and associated LNs were important to induce Ab responses against HPV16-VLPs, the same group observed later that in order to have SIgA in the female genital tract, HPV16-VLPs should target the lower respiratory tract (138). Similar findings were observed in humans. Specifically, Nardelli-Haefliger D et al have shown that the aerosol administration route (which reaches the lower respiratory tract) with HPV16-VLPs was superior at inducing specific IgA AFCs as well as SIgA when compared to nasal vaccination targeting the upper airways (131). In agreement with these findings, we also observed that MLNs draining the lung are the major inductive site of Ab response upon i.n. administration (161). Moreover, experiments performed in rats showed that Qβ-VLPs administration into the nose is inefficient at induce Ab responses (our unpublished data).
Altogether, these data suggest that VLPs technology may represent a promising choice for mucosal vaccination and points to the inhalation route as a potent alternative to parenteral injections.

### 5.1.1 Do the levels of mucosal IgA correlates with protection?

The biological importance of IgA in protecting against viral infection is a matter of debate. One reason is that IgA deficient individuals are normally asymptomatic. In addition, IgA was not required for prevention of influenza virus infection and disease in experimental models using IgA deficient mice (IgA\(^{-/-}\)) (196). Specifically, the authors used three approaches to determine the role of IgA. By comparing the susceptibility to influenza virus infection in naïve mice (non-immunized), the authors determined that the levels of pulmonary virus replication and the rate of clearance were similar in IgA\(^{-/-}\) and IgA\(^{+/+}\) mice. Secondly, they addressed the importance of Ag-specific IgA in protecting against viral challenge. To this end, they immunized mice i.n. with influenza virus vaccine containing cholera toxin and observed that nasal and pulmonary protection against influenza virus infection was achieved in IgA\(^{-/-}\) mice. Last, they determined by passive immunization that influenza-specific pIgA as well as IgG1, IgG2a and IgM equally prevented virus infection in the lung and nose (196). However, what is usually not taken into consideration is that in IgA\(^{-/-}\) mice the immune system has a backup mechanism. In another words, it has been clearly shown that in the absence of IgA, immunoglobulins from other isotypes (i.e. IgM and IgG) are overproduced most probably as a compensatory mechanism (196, 197), as shown in IgA-deficient humans (198, 199). However, Renegar et al has elegantly shown that both SIgA and systemic IgG are important to mediate protection against influenza infection in the respiratory tract. While SIgA protects the upper nasal compartment, IgG is more important for protection of the lungs (150).

Also in humans the importance of IgA is controversial. Although humans carrying selective IgA deficiency are apparently asymptomatic, they can eventually suffer from respiratory and gastrointestinal infections, respond poorly to TI antigens and develop B-cell lymphoproliferative disorders and autoimmunity (157, 200). These same symptoms can also be observed in TACI deficient mice, a class-switch-inducing receptor involved in TI IgA responses (26, 167).

In health individuals, it has been shown that upon vaccination with cold-adapted influenza virus, resistance to infection has been correlated with anti-hemagglutinin (anti-HA) IgA levels in the nasal washes (201). Also in the mouse model, anti-influenza immunity has been shown
to directly correlate with the levels of nasal IgA (202). Balmelli et al also have shown that following i.n. vaccination with human pappilomaviris type 16 (HPV16) VLPs, mice generated high titers of mucosal IgA in the genital secretions which translated in to high viral neutralization (127). Additionally, SIgA has an intrinsic capacity to mediate cross-protection and therefore it has been shown in many studies that SIgA is highly effective against heterologous virus infection, showing its advantage over anti-viral systemic IgG (148, 203, 204). Other groups have further correlated the induction of SIgA with protection against several upper respiratory tract pathogens, including influenza in humans (205, 206) and mice (207-209). An important role of IgA has also been demonstrated for mediating protection against pathogenic bacteria (98, 149). These results clearly confirm the importance of IgA in preventing virus and bacterial infections. Overall, we believe that in IgA-/- as well as in patients with IgA deficiency, the increased levels of IgG and IgM mask the importance of IgA. But in healthy individuals, IgA most likely plays an important role in mediating protection.

To date, we still could not directly investigate the real importance of IgA in a infection model. Although we have observed in mice that upon i.n. vaccination with VLPs expressing the ectodomain of M2 (a protein which is highly conserved in all known human influenza A strains), M2-specific IgA was induced in BAL and mice were protected against viral challenge (161), it is unlikely that the protection observed was accomplished by neutralizing M2-specific IgA. This is because we have previously seen that ADCC is the mechanism which confers protection against influenza virus infection upon M2-VLPs vaccination (134). Additionally, IgA-/- mice vaccinated with VLP-M2 were protected upon a lethal dose of influenza virus infection (Schmitz, unpublished data). In order to directly assess the neutralization capacity of mucosal IgA induced by VLPs vaccination, we are currently developing vaccines based on hemagglutinin (HA), the most abundant surface glycoprotein of influenza virus and therefore can be neutralized by SIgA antibodies.

5.1.2 Is long-term humoral immunity in the mucosa provided by long-lived plasma cells?

The hallmark of vaccination is the maintenance of memory antibody levels which can be provided either by memory B cells that, upon antigen re-stimulation differentiate into short-lived antibody-secreting cells or by long-lived plasma cells (210, 211). Although a large body of evidences has shown that both, memory B cells and long-lived plasma cells are responsible
to maintain the serum antibody levels (210), little is know about the humoral memory at mucosal surfaces, in particular about the maintenance of secretory IgA levels.

In this regard, Hou and colleagues have seen that upon influenza infection high numbers of long-lasting influenza-specific IgA AFCs are found in the NALT as well as in the bone-marrow (139). Later, the same group showed that i.n. immunization with inactivated respiratory syncytial virus (RSV) in combination with bacterial adjuvants was able to induce IgA plasma cells which mediated long-term protection against RSV infection (212). However, it remains elusive whether these IgA AFCs are short-lived and are constantly generated due to stimulation by persisting Ag or whether they constitute a pool of long-lived plasma cells residing in survival niches. The fact that the presence of bacterial component as adjuvant was essential to generate this pool of AFCs favours the idea that a chronic stimulation is occurring. On the another hand, the fact that high numbers of specific-AFCs were found in different compartments early post-infection, but were maintained only in the NALT, suggests that these AFCs indeed might represent long-lived PCs which have found in NALT survival niches.

We also have seen in mice that upon a single i.n. immunization dose of VLPs, the levels of VLP-specific IgA AFCs are sustained in the BM up to 120 days post-immunization (161). At the same time-point, VLP-specific-IgA AFCs could also be detected in the MLN draining the lung (161) followed by specific-IgA Abs in the BAL (Bessa, data not shown).

Notably VLPs can not replicate in the host and do not require administration in adjuvant (therefore do not prolong the immune response by persisting in a depot). Nevertheless, high numbers of IgA-AFCs can be detected. This certainly favours the hypothesis of long-lived IgA PCs being generated in mucosal compartments. Indeed, studies carried out by our group showed that the depletion of FDCs and its association with antigen had a major impact only in the early phase of the B cell response. In contrast, the depletion of FDCs was largely irrelevant after the establishment of the Ab response because the serological Ab levels were maintained even after the depletion of FDCs late after immunization. Thus, VLPs trapped on FDCs is required only for establishing the pool of memory B cells and long-lived PCs after VLPs immunization. We have shown that the serum IgG levels are mainly maintained by a very slowly declining PCs population in the BM (130). Although this has not been formally demonstrated for IgA, we believe that also the VLP-specific IgA AFCs found in the spleen and in BM at late time-points are responsible to maintain the IgA Ab levels.
Since this is currently only a hypothesis it will be interesting to directly assess the life-span of mucosal IgA PCs. Another important question is whether the IgA AFCs detected in the BM are originating in the mucosal compartments or whether these cells have a systemic origin. These questions are extremely relevant in terms of the duration of protection provided by the mucosal versus parenteral vaccination.

5.2 Regulation of IgA responses by virus-like particles: analogies and differences to other antigens

5.2.1 TD IgA switching

Antibody class switching is a process in which the IgH constant (C) region μ (C_μ) and C_δ genes encoding IgM and IgD are replaced by any of the downstream IgH C region exons such as C_γ, C_α or C_ε encoding IgG, IgA and IgE respectively (32). IgA class switching can occur via both, T cell dependent (TD) and T cell independent (TI) mechanisms. Factors such as the antigen nature and the site where the humoral immune response is initiated will determine whether IgA responses will require T cell help or not.

The most common experimental antigen (Ag) used to evaluate the mechanisms underlying IgA responses are intestinal bacteria. This is because the gut mucosa hosts approximately 80% of all antibody forming cells (AFCs) secreting IgA (213, 214). However, these models cannot be readily used for understanding the mechanisms controlling IgA responses in the context of mucosal vaccine design. There is therefore a need to understand the mechanisms regulating IgA responses against antigens other than gut commensal and pathogenic bacteria. Similarly to TD IgG antibody responses, also TD IgA responses occur after recognition of specific antigens by B cells which leads to germinal center (GC) reactions in follicular compartments of secondary lymphoid organs (31). It is during the GC reaction that B cells undergo the two main events of antibody maturation such as class-switch recombination (CSR) and somatic hypermutation (SHM) (215, 216). CSR is a process dependent on the DNA-editing enzyme activation-induced cytidine deaminase (AID) (217). AID deaminates cytosine residues on both strands of the S-region DNA, thereby generating DNA “cuts” that will be processed into double-stranded DNA breaks (43, 216). The second event introduces point mutations at high rates into V_HDJ_H and V_LJ_L exons, thereby providing the structural correlate for selection of high affinity antibodies (157, 215).
The hallmark of GC reactions is the generation of long-lived memory B cells and AFCs after B cell interaction with CD4$^+$ T cell via CD40L (CD154) and CD40 engagement and recognition of antigen trapped on the surface of follicular dendritic cells (FDCs) (56). Not only CD40L plays a critical role for TD IgA CSR. Also TGFβ1 has been shown to be essential for IgA TD responses. While CD40-CD40L ligation is crucial for the initiation of the switch process by inducing AID (an essential requirement for IgA CSR) expression in B cells, TGFβ1 seems to play its major role by inducing the transcription of the Cα gene (58, 74, 157).

In the present study we observed that the mucosal IgA responses against VLPs were dependent on T cell help as well as the cognate interaction between T-B cells. This suggests that most likely mucosal administration of VLPs might generate IgA with memory as well as long-lived PCs fate.

### 5.2.2 Role of TGFβ1

Transforming growth factor-β is considered to be an essential IgA-switch factor (218). However its in vitro importance is sometimes controversial and strongly depends on the different systems applied (219).

The critical role for TGFβ1 in regulating IgA responses has been shown in vivo firstly in TGFβ1$^{-/-}$ mice (220). By comparing the immunoglobulin isotypes in serum as well as in mucosal samples of untreated TGFβ1$^{-/-}$ and control mice, van Ginkel et al observed that while IgG and IgM Abs were elevated, the levels of IgA were consistently reduced in TGFβ1$^{-/-}$. The authors associate the decreased levels of IgA (which is considered an antiinflammatory Ig) at mucosal surfaces and the increased levels of IgG and IgM (proinflammatory Igs due to complement activation) with their high susceptibility to inflammation and early death observed in the TGFβ1$^{-/-}$ (220). Due to the severe disease developed rapidly by these mice, they are not suitable to evaluate the role of TGFβ1 in regulating IgA responses upon Ag immunization.

In order to overcome this problem, Cazac and Roes generated a mouse strain in which TGFβ receptor II (TβRII) is gene deleted on B cells (TβRII-B) (110). Systemic antigen-specific Ab responses were assessed in TβRII-B mice upon immunization with nitrophenyl coupled to chicken globulin (NP-CG) in alum, a prototype TD antigen. The authors observed that in contrast to other Ig isotypes, serological anti-NP IgA levels were almost absent in TβRII-B mice (110). The same mouse has been further used to evaluate the mucosal IgA response upon i.n. immunization (163). TβRII-B mice immunized i.n. with β-galactosidase containing
MALP-2 as adjuvant elicited almost undetectable β-gal-specific IgA responses in mucosal secretions compared to control mice (163). Therefore, both studies elegantly showed in vivo the critical role of TGFβ in driving both systemic and mucosal IgA responses against TD antigens.

As mentioned above, VLPs are TI-1 antigens but at the same time they have features of TD Ags (i.e. they are protein). In the present study we evaluated the role of TGFβ in driving systemic and mucosal IgA responses against VLPs in vivo. Our results showed that the systemic IgA responses against VLPs elicited by s.c. immunization in TβRII-B mice is largely independent of TGFβ signaling in B cells. In contrast, VLP-specific IgA titers in BAL were indeed reduced in TβRII-B compared to the control group, but not to the same extent as observed previously by Borsutzky et al. Thus, collectively these findings show that the role of TGFβ in regulating IgA responses largely depend on the nature of Ag as well as site of Ag exposure.

Besides TGFβ, also other Th2 cytokines such as IL-5 and IL-10 have been implicated in driving IgA responses by promoting IgA B cells expansion and terminal differentiation (58). Also IL-6 has been linked to IgA responses. Furthermore, it has been shown that Payer’s patches (PPs) DCs have the ability to induce IgA production by naïve B cells. This was associated with the better capacity of PP DCs to produce IL-6 when compared to splenic DCs (221). It has also been shown that in the lung and MLNs of IL-6−/− mice, the number of IgA AFCs were much lower when compared to IL-6+/+ mice. I.n. infection with vaccinia virus expressing IL-6 could restore this number (222). Furthermore, IL-6 is generally considered an important cytokine for B cell responses which provides crucial PCs survival signals (47).

Upon i.n. immunization with VLPs, we observed that the levels of VLP-specific IgA elicited in BAL was similar between IL-6−/− and control mice. The same was true for IL10−/− mice. Based on these findings, we believe Th2 cytokines play a minor role in driving IgA responses against VLPs. A possible explanation for this is that VLPs are loaded with bacterial RNA, a TLR7 ligand. It has been shown that TLR signaling enhances TACI expression in B cells (223) and that TACI is involved in IgA antibody production and PCs survival (91, 169, 224, 225). Thus, it may be that signaling via TACI is sufficient to provide IgA AFCs survival factors and IgA production in the absence of IL-6.
5.2.3 TI IgA switching

The TI class-switching towards IgA is generally a rapid process. There is a large body of evidences showing that in mice the intestinal TI IgA responses are mediated by B1 B cells from the peritoneal cavity (226-228). Indeed, B1 B cells express unmutated IgA Abs which means that they have not been subjected to SHM (157). Not only B1 B cells can account for IgA TI CSR. Indeed, systemic TI IgA responses are usually mediated by splenic marginal zone B cells (73, 76) which, similarly to B1 B cells, can also express polyreactive IgA Abs. Using VLPs, we observed that only the systemic IgA responses elicited upon s.c. immunization elicited IgA responses in the absence of T cell help. Under these circumstances it is likely that MZ B cells (which are the first B cell population interacting with blood-borne Ags) and perhaps also splenic B1 B cells are the source of systemic IgA Abs.

TI IgA responses can be initiated by linking B cells with different innate immune pathways. Signaling through TLR is one mechanism by which TI Ags can mediate TI IgA CSR (28). Polysaccharides can also activate B cells independently of T helper cells by mediating BCR cross-linking (229). Our data now suggest that TLR7 signals may be sufficient to directly trigger IgA CSR.

Not only TLR signaling and BCR cross-linking can induce TI IgA responses. DCs-derived factors such as BAFF and APRIL have also been strongly implied in TI IgA responses (24, 95).

5.2.4 Role of APRIL and BAFF

APRIL and BAFF secreted by DCs directly mediate TI IgA CSR (24, 26). The importance of APRIL in regulating TI IgA responses became evident since APRIL−/− mice immunized i.n. with TI antigens failed to mount Ag-specific mucosal IgA responses (168). Furthermore, it has been shown in vitro that both BAFF and APRIL are able to induce CD40-independent IgA CSR in murine splenic naïve B cells, even in the absence of BCR stimulation (26). BAFF and APRIL bind to their receptor TACI and BCMA expressed on B cells and BAFF additionally binds to BAFF-R (Figure 3.1). However, only TACI has been shown to have a critical role in the induction of IgA CSR by APRIL and BAFF in mouse as well as human system (26, 168, 169).
Regarding the mucosal Ab responses elicited by VLPs, we observed that upon i.n. immunization IgA as well as IgG responses are significantly reduced in TACI;BCMA\textsuperscript{-/-} as well as in TACI\textsuperscript{-/-} mice. This seems a bit confusing and contradictory if we take into account that TACI is involved in the regulation of TI rather than TD Ab responses (89, 167). Importantly, after i.n. immunization, IgA responses are TD. However, \textit{in vitro} studies have shown that TACI enhances the differentiation of B cells activated under limiting conditions of CD40 ligation into AFCs, suggesting that TACI is also important for TD Ab responses (91). Thus, it might be that upon i.n. immunization with VLPs, T helper cells, CD40-CD40L engagement and TGF\textbeta are responsible for CSR whereas TACI provides B cell differentiation into AFCs.

With regard to the systemic Ab response, we have seen that upon s.c. immunization with VLPs, both specific-IgG and IgA are independent on signaling via TACI. However, it has been shown that systemic Ab responses elicited by NP-ficoll (prototype TI-2 antigen) but not by NP-CGG (a TD Ag) are strongly regulated by TACI (89, 167). This difference can be explained either by the difference of Ag nature, i.e. whereas NP-ficoll is a TI-2 antigen, VLPs are TI-1 antigen and therefore with stronger capacity to generate B cell responses by its own. Another possibility is that the RNA present on VLPs overcomes the activation signals...
required via TACI. In addition, VLPs might acquire help from Th cells, which may overcome
the TACI-dependence for systemic IgA responses. In another words, since the role of Th cells
and TACI were assessed separately we can not exclude the possibility that systemic IgA
responses are dependent either from Th cells or from signaling via TACI.

5.2.5 Role of TLR signaling in IgA responses and its implication as mucosal adjuvant

Cholera toxin (CT) and *E. coli* heat-labile toxin are considered the most potent mucosal adjuvants after numerous animal studies showing their stimulatory properties (113, 115, 188). However there is a high degree of toxicity which limits their use in humans. For instance, it has been shown in mice that upon i.n. administration, CT reaches the olfactory bulb possibly causing adverse effects in the central nervous system (230). Moreover, in Switzerland, a nonliving influenza vaccine containing heat-labile enterotoxin (LT) of *E. coli* as adjuvant (Nasalflu) when given i.n. in humans was associated with cases of Bell’s palsy, a paralysis of the facial nerve. The vaccine had to be withdrawn from the market after 1 year (231, 232). It is therefore important to develop new mucosal adjuvants better tolerated by humans.

We have seen that bacterial ssRNA has the ability to induce mucosal IgA responses against VLPs administered i.n. This was not the case in mice immunized with empty VLPs as well as in MyD88 and TLR7\(^{-/-}\) mice. These results points to ssRNA as a promising mucosal adjuvant candidate for enhancing mucosal IgA responses.

In line with this, it has been shown that upon i.n. immunization, inactivated influenza virus (a single stranded RNA virus) enhances the systemic as well as mucosal IgA responses to coadministered simian-human immunodeficiency (SHIV) VLPs (233). Although the enhancement of the immune response by coimmunization with inactivated influenza virus was similar to with is seen upon addition of CpGs DNA, the authors concluded that the inactivated influenza virus elicits their role as adjuvant due to their capacity to form aggregation with VLPs (233). Other groups have demonstrated the adjuvant capacity of poly (I:C) (a synthetic ds RNA ligand of TLR3) when administered i.n. in combination with haemagglutinin-modified recombinant H5N1 influenza virus (234). In this report the authors showed that the mechanism of action of poly (I:C) is due to its capacity to enhance the expression of TLR3 and TLR7 in the NALT and spleen, which in turn may affect cytokines expression and antibody production (234).
The macrophage-activating lipopeptide-2 (MALP-2), an antagonist of TLR2/6 has been shown to have a potent activity as mucosal adjuvant and improve both, humoral and cellular immunity (235, 236). Borsutzky et al showed later that the adjuvant effect of MALP-2 is via direct TLR2 stimulation in B cells (237). Indeed the authors showed that IgM and IgG AFCs could be generated in cultures containing only MALP-2 and B cells. However the detection of IgA AFCs was possible only in the presence of accessory cells (237). This fits with our findings with i.n. administered VLPs. Whereas TLR7 stimulation in B cells is sufficient to induce VLP-specific IgG2a responses, the IgA levels upon i.n. immunization did not depend on TLR7 activation directly in B cells but in alveolar macrophages and lung DCs instead.

Taken all together, based on our findings one could imagine that not the virus-VLP aggregation (233) and the increase of TLR expression in lymphoid tissues have an adjuvant effect, but rather the secretion of BAFF and APRIL by lung DCs and alveolar macrophages upon TLR stimulation.

5.2.6 Role of epithelial cells in triggering immunoglobulin class switching upon TLR stimulation

Epithelial cells (ECs) have long been considered only a physical barrier separating the sterile internal milieu of the body from the contaminated external environment. However, it has been shown recently that airway ECs produce BAFF and APRIL, both B cell-activating factors, in response to TLR3 ligand stimuli (238). In this report the authors speculate that BAFF and APRIL production by airway ECs may contribute to local accumulation, activation, CSR and Ab production by B cells residing in the airways (238). The hypothesis that BAFF secreted by ECs would be involved in CSR was further confirmed by Cerutti and colleagues (84). In summary, the authors showed that human tonsillar ECs induce IgG and IgA CSR through a TLR-inducible pathway after sensing viral RNA. In consequence, ECs released BAFF which stimulate sub-epithelial B cell to undergo CSR and secrete broadly reactive IgG and IgA Abs (84).

Since we observed that TLR signaling was a pivotal factor for mucosal IgA responses after i.n. immunization, we were wondering whether lung ECs after sensing VLPs loaded with RNA also would induce local IgA CSR. However, our BM transplantation experiments clearly revealed that TLR signals in hematopoietic cells was responsible for mediating mucosal IgA responses against VLPs. The fact that wild type mice reconstituted with MyD88⁻/⁻ BM was unable to generate mucosal IgA excludes the possibility that ECs alone are
sufficient to induce IgA CSR upon immunization with VLPs. This finding goes in line with the notion that upon i.n. immunization with Qβ-VLPs, mucosal B cell responses are initiated in MLNs after Ag transport by lung DCs and alveolar macrophages rather than locally in the lung and might be an explanation why in our system, airway ECs do not count for IgA CSR (84) and Kato (238).

5.3 Conclusions

Collectively, the present study elucidating the mechanisms regulating IgA responses against VLPs (Figure 2.18) provides helpful insights in terms of using VLP technology for development of effective and safe mucosal vaccines for use in humans.

Overall, the combination of TD and TI mechanisms has two major advantages. The TI mechanism mediated by TLR and BCR signaling can occur relatively quickly, providing therefore a rapid systemic Ab protection against pathogens which have breached the mucosal barrier. While this occurs, the mucosal system has time to generate a more elaborated IgA response which relies on T cell help, CD40L, TACI and TGFβ and therefore can mount a GC reaction which will possibly lead to the generation of IgA long-lived plasma-cells and IgA memory B cells. These two cell types are responsible for maintaining the humoral memory levels which is the hallmark of vaccination.
6 APPENDIX

6.1 References


6.2 Abreviations

Ab – antibody
ADCC – antibody dependent cell-mediated cytotoxicity
AFC – antibody forming cells
Ag – antigen
APRIL – a proliferation-inducing ligand
BAFF – B-cell activating factor of the tumour necrosis factor family
BAL – bronchus-alveolar lavage
BCR – B cell antigen receptor
BM – bone-marrow
C – complement
CT – cholera toxin
CTL – cytotoxic T cell
ECs – epithelial cells
ELISA – enzyme-linked immunosorbent assay
ELISPOT - enzyme-linked immunospot
FO – follicular
gC – germinal center
HA – hemagglutinin
i.n. – intranasal(ly)
iNOS – inducible nitric oxide synthase
MALP-2 – macrophage-activating lipopeptide-2
MHC – major histocompatibility complex
MLN – mediastinal lymph node
MZ – marginal zone
NALT – nasopharynx associated lymphoid tissue
s.c. – subcutaneous(ly)
PC – plasma-cells
pIgR – polymeric immunoglobulin receptor
PNA – peanut agglutinin
PP – Payer’s patches
SD – standard deviation
SEM – standard error of the mean
SIgA – secretory IgA
TACI – transmembrane activator and calcium-modulating cyclophilin-ligand interactor
TD – thymus dependent
Th – T helper
TI – T cell independent
TLR – toll-like receptor
TNF – tumour necrosis factor
VLP – virus-like particles
WT – wild-type
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