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Alveolar Macrophages and Lung Dendritic Cells Sense RNA and Drive Mucosal IgA Responses

Juliana Bessa,* Andrea Jegerlehner,* Heather J. Hinton,* Paul Pumpens,† Philippe Saudan,* Pascal Schneider,‡ and Martin F. Bachmann*1

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 vs mucosal IgA responses in mice were differently regulated. Systemic IgA responses following s.c. immunization were T cell independent and did not require TACI or TGFβ, whereas mucosal IgA production 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 whereas mucosal IgA required TLR7 signaling to lung dendritic cells and alveolar macrophages. 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. The Journal of Immunology, 2009, 183: 3788–3799.

Successful vaccines mediate protection via neutralizing Abs (1, 2). Parenteral routes of immunization are very efficient in inducing neutralizing IgG responses that can be maintained as humoral immunological memory over long periods of time (3, 4). However, these routes usually induce poor IgA responses, especially at mucosal surfaces. For this reason, intranasal (i.n.)2 administration might be an attractive route of vaccination, because 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 (5, 6). IgA responses induced by TD Ags such as proteins require CD4+ T cell help mediated by CD40 ligand (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 (a proliferation-inducing ligand; secreted mainly by activated dendritic cells (DCs) and macrophages) binding to TACI (transmembrane activator, calcium modulator, and cyclophilin ligand interactor) on B cells and are mediated mainly by B1 B cells (7, 8).

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 (9). Retinoic acid 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 GALT-DC-derived IL-6 and IL-5, retinoic acid can promote IgA secretion (10). Recently, NO secreted by DCs has also been implicated in the regulation of IgA production in the gut (7).

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 (11).

Many reports have investigated the role of TLR signals in B cell responses, including memory B cell responses (12, 13) as well as CSR and induction of IgG Abs (14–20). The role of TLR signals in the induction of IgA responses is a subject of investigation. The Medzhitov group has detected normal levels of serum IgA in MyD88−/− mice, suggesting that TLR signaling is dispensable for IgA responses (16). However, more recently it was shown that TLR stimulation either on gut DCs (7) or in intestine epithelial cells (21) promotes IgA responses both in sera and intestinal content, indicating a potential role for TLR and MyD88 in regulating IgA responses.

Virus-like particles (VLPs) are highly effective immunogens that can induce strong Ab responses. VLPs are classic TI type 1 Ags that 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 s.c. immunization with VLPs derived from the bacteriophage Qβ, Qβ-VLPs (22), are efficient immunization routes for the induction of IgA responses (23). Qβ-VLPs are packaged with RNA derived from Escherichia coli during its self-assembly process. Thus, Qβ-VLPs typically mediate TLR3/7 signals that 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 to Qβ-VLPs both at the systemic level and in the respiratory tract mucosa. We found that depending on the site of Ag sampling, TLR7 signaling was required either to lung DCs and alveolar macrophages or to B cells and resulted in TD vs TI IgA responses, respectively.

Materials and Methods

Mice

C57BL/6 mice were purchased from Harlan. TLR7−/− (24), MyD88−/−, MHC II−/−, CD40−/−, TLR7-B, TACI−/− and TACI;BCMA−/− (where BCMA is B cell maturation Ag) were generated at and provided by Biogen Idec (32). All animals were kept under specific pathogen-free conditions at BioSupport and were used at 8 to 12 wk of age. Experiments were conducted in accordance with protocols approved by the Swiss Federal Veterinary Office (Bern, Switzerland).

Immunization and Ag

To evaluate the Ab response induced by Qβ-VLP, C57BL/6, TLR7−/−, MyD88−/−, MHC II−/−, TLR7-B, TACI−/− and TACI;BCMA−/− mice were immunized either i.n. or s.c. with 50 μg of Qβ-VLP containing Escherichia coli-derived sRNA.

For i.n. immunization with Qβ-VLP, mice were anesthetized with isoflurane and vaccine was administered using a 200-μl pipette. The 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 100 μl (2 × 50 μl).

Capsids of the RNA phage Qβ were cloned into the pQβ10 vector and purified as described elsewhere (22). The AP205 coat protein (33) was cloned into the pQβ10 vector (34) and expressed and purified similarly as Qβ.

ELISA

ELISA plates (Nunc-Immuno MaxiSorp; Nunc) were coated overnight with 100 μl of Qβ-VLP (1 μg/ml) and ELISA was performed according to standard protocols using the following HRP-conjugated secondary Abs: goat anti-mouse IgG (Fcγ specific; Jackson ImmunoResearch); goat anti-mouse IgA (α-chain specific; Sigma-Aldrich); 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 (GE Healthcare). 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 bronchoalveolar lavage (BAL), undiluted samples were used. Anti-Qβ-VLP IgG titers in serum and in BAL are indicated as dilutions reaching a final OD of the OD of the naive sample. Cells were washed off and plates were treated the ss-cDNA with 2U of RNase H (New England Biolabs) at 37°C for 20 min. The cdNA was then used as template for real-time quantitative RT (Reverse transcription)-PCR (Cycler instrument; Bio-Rad) with the gene-specific primers β-actin-F (5′-CCCTGAAAGTACCCCACTTGAA-3′), β-actin-B (5′-CTTTT CACGGTGGCCTTAG-3′), APRIL-F (5′-GGGGAGGAGGATGTCA GATG-3′), APRIL-B (5′-GGGAGGAGGATGTCA GATG-3′), and BAFF-F (5′-AGGGCTGGAAGAGGATGAGG-3′) and BAFF-B (5′-CAGAG AAGCGAGGGAGG-3′) (where BAFF stands for B cell-activating factor of the TNF family, F stands for forward, and B stands for backwards) using Brilliant SYBR Green QPCR Master Mix (Stratagene) according to the manufacturer’s protocol.

Results

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

VLPs are classified as 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 TD Ag, because 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 (19, 36). In this study we assessed whether the IgA response against Qβ-VLP would require T cell help or whether, similarly as the IgM response, IgA could also be induced by a TI mechanism. To address this question, we immunized 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 compared with WT mice. In marked contrast, the levels of systemic IgA were similar for MHC II$^{-/-}$ and WT mice immunized s.c. (Fig. 1A). 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 with the WT group, whereas the systemic IgA titer elicited in CD40$^{-/-}$ mice immunized s.c. was comparable to that of the WT group (Fig. 1B). These observations show that IgA responses against Qβ-VLP are differentially regulated depending on the site of Ag 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 (data not shown).

It has been previously shown that a high Ag dose can be trapped in the splenic marginal zone and induce TI Ab responses (37). We therefore assessed whether the TD IgA observed after i.n. immunization was not simply reflecting the low dose of Ag sampled following this immunization route. To address this hypothesis, we administered high doses of Qβ-VLP i.n. and measured IgA titers in BAL and serum. Fig. 2A shows that no Qβ-VLP-specific IgA could be detected in BAL of MHC II$^{-/-}$ mice, even when 1 mg of Qβ-VLP was administered. In serum, we observed that low levels of IgA could be induced in MHC II$^{-/-}$ mice following a high Ag dose (Fig. 2B). This low TI IgA titer in serum might be due to some Ag leakage in the blood circulation following a high dose of Qβ-VLP i.n. (unpublished data). Importantly, the TI IgA titer following 1 mg of Qβ-VLP was significantly lower compared with the TD IgA induced with only 50 μg of Qβ-VLP. Thus, the T cell help dependency for the induction of IgA following i.n. immunization is not due to the impact of the Ag dose. In contrast to the IgA response, the Qβ-VLP-induced IgM response in serum was almost completely TI, corroborating the notion that the regulation of IgA Abs is governed by a different mechanism than IgM responses (Fig. 2C).

**Differential requirements for TGFβ and TACI signaling for mucosal and systemic TI IgA production**

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 (7, 38). Evidence for its in vivo role was provided by experiments showing that mice deficient for the TGFβ receptor TβRII selectively on B cells (TβRII-B) were almost completely devoid of IgA, both in serum and in mucosal washes (39, 40). 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 not significantly reduced compared with the control group. In contrast, the mucosal IgA titer in BAL of TβRII-B mice was significantly lower when compared with that of control mice. This demonstrates that the systemic TI IgA response to Qβ-VLP is not dependent on TGFβ signaling to B cells, whereas TGFβ plays an important role for the induction of mucosal IgA response (Fig. 3A).

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, a B lymphocyte stimulator protein (also known as BLyS,
TALL-1, THANK, and zTNF4), produced by DCs that interact with their cognate receptors, TACI and BCMA, expressed on B cells (8, 41). 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 TI IgA levels in TACI;BCMA−/− mice were not significantly reduced compared with those in 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 whereas TI systemic IgA responses occurred in a TACI/BCMA-independent manner (Fig. 3B). To investigate whether TACI or BCMA signaling was required for mucosal IgA responses, we also administered Qβ-VLP i.n. to TACI−/− mice. Similarly as 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 require TACI-mediated signals.

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

Our previous data demonstrated that the mucosal IgA production in response to i.n. immunization required Th cell-derived signals (CD40L) in addition to TACI-mediated signals. In contrast, the systemic IgA response elicited by s.c. immunization did not require Th cells or TACI signaling. Thus, the IgA responses against VLPs are differentially regulated depending on the site of Ag exposure. We therefore sought to understand which additional factor, other than Th cells and 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 the sera 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-VLPs with or without packaged RNA and the response of WT mice to TLR7- or MyD88-deficient mice. As shown in Fig. 4, A and 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 (19). In contrast, TLR7 signaling had no influence on specific IgG1 titers following i.n. immunization (Fig. 4A). In the BAL, similarly as the systemic Ab response, the levels of IgA and IgG2a were completely abolished in the absence of TLR7 signaling (Fig. 4C).

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

We have previously seen that MLNs are the major inductive site of the Ab response upon i.n. immunization against Qβ-VLP (23). Next, the impact of TLR signaling on the number of AFCs secreting Qβ-VLP-specific IgA in these LNs was investigated. Similarly as the Ab titer, the number of AFCs secreting IgA was strongly reduced in mice immunized with Qβ-VLP devoid of RNA as well as in TLR7−/− and MyD88−/− mice compared with control mice (Fig. 4D). The same was true for the spleen of s.c. immunized mice (Fig. 4E).

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.

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 WT C57BL/6 mice with MyD88−/− 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. 5A). 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 (19). 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, B cell-deficient (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. This model allowed us to generate chimeric mice containing only TLR7 WT or deficient B cells with all other hematopoietic cells derived from JH−/− mice expressing TLR7. Our results showed that 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. 5, B and C). This shows that TI 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 in which the B cells were TLR7 deficient (Figs. 5, B and C). Based on this finding, we concluded that not only TLR9 and TLR4 signaling in B cells (13, 16, 19) 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. Thus, IgG2a and IgA CSR are differently regulated.

Lung DCs and alveolar macrophages are the major cell populations transporting Qβ-VLP from the lung to the draining LN

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 that drain the lower airways are the major sites to which Qβ-VLPs are transported and where the Ab response is initiated (23).

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+ cells are lung DCs (42). By analyzing Qβ− cells in the lung, we observed that most of the Qβ+ cells were CD11c+CD11b− cells and a minor proportion were CD11c+CD11b+ cells, identifying the major cell population interacting with Qβ-VLPs as alveolar macrophages (Fig. 6A). We also found a few Qβ-VLPs in association with CD11c−CD11b+ cells and B cells in the lung (Fig. 6A and data not shown).

In MLNs of mice that received Qβ-VLPs i.n., the two major cell populations bearing Qβ-VLPs were again alveolar macrophages and DCs (Fig. 6B). Almost no CD11c−CD11b+ cells positive for Qβ-VLPs were found in MLNs, indicating that this cell population is nonmigratory and remains in the lung. Thus, it is possible that alveolar macrophages and DCs take up Qβ-VLPs in the lung, sense the endogenous ssRNA, and transport the Ag to

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**FIGURE 4.** Role of TLR7 signaling in regulating IgA responses against Qβ-VLP. A and B, Mice were immunized either i.n. (A) or s.c. (B) with 50 μg 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 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. D and E, Number of AFCs secreting Qβ-VLP IgA was determined by ELISPOT in MLNs of i.n. (D) and spleens of s.c. (E) immunized groups. ELISPOT data show the mean values + SEM. The data shown are pooled from two independent experiments; n.d., not detectable. Statistical significance was assessed by unpaired Student’s t test (*, p < 0.05; and **, p < 0.01).
MLNs. 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-VLPs are carried to MLNs by alveolar macrophages and

**FIGURE 5.** 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 and C, JH−/- mice were lethally irradiated and subsequently reconstituted with BM cells isolated from JH−/− mice (80%) mixed with 20% of BM cells isolated from either C57BL/6 (WT B cells) or 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.d., not detectable. The data shown are pooled from two independent experiments. Statistical significance was assessed by unpaired Student’s t test (*, p < 0.05; and **, p < 0.01).
Following s.c. administration, a large fraction of Qβ-VLPs enter 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. 6C and data not shown).

By comparing the numbers of AFCs secreting Qβ-VLP-specific Abs 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. 6D). This suggests that CD11c+ cells that take up Qβ-VLP and migrate to MLNs are the key cells for the induction of mucosal IgA responses.

**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 DC and alveolar macrophage 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 to induce mucosal IgA responses. It has been shown that upon CpG and dsRNA stimulation, mucosal DCs increase BAFF and APRIL expression (7, 43). We were therefore wondering whether ssRNA signaling via TLR7 expressed in lung DCs and alveolar macrophages has a similar effect.

To address this question, we sorted the Qβ+ cells from MLNs of WT and TLR7−/− mice that received Qβ-VLP i.n. and compared the expression level of APRIL and BAFF. Fig. 7A shows that the levels of APRIL and BAFF were reduced 3- and 2-fold, respectively, in the Qβ− cells isolated from the TLR7−/− mice when compared with 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**

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 that requires TLR7 signals to promote IgA responses, we generated mixed BM chimeras using BM cells from CD11c-DTR-transgenic (CD11c-DTR) mice. CD11c-DTR mice have the CD11c promoter driving the expression of the monkey DTR, which allows the conditional depletion of CD11c+ cells following DT administration (30). We therefore adopted this strategy 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. CD11c− DTR mice have the CD11c promoter driving the expression of the monkey DTR, which allows the conditional depletion of CD11c+ cells following DT administration (30). We therefore adopted this strategy 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. CD11c+ cells from MLNs of i.n. and s.c. immunized mice were determined by ELISPOT on day 20 postimmunization. Mean values ± SEM are indicated. The data shown are pooled from two independent experiments. Statistical significance was assessed by the unpaired Student t test (**, p < 0.01).
in CD11c

We hypothesized that TLR7 deficiency

serum IgA in response to TI type II Ags (45). Furthermore,

A

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titers in response to Q

signaling were dispensable for the induction of systemic TI IgA

mucosal Q

IgA following s.c. immunization does not require Th cells or

via mucosal or systemic routes. Our data show that systemic

the most abundant Ig isotype in humans and understanding its

cause local infections at mucosal sites. Secretory IgA plays a crit-

Most pathogens invade the body through the mucosa, and some

In the present study we dissected the mechanisms involved in

IgA responses against Qβ-VLP administered

via mucosal or systemic routes. Our data show that systemic

IgA following s.c. immunization 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 (44). 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
isotype switching and IgA production by B cells (48, 49). Surpris-
ingly, however, TACI was important for driving a TD IgA re-

response rather than TI IgA responses. This is not consistent with the
dogma that TACI mediates TI IgA CSR (45, 46). Importantly, the
mucosal IgA levels in BAL were completely abolished in both
MHC II−/− as well as in CD40−/− mice, whereas the reduction
was only partial in TACI;BCMA−/− mice. A dominant role for TACI confirms
previous findings showing that TACI is important for mediating
isotype switching and IgA production by B cells (48, 49). Surprising-
ly, however, TACI was important for driving a TD IgA re-

response rather than TI IgA responses. This is not consistent with the
dogma that TACI mediates TI IgA CSR (45, 46). Importantly, the
mucosal IgA levels in BAL were completely abolished in both
MHC II−/− as well as in CD40−/− mice, whereas the reduction
was only partial in TACI;BCMA−/− mice. This possibly indicates the
synergistic role of Th cells and TACI signaling. Although,
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 cul-
tures containing limiting conditions of CD40 ligation, suggest-
ing that TACI may be important also for the Ab response to TD
Ags (50).

TGFβ has been shown to be an important cytokine for IgA
CSR in vitro (38, 51, 52). In vivo, it was found that the IgA
response, both in serum and in mucosal washes, against TD Ags
was completely abolished in mice that lack TGFβ-RII in B cells
(39, 40). More recently, it was found that expression of TGFβ-RII
on naive B cells is induced by inducible NO synthase
FIGURE 8. TLR7 regulates appropriate IgA responses against VLPs directly through signaling in B cells and indirectly through activation of CD11c⁺ lung DCs and alveolar macrophages (Mo). A. Mucosal IgA responses require lung DC 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 TI systemic IgA responses simply require strong BCR cross-linking and TLR7 signaling directly in B cells that are provided by direct interaction with VLPs.

(iNOS) with IgA CSR being impaired in iNOS⁻/⁻ mice (7). 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. that lack TGFβRII in B cells, whereas systemic TI IgA levels were not significantly 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 Th cells in addition to BAFF or APRIL secreted by DCs. In contrast, the 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 fact that mice deficient in TGFβRII expression in B cells previously have been shown to generate reduced IgA responses to chicken γ-globulin (36) may reflect differences in Ag size (soluble protein vs VLP) or the absence of TLR ligands or may even be due to differences in the genetic background and/or rearing conditions that potentially affect the composition of the mucosal microbiota.

The role of TLR ligands as a third signal for Ab class switching has been previously described in vitro (17). In vivo, several reports have indicated a role for TLR signaling in B cells as a part of the Igs switching factor (13, 16, 18–20). 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.

In accordance with this notion, other groups have also shown that microbial Ags are of ultimate importance in activating B cells and inducing IgA responses at both the serological and the mucosal level. Tezuka et al. reported that NO plays a crucial role in IgA class switching and secretion (7). Interestingly, iNOS expression has been shown to be dependent on TLR stimulation, as DCs from germfree or MyD88⁻/⁻ mice express reduced levels of iNOS. Interestingly, it has been shown that BCR-deficient mice could develop a germinal center in the GALT (53). However, germinal centers failed to develop in mice maintained on antibiotics treatment, indicating a major dependence on the intestinal bacterial flora. More recently, it has also been shown that chronic epithelial TLR activation resulted in increased B cell recruitment and, consequently, fecal IgA content (21).

Our experiments with BM chimeras demonstrated that TLR7 signaling in B cells was required for TI 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 different lymphoid organs, including the spleen (our unpublished data). This might facilitate the direct interaction of Qβ-VLP with B cells. In contrast, mucosal IgA production requires TLR7 signaling by alveolar macrophages and lung DCs rather than directing TLR signaling to B cells. Thus, under these conditions, B cells may have little direct contact with VLPs, and IgA is induced indirectly, via CD40-CD40L, TGFβ-TGFβRII, and APRIL/BAFF/TACI signaling. Indeed, APRIL and BAFF, the ligands for TACI, were up-regulated in alveolar macrophages and lung DCs upon TLR7 triggering (Fig. 8A). These data are 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 (43). Perhaps soluble ssRNA not in association with viral particles also would trigger similar mechanism, because most likely it would target and be sampled by a cell population other than that of alveolar macrophages and lung DCs.

The observation that alveolar macrophages are a key population for induction of IgA seems counterintuitive, because macrophages are usually nonmigratory. Alveolar macrophages, however, seem to be different because it is an intrinsic feature of CD11c⁺ alveolar macrophages (42) to migrate to MLN and initiate immune responses against particulate Ags (54). 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. Because different types of DCs and macrophages have distinct roles in the initiation and maintenance of immune responses (55), it might well be that the mechanism described here is not...
applicable for IgA induction in other mucosal sites because alveolar macrophages and lung DCs are the cell populations restricted to the lung tissue.

In summary (Fig. 8), our data demonstrate that for T1 systemic IgA responses, direct TLR7 signaling to B cells is crucial, whereas for TD mucosal IgA responses, regulation occurs indirectly through the ability of TLR7 signaling to activate alveolar macrophages and lung DCs, leading to increased BAFF and APRIL production, Th 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 have similar properties. We have demonstrated for the first time that direct TLR signaling to B cells in combination with multivalent Ag results in T1 IgA CSR.

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Disclosures
J.B., A.J.H.J., P.S., and M.F.B. are all employees of Cytos Biotechnology AG and may hold shares or share options in the company.

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