Inactivated influenza vaccine adjuvanted with Bacterium-like particles induce systemic and mucosal influenza A virus specific T-cell and B-cell responses after nasal administration in a TLR2 dependent fashion

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Background: Nasal vaccination is considered to be a promising alternative for parenteral vaccination against influenza virus as it is non-invasive and offers the opportunity to elicit strong antigen-specific responses both systemic and locally at the port of entry of the pathogen. Previous studies showed that non-living bacterium-like particles (BLPs) from the food-grade bacterium Lactococcus lactis are effective stimulators of local and systemic immune responses when administered intranasally. Moreover, in vitro, BLPs specifically interact with human Toll-like receptor 2 (TLR2), suggestive of a role for TLR2 dependent immune activation by BLPs. Methods: In the present study, we examined the role of TLR2 in vivo in immune activation after nasal administration of BLP mixed with split influenza vaccine (BLP-SV) of influenza A virus (IAV) using TLR2 knockout mice. Results: The systemic Th1 cell and subsequent B-cell responses induced after intranasal BLP-SV vaccination depended on the interaction of BLPs with TLR2. Notably, the BLP-SV-induced class switch to IgG2c depended on the interaction of BLP with TLR2. Local induced IAV-specific Th1 cell responses and the mucosal B-cell responses also depended on interaction of BLP with TLR2. Strongly reduced Siga levels were observed in TLR2 knockout mice both in the nasal and vaginal lavages. In addition, detailed analysis of the T-cell response revealed that nasal BLP-SV vaccination promoted Th1/Th17 immune responses that coincided with increased IAV-specific IgG2c antibody production. Discussion: Altogether these results indicate that nasal BLP-SV vaccination induces IAV-specific T-cell and B-cell responses, both systemically and at the site of virus entry in a TLR2-dependent manner.

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1. Introduction

Infection with influenza A virus (IAV) causes a contagious disease that affects mainly the upper respiratory tract and is still one of the leading causes of mortality and morbidity worldwide [1,2]. Most vaccines against influenza A and B in use today are administered via the parenteral route. Although these vaccines can induce virus-specific systemic immune responses, they barely activate the mucosal immune system, the port of entry of the influenza viruses [3,4]. Nasal vaccination therefore might be a promising alternative for parenteral vaccination against influenza virus, since this route of vaccination resembles more closely natural infection and it is known to elicit both systemic and mucosal immune responses [4,5]. In addition, nasal vaccination might enhance vaccine efficacy in contrast to parenteral vaccination since nasal vaccination is associated with secreted Iga (Siga) antibody production at the mucosal surfaces [5–7]. Because Siga forms a first line of defence against invading pathogens at the portal of entry [8–10], it may help to prevent penetration and replication of influenza virus in the respiratory tract mucosa early after host cell invasion.

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The potential advantages of the nasal route of vaccination renders it attractive in the protection against respiratory infections, however, effective stimulation of the nasal mucosal immune system with non-replicating vaccine antigens usually requires the use of an antigen carrier system and/or adjuvant [11,12]. However, this route of immunization is associated with the occurrence of facial nerve paralysis (Bell’s Palsy) as a result of the use of Escherichia coli heat-labile toxin (LT) or mutants thereof, as adjuvant. Clearly, the use of toxins or toxoids should be avoided as nasal adjuvant. An example of a recently developed nasal immunostimulatory system is the bacterium-like particle (BLP) derived from the food-grade bacterium Lactococcus lactis [13,14]. BLPs are obtained by an acid pre-treatment, which degrades all cellular components, including DNA and proteins but leaves the peptidoglycan shell intact. The result is a non-living particle that still has the shape and size of an untreated bacterium. The procedure is applicable to all Gram-positives, hence the name that was formerly used: Gram-positive Enhancer Matrix (GEM) [13,14]. Because of their safe use and adjuvant activity [15,16], BLPs are an attractive adjuvant candidate for the development of nasal influenza vaccines.

Previously, we showed that intranasal (i.n.) immunization with influenza monovalent subunit vaccine of strain A/Wisconsin (H3N2) mixed with BLPs strongly potentiate immunogenicity of influenza subunit vaccine resulting in both local and systemic immune responses [15,16]. In vitro studies using a panel of human Toll-like receptors (TLRs) expressed in HEK293 cells suggest that BLPs have the capacity to mediate TLR2 signalling. Also, TLR2-specific blocking antibodies reduced the BLP-induced IL6 production by murine CD11c+ DCs in vitro [17]. However, it is currently unclear if TLR2 activation via BLPs is fully responsible for the enhanced activation of the adaptive immune system in vivo as measured by T-cell and B-cell activation. First of all, TLR2 can form heterodimers with other TLRs, specifically TLR1 and TLR6 [18,19]. Especially TLR2/TLR1 heterodimers were shown important in the induction of a protective mucosal Th17 immune response in vivo, whereas TLR2/TLR6 heterodimers were not [20]. In addition, TLR2 is expressed on the surface of a large number of immune cells including macrophages [21], monocytes and dendritic cells [22], M cells [23], B cells [24] and T cells [25] including regulatory T cells [26] capable of differentially regulating the immune response. Although there is ample evidence that vaccination with BLP adjuvanted vaccines induces protective immunity, it remains to be proven whether TLR2 mediated effects are responsible for the observed activation of the adaptive immune response in vivo.

To address the proposed role of TLR2 in vivo in the BLP-dependent activation of the adaptive immune system, we explored local and systemic influenza A virus specific T-cell and B-cell responses in TLR2 knockout (TLR2KO) and wild-type control mice after i.n. administration of BLPs supplemented with inactivated influenza vaccine.

2. Materials and methods

2.1. Mice

Female BALB/c wild-type (wt) mice (6–8 weeks) were purchased from Harlan Laboratories, Zeist, The Netherlands. Six to eight weeks old C57BL/6j (wt) and B6.129–Tlr2tm1Krf/j mice (TLR2KO) were purchased from Jackson Laboratories, France. All mice were kept under standard housing conditions at the University of Groningen, The Netherlands. Animal experiments were evaluated and approved by the Committee for Animal Experimentation of the University of Groningen, The Netherlands, according to the guidelines provided by Dutch Animal Protection Act.

2.2. Preparation of BLP-SV

Influenza monovalent split vaccines of strain A/Beijing/262/95 (H1N1) and A/Sydney/5/97 (H3N2) were purchased from Adimmune Corp. Taiwan (egg derived, formalin inactivated). The concentration of the haemagglutinin (HA) in the vaccine was determined using the single radial immunodiffusion assay. The standard BLP-SV vaccines consisted of influenza monovalent SV containing 5 μg HA antigen mixed with BLPs (0.15 mg dry-weight). BLPs were prepared as described before [13,14]. BLPs were stored at −80 °C until use. BLPs and SV, were mixed just prior to i.n. administration. All i.n. vaccine doses were delivered in a final volume of 10 μl of PBS.

2.3. Immunization

Mice to be i.n. immunized were lightly anaesthetized with 2.5%, v/v, isoflurane over oxygen (0.8 L/min). Once anaesthetized, the mice were vaccinated i.n. every 10 days with 10 μl of sterile PBS containing BLP-SV (BLPs mixed with the influenza A strain (A/Beijing/262/95 (H1N1)) or SV alone and sacrificed at day 34 of the experiment. Mice were vaccinated i.n. 3 times on day 0, 14 and 28 with 10 μl of sterile PBS containing BLP-SV (BLPs mixed with the influenza A strain (A/Sydney/5/97(H3N2))) or SV alone and sacrificed at day 42 of the experiment. SV without BLPs was administered i.m. in 50 μl of PBS as a positive control for the immunogenicity of the antigenic materials.

2.4. Serum collection, nasal, lung and vaginal lavages

Blood was collected via puncture of the orbital plexus for antibody measurements and the mice were sacrificed on day 34 or 42 via exsanguination by heart puncture under O2/isoflurane anaesthesia. Subsequently, nasal, lung and vaginal washes were conducted for S IgA antibody measurements. For nasal and lung lavages, 1 ml PBS that contained Roche “complete” protease inhibitor (according to manufacturer’s description) was used. The tube containing the lavage fluid was placed on ice and centrifuged at 300–400 × g for 5 min at 4 °C and supernatants were collected. Vaginal lavages were conducted by repeated pipetting of 0.2 ml of PBS supplemented with Roche “complete” protease inhibitor. All lavage samples were stored at −20 °C.

2.5. IAV-specific IgG, IgG1, IgG2c and S IgA ELISA

ELISA was performed as previously described [27]. Briefly, ELISA plates (Greiner, The Netherlands) were coated overnight at 4 °C with influenza monovalent split vaccines of strain A/Sydney/5/97 H3N2 or A/Beijing/262/95 H1N1 (Adimmune). The plates were washed twice and blocked in 200 μl of a 2.5% solution of Protifar Plus (Nutricia™) in coating buffer (0.05 M carbonate-bicarbonate pH 9.6–9.8) for 45 min at 4 °C and washed four times before samples were applied. Sera were applied in serial two-fold or triple-fold dilutions and a mouse control serum sample positive for A/Sydney/5/97 or A/Beijing/262/95 H1N1 was included on each plate. For detection of S IgA, 100 μl of the lavage was used undiluted in the first well and subsequently serial two-fold diluted. The plates were incubated for 1.5 h at 4 °C, washed 3 times and incubated for 1 h at 4 °C with anti-mouse IgG-HRP conjugates (Southern Biotech). After incubation, the plates were washed 3–4 times and incubated for 30 min with 100 μl staining solution (1 tablet of OPD (o-Phenylenediamine dihydrochloride) dissolved in 100 ml 0.05 M phosphate-citrate buffer pH 5.0 and 40 μl H2O2). After incubation the reaction was stopped by adding 50 μl 2 M H2SO4 per sample and the absorbance was determined at 492 nm.
2.6. IAV-specific IFN-γ T-cell and B-cell ELISPOT

The IAV-specific IFN-γ T-cell and IAV-specific B-cell response in the spleen and local draining cervical lymph nodes (CLN) or inguinal lymph nodes (ILN) after i.n. BLP-SV or i.m. SV vaccination, respectively, was assessed by ELISPOT. For detection of IAV-specific B-cells, cells were directly cultured in high protein binding filter plates (MultiScreen-IP, Millipore) that were pre-coated with Vaxigrip® suspension for injection; strains 2009/2010, Sanofi Pasteur MSD, lot: E7068 at 1 μg per well dissolved in 50 μl of PBS. For detection of IAV-specific IFN-γ T-cells, cells were cultured in the presence of HA antigen or IMDM (Gibco, Invitrogen) medium as a control that was supplemented with heat-inactivated 5% FCS (Bodinco, The Netherlands), 5 × 10⁻⁵ M 2-mercaptoethanol, penicillin (100 units/ml) and streptomycin (100 μg/ml) (Gibco, Germany) for 72 h at 37 °C in high protein binding filter plates (MultiScreen-IP, Millipore) that were pre-coated with a rat anti-mouse IFN-γ monoclonal antibody (clone AN-18, purchased at BD Biosciences, Pharmingen) at 0.1 μg per well dissolved in 50 μl of PBS for 48 h at 37 °C.

After incubation, spot forming units of IAV-specific B- and T-cells were detected with goat-anti-mouse IgG-biotin (Sigma) and Avidin-AP (Sigma). Plates were developed with NBT-BCIP (Roche) and analyzed by using the Aelvis spotreader and software. Data are shown as IAV-specific IFN-γ T-cell or the IAV-specific B-cell count per 10⁶ cells above background.

2.7. Luminex

Single cell suspensions were prepared from spleen and draining lymph nodes and cells were cultured for 72 h in the presence of ConA at 2.5 μg/ml or IMDM (Gibco, Invitrogen) at 37 °C. Analyzing the culture supernatants assessed the amount of cytokine secreted during a 72 h T-cell re-stimulation. Briefly, fluorescently microbeads coated with capture antibodies for simultaneous detection of IL-17A (TC11-18H10) and IL-5 (TRFK5) were added to 50 μl of culture supernatant. Cytokines were detected by biotinylated antibodies IL17 (DuoSet ELISA kit, R&D systems Europe Ltd, the U.K.), IL-5 (TRFK5) and PE-labelled streptavidin (BD Biosciences Pharmingen). Fluorescence was measured using a Luminex model 100 XYP (Luminex, USA). Data are shown as the cytokine concentration above background in pg/ml.

2.8. Statistics

Statistical analysis was performed with Prism software (Graphpad Software Inc., San Diego, version 4.00). An unpaired two-tailed t-test was used in Fig. 2. One-way ANOVA followed by a Bonferroni’s multiple comparisons test was used in Fig. 4C. One-way ANOVA followed by a Kruskal–Wallis test and Dunn’s multiple comparison test was used in all other experiments.

3. Results

To investigate the role of TLR2 in BLP-mediated local and systemic IAV-specific T-cell and B-cell activation, B6.129-Tlr2¹²¹/KI/J mice (TLR2KO) and C57BL6/J (wt controls) were immunized i.n. with BLP-SV (A/Sidney/5/97, H3N2). As a control, wt mice were i.m. immunized with SV alone. Fourteen days after the last immunization, cells from the draining lymph nodes (dLN) and spleen were isolated and analyzed for IAV-specific IFN-γ producing cells and IAV-specific B-cells.

In the local dLN significantly reduced numbers of IAV-specific IFN-γ producing T-cells (Fig. 1A) and lower numbers of IAV-specific B-cells (Fig. 1B) were observed in TLR2KO mice compared to the number of cells in wt control mice. Similar to the observations made in the local dLN, also significantly lower numbers of IAV-specific IFN-γ producing T-cells (Fig. 1C) and a slight reduction in IAV-specific B-cell numbers (Fig. 1D) were observed in the spleen of TLR2KO mice compared to vaccinated wt mice. These data indicate that induction of IAV-specific IFN-γ T-cell and B-cell responses both in the local dLN and spleen requires interaction of BLP with TLR2.

The IAV-specific IFN-γ T-cell responses in the dLN of wt controls were slightly higher after i.n. BLP-SV immunization compared to the responses after i.m. immunization with SV alone although this did not reach statistical significance. The systemic IFN-γ T-cell response observed in spleen was similar after i.n. and i.m. immunization (Fig. 1). Similar observations were made when BALB/c mice were immunized i.n. and i.m. with BLP-SV and SV, respectively (Table 1).

To investigate how i.n. BLP-SV vaccination affects systemic T-cell differentiation we analyzed IL-5 and IL-17A production of activated splenocytes. After i.n. BLP-SV vaccination the enhanced IAV-specific IFN-γ T-cell responses coincided with a slightly increased production of IL-17A cytokine (Fig. 2A) and significantly decreased secretion of IL-5 cytokine (Fig. 2B) compared to SV i.m. vaccinated mice. Together these results indicate that the IAV-specific T-cell and B-cell responses induced after i.n. BLP-SV administration are TLR2 dependent and results in Th1/Th17 skewing.
Table 1
Local and systemic T-cell responses induced after vaccination in BALB/c mice.

<table>
<thead>
<tr>
<th>Vaccine formulation</th>
<th>Route of administration</th>
<th>IFN-γ producing cells (per 10^6 cells)</th>
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<tr>
<td></td>
<td></td>
<td>Spleen</td>
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<tr>
<td>BLP-SV</td>
<td>Intranasal</td>
<td>93 ± 12</td>
</tr>
<tr>
<td>SV</td>
<td>Intrasubcutaneously</td>
<td>114 ± 30</td>
</tr>
</tbody>
</table>

BALB/c mice were vaccinated three times with 10 μl of BLP-SV (A/Beijing/262/95) or SV (A/Beijing/262/95) in PBS, i.n. or i.m, respectively. dLNs and spleens were isolated and single cells were re-stimulated ex vivo with HA antigen for 48 h at 37 °C in anti–IFN-γ pre-coated ELISPOT plates for IAV-specific T-cell detection. Data are shown as the IAV-specific IFN-γ T-cell count per 10^6 cells above background (spots counted on medium coated plates). Data are shown as the mean ± SEM with n = 5 mice per group.

Thus, both IAV-specific systemic Th1 cell and subsequent B-cell responses that were associated with enhanced IgG2c antibody production induced after i.n. BLP-SV vaccination depended on interaction of BLP with TLR2.

4. Discussion

Earlier studies have demonstrated in vitro that BLPS can activate TLR2 signalling in human TLR-transfected HEK cells and mouse dendritic cells [17]. This implies that TLR2 activation by BLP could be responsible for enhancing adaptive immune responses in vivo, but formal proof for this was lacking. Previous studies showed that the effect of TLR2 triggering on the outcome of the immune response in vivo is variable and depends on several unknown factors: TLR2 can form heterodimers with other TLRs, specifically TLR1 and TLR6 [18, 19] and TLR2 is expressed by a plethora of immune cells [21–26]. Furthermore, the immunostimulatory activity of BLPS in vivo could be the result of activation of innate receptors different from TLR, for example, NOD receptors. Here, we provided clear evidence for an essential role of TLR2 in the BLP-dependent activation of the IAV-specific adaptive immune responses in vivo upon nasal vaccination.

Moreover, we showed that both local and systemic IAV-specific IFN-γ T-cell response (Fig. 1A and C) and B-cell responses (Fig. 1B and 1D) required the activation of TLR2 by BLPS given that the number of cells was substantially reduced in TLR2KO mice after i.n. BLP-SV vaccination compared to wt control mice. Since IFN-γ producing Th1 cells are known to promote IgG2c production by B-cells [28], we explored if the IgG class switch to IgG2c also depended on the interaction of BLPS with TLR2. The data showed a significantly reduced IAV-specific IgG2c antibody production in TLR2KO mice after i.n. BLP-SV vaccination compared to wt control mice (Fig. 4C) that correlated with reduced numbers of IFN-γ-producing T-cells. Therefore, we suggest that the enhanced IgG class switch to IgG2c was mediated by IAV-specific IFN-γ producing T-cells and this required the interaction of BLPS with TLR2. Since interaction of BLPS with TLR2 skewed the responses towards Th1 type, i.n. BLP-SV vaccination, as expected, did not affect IgG class switch to IgG1 (Fig. 4D).

In addition, we found that i.n. BLP-SV vaccination also modestly enhanced the response towards Th17 type (Fig. 2A). The role of Th17 and other IL-17 producing cells in protection against influenza infections is still not completely clear [29]. However, IL-17 producing cells might be beneficial in protection against severe influenza infections, since enhanced numbers of IL-17 producing influenza specific T cells can protect the host against an, otherwise lethal, influenza infection [30]. Surprisingly, the influenza A virus itself has been described to inhibit Th17-mediated immunity thereby enhancing the risk of complicating secondary Staphylococcus aureus infections [31].

TLR ligands have been studied previously in influenza virus studies and i.n. pre-treatments with especially TLR2 and TLR4 ligands were found to protect mice against lethal influenza pneumonia in an antigen independent manner [32]. Moreover, i.n. immunization with influenza-derived peptides coupled to bacterial-derived lipids induced DC maturation via TLR2 binding and enhanced
activation of IFN-γ secreting CD8+ T-cells at the site of infection after i.n. exposure to influenza virus [33]. Earlier it was shown that nasal immunization with BLP activated and enhanced the maturation of dendritic cells (DCs) that enhanced the activation of IFN-γ producing CD4+ T-cells [17]. However, the BLP interaction with TLR2 in vivo might involve other cell types since TLR2 is expressed on many immune cells, including B-cells [24]. For example, B-cell intrinsic MyD88 signals can also drive IFN-γ production from T-cells and result in enhanced T-cell dependent IgG2c antibody responses [34]. Therefore, we suggest that the interaction of BLPs with TLR2 expressed by antigen presenting cells, such as dendritic cells but also B cells, requires further investigation to understand the mechanism that drives the immunological outcome after nasal vaccination.

SLgA at mucosal tissues forms a first line of defence against invading pathogens at the port of entry [8–10]. Therefore, we explored if the induction of mucosal SLgA responses after i.n. BLP-SV vaccination required BLP interaction with TLR2. Indeed, the data showed that SLgA responses measured in nasal (Fig. 3B) and vaginal lavages (Fig. 3C) were TLR2 dependent. Previously, it was shown that i.n. vaccination with BLP vaccines induced enhanced SLgA at mucosal tissue in BALB/c mice compared to parenteral vaccination [15,35]. The potency to induce a mucosal SLgA response was independent of the mouse strain tested, as both C57BL6/J and BALB/c mice induced strong responses (Fig. 3). Similar to the local immune response induced by BLP adjuvanted vaccination, also systemically induced immune responses in BALB/c and C57BL6/J are comparable as shown by enhanced IFN-γ producing cells and IAV-specific IgG titres [17,35].

Although the IL-5 cytokine is a differentiation marker for B-cells that produce IgA [36] we did not detect significant IL-5 cytokine secretion after i.n. BLP-SV vaccination (Fig. 2B). Since TLR2 signalling can also trigger IgA production by human B-cells directly [37], we suggest that the SLgA responses are at least partly enhanced due to the interaction of BLP with TLR2 on B cells (Fig. 3B and C). Previously, it has been shown that BLP adjuvanted vaccines induce protective immunity to subsequent infection [15,17]. Moreover, recent data showed that i.n. vaccination with a BLP adjuvanted influenza vaccine results in improved protection against both homologous and heterologous influenza challenge infections as compared to protection levels observed after conventional parenteral influenza vaccination [35]. These data underline that enhanced systemic and mucosal B-cell responses induced by i.n. vaccination with BLPs result in a strong protective and broad immune response.

In conclusion, the interaction of BLPs with TLR2 in vivo is required for the enhanced activation of systemic and local IAV-specific adaptive immune responses as observed after i.n. BLP-SV vaccination. Especially the ability to induce local IAV-specific immune responses, in particular elevated levels of IAV-specific
IFN-γ producing T-cells and IgA antibody secreting B-cells, make BLPS an attractive immune stimulator to be used in nasal vaccination against influenza infection.

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