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Safety evaluation of pulmonary influenza vaccination in healthy and ''asthmatic'' mice

Antoine Minne[a], François Huaux[b], Joanna Jaworska[c], Ro-Dug Rha[c], Eckard Hamelmann[c,1], Rita Vanbever[a,*,1]

[a] *Department of Pharmaceutical Technology, Université catholique de Louvain, Avenue E. Mounier, 73 UCL 73.20, Brussels B-1200, Belgium*
[b] *Department of Industrial Toxicology and Occupational Medicine, Université catholique de Louvain, Brussels B-1200, Belgium*
[c] *Department of Pneumology and Immunology, Charité University Children's Hospital, Berlin D-13353, Germany*

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Abstract The present study reports animal immuno-toxicological data of pulmonary vaccination against inactivated seasonal influenza. Its aims were (i) to monitor the temporal kinetics of lung inflammation in normal mice over a period of 2 weeks following pulmonary vaccination in order to assess the risk of chronic lung inflammation, (ii) to evaluate the impact of pulmonary vaccination on the asthmatic phenotype in an established allergen-sensitized murine model of asthma. Both sets of experiments were performed using high doses of split influenza virus vaccine. In the first part of this study, we showed that pulmonary influenza vaccination induced a slight local inflammatory response which was limited in duration since it was no longer observed at 2 weeks post-vaccination. At this time point, it has previously been shown that the immunogenic efficacy was maintained. In the second part, we demonstrated that pulmonary influenza vaccination did not significantly exacerbate the cardinal features of asthma, i.e., allergen-specific IgE formation, the development of airway hyperreactivity (AHR) and eosinophilic airway inflammation. Our data therefore suggest that the overall immuno-toxicological profile of pulmonary vaccination against seasonal influenza was acceptable, even in an animal model of pulmonary hypersensitivity.

Introduction

Influenza virus is a respiratory pathogen that causes yearly epidemics resulting in high rates of morbidity and mortality [1]. Moreover, a virulent strain of avian influenza A (H5N1) currently represents a major pandemic threat [2]. In this light, effective prevention of influenza is the ultimate goal,

* Corresponding author. Tel.: +32 2 764 73 25; fax: +32 2 764 73 98.
E-mail address: rita.vanbever@uclouvain.be (R. Vanbever).
[1] Eckard Hamelmann and Rita Vanbever jointly supervised this project.

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and new vaccination strategies are being investigated to improve efficacy, coverage and safety. As influenza virus naturally infects the host through the mucosal surface of the upper respiratory tract, vaccines delivered by the respiratory route are promising since they might not only induce systemic but also mucosal immunity [3,4].

Aerosol administration of drugs or vaccines is technically not demanding, non-invasive, and thus readily accepted by patients. This is best illustrated by the fact that more than 4 million school-age children in Mexico have received measles vaccination by pulmonary aerosol with no significant adverse events reported [5,6]. Pulmonary aerosol vaccination could potentially be easily used in rapid mass-immunization campaigns. Therefore, it could be extremely valuable in the case of an influenza pandemic, or for universal vaccination of children with the seasonal influenza vaccine, which could significantly reduce the spread of influenza in the community [7—9]. Interestingly, intranasal live-attenuated influenza vaccine (LAIV) appears to have superior efficacy to existing injectable vaccines, and is safe for children at least 18 months of age [10,11]. Pulmonary inactivated influenza vaccination is another strategy which, although still more experimental, has already shown great promise [12,13].

One of the main safety issues regarding pulmonary influenza vaccination is related to the induction of an immune response in the respiratory tract, since this could possibly trigger chronic inflammatory responses or exacerbate pre-existing lung diseases, such as asthma. An additional safety concern for this route of immunization will arise from the need of an adjuvant for increasing the immunogenicity of pandemic H5 influenza antigen [2]. Any new vaccination strategy targeting the respiratory tract should therefore be cleared of the allegation of deleterious inflammation as well as exacerbation of chronic inflammatory airway disorders. In the case of LAIV, safety concerns in asthma patients have been put to rest by a large study by Fleming et al. [14]. However, for pulmonary vaccination with inactivated split influenza virus, there are currently no clinical or experimental data available.

We therefore aimed to address two major safety concerns: first, an inflammatory reaction to the seasonal vaccine should be limited in duration and magnitude; second, intra-pulmonary vaccination should not exacerbate a pre-existing asthmatic airway disease. To address the first question, we monitored the temporal kinetics of lung inflammatory markers in normal mice over a period of 2 weeks following pulmonary vaccination. The second set of experiments was performed in a well established murine model of allergen-induced airway disease. High doses of split virus seasonal influenza vaccine were applied in both studies.

Materials and methods

Treatment protocol

All experimental procedures were approved by the institutional animal ethics committee. Female BALB/c mice (6—8 weeks, Harlan) were immunized as described in Table 1. To investigate the short- and long-term safety of pulmonary influenza vaccination in non-asthmatic animals, mice were first primed on day 0 by intranasal (i.n.) instillation with a combination of three inactivated whole virion influenza strains (5 μg hemagglutinin (HA)/strain; A/Wyoming/3/2003, A/New Caledonia/20/99, B/Jiangsu/10/2003, GSK Biologicals, Belgium) to generate a background immunity (IN priming). The actual influenza vaccination was performed on day 39 by an intratracheal (i.t.) booster immunization with high vaccine doses of either a combination of three split virion influenza strains (3 μg HA/strain, 50 μl/instillation, same strains as for IN priming) or one split virion influenza strain (12 μg HA, 50 μl/instillation, A/Wyoming/3/2003) (IT boost).

Allergen-sensitization

In a second set of experiments, we investigated the safety of pulmonary influenza vaccination in an established murine model of asthma [15—17]. The mice were vaccinated against influenza according to the protocol mentioned above, to which an allergen-sensitization/challenge protocol was added. Mice were systemically sensitized on days 10 and 23 by intraperitoneal injections of 20 μg ovalbumin (OVA, Grade VI, Sigma, USA), emulsified in 2 mg of aluminium hydroxide (AlumInject; Pierce, Rockford, IL, USA) in a total volume of 200 μl (sensitization). All OVA-sensitized groups were challenged with aerosolized OVA (100 mg/10 ml, Grade V; nebulized by Medical Assistance System) for 20 min each day, on days 37—39 (challenge).

Negative controls involved exposure to PBS instead of OVA or influenza vaccine. Positive controls for lung inflammation were sham-primed with PBS and i.t. instilled with LPS (20 μg, 50 μl of a 400 μg/ml solution, *Escherichia coli* O111:B4, Sigma).

Broncho-alveolar lavage (BAL)

At different time points, i.e., 4 h, 36 h, 72 h, 7 days and 14 days after the i.t. booster immunization in the normal mice, or on day 41 in the allergen-sensitized mice, the lung was lavaged twice with 0.8 ml PBS, as previously described [15]. Cells of both aliquots were pooled; the total number of live cells recovered by lavage was determined by the trypan blue exclusion method, and differential cell counts were performed on cytocentrifuge preparations stained with Diff-Quick (Dade NV/SA).

Measurement of soluble biochemical components of BAL

All assays were performed in the BAL supernatants of the first aliquot. TNF-α and serum albumin levels were measured by ELISA according to the manufacturers' instructions. TNF-α ELISA was performed using a Pharmingen OptEia kit (PharMingen) with a detection limit of 23 pg/ml. Mouse albumin ELISA was performed using a commercial kit (Bethyl Laboratories, Montgomery, TX, USA) with a detection limit of 11 ng/ml. BAL total protein content was assessed spectrophotometrically using a commercial kit (Systemes Technicon, Doumon, France). BAL lactate dehydrogenase (LDH) activity was assessed spectrophotometrically by monitoring the reduc-

Table 1 Immunization and sensitization protocols

Group	IN priming (day 0)	IT boost (day 39)
A		
PBS	PBS	PBS
Flu 9 μg	Flu	Flu 9 μg (trivalent)
Flu 12 μg	Flu	Flu 12 μg (monovalent)
LPS	PBS	LPS

Group	IN priming (day 0)	Sensitization/challenge (days 10,23/37—39)	IT boost (day 39)
B			
PBS/OVA/PBS	PBS	OVA	PBS
Flu/OVA/PBS	Flu	OVA	PBS
Flu/OVA/flu 9 μg	Flu	OVA	Flu 9 μg (trivalent)
Flu/OVA/flu 12 μg	Flu	OVA	Flu 12 μg (monovalent)
PBS/OVA/LPS	PBS	OVA	LPS

(A) To investigate the safety of pulmonary influenza vaccination in naïve animals, mice were primed on day 0 by intranasal (i.n.) instillation of three inactivated whole virion influenza strains (5 μg hemagglutinin (HA)/strain; A/Wyoming/3/2003, A/New Caledonia/20/99, B//Jiangsu/10/2003) (IN priming). Vaccination was performed on day 39, the mice then received an intratracheal (i.t.) booster immunization with high vaccine doses of three split virion influenza strains (3× 3 μg HA/strain, 9 μg HA in total, 50 μl/instillation, same strains as for IN priming) or one split virion influenza strain (12 μg HA, 50 μl/instillation, A/Wyoming/3/2003) (IT boost). (B) To investigate the safety of pulmonary influenza vaccination in allergen-sensitized mice, animals were immunized as mentioned above. Additionally, mice were systemically sensitized against ovalbumin (OVA) on days 10 and 23 (sensitization), and were challenged with aerosolized OVA on days 37—39 (challenge). Negative controls involved exposure to PBS instead of influenza vaccine or OVA, and a positive control for lung inflammation was sham-primed with PBS and i.t. instilled with LPS (20 μg).

tion of NAD^+ at 340 nm in the presence of lactate [18].

Lung histology

36 h after the i.t. booster immunization in normal mice, the lung lobes were instilled with 1 ml 50% TissueTek (Reichart-Jung, Nuβloch, Germany) in PBS. The left lobe was removed, embedded in TissueTek cryomatrix and frozen by rapid immersion in liquid nitrogen, and preserved at −70 °C. Sections (8 μm) were stained with haematoxylin—eosin, coverslipped, and examined by light microscopy.

Immunoglobulins

Total IgG and IgA titres in BAL supernatants were determined by ELISA, as described [13]. Standard curves were constituted of mouse IgG (Chrompure, Jackson ImmunoResearch) or purified mouse myeloma protein IgA (MP Biomedicals, Aurora, OH, USA). The quantification limits of the IgG and IgA assay were 0.9 ng/ml and 2.4 ng/ml, respectively. Serum levels of OVA-specific IgE were measured by ELISA, as previously described [15]. Levels of OVA-specific IgE were calculated in relation to pooled standards generated in our laboratory and expressed as arbitrary lab units per ml (LU/ml). The detection limit of this assay was 12 LU/ml.

In vivo airway reactivity

On day 40, lung function was measured by whole-body plethysmography (WBP, EMKA Technologies, F), as previously reported [16]. Animals were exposed to aerosolized PBS for baseline reading and then to increasing concentrations of methacholine (MCh) (6—50 mg/ml). Airway reactivity was expressed as the increase of enhanced pause (Penh) values for each concentration of MCh relative to baseline Penh values.

Statistical analysis

Values for all measurements were expressed as mean ± standard error of the mean. Groups of mice were comprised of four to six animals. Data were analyzed by using the JMP version 4.0.2 and GraphPad Prism version 4.00 software programs. Pairs of groups were compared by one-way ANOVA with Tukey's multiple comparison post-test. Statistical significance was set at $P < 0.05$.

Results

Effect of pulmonary influenza vaccination in normal mice

BAL cell population and lung histology

As a marker for pulmonary inflammation, we analyzed the cellular distribution in BAL fluid after vaccination. Pulmonary influenza vaccination in normal mice increased BAL total cell counts, but this was only statistically significant at days 7 and 14 after vaccine administration (Fig. 1A). This increase in total cells was due to the recruitment of lymphocytes, which steadily increased over time to reach a peak 7 days post-immunization (Fig. 1B). Pulmonary delivery of influenza vaccine induced a slight and transient influx of neutrophils that peaked at 36 h and faded by day 7 (Fig. 1C). This influx was not pronounced on histological slides from the lung at 36 h (Fig. 2). In contrast to pul-

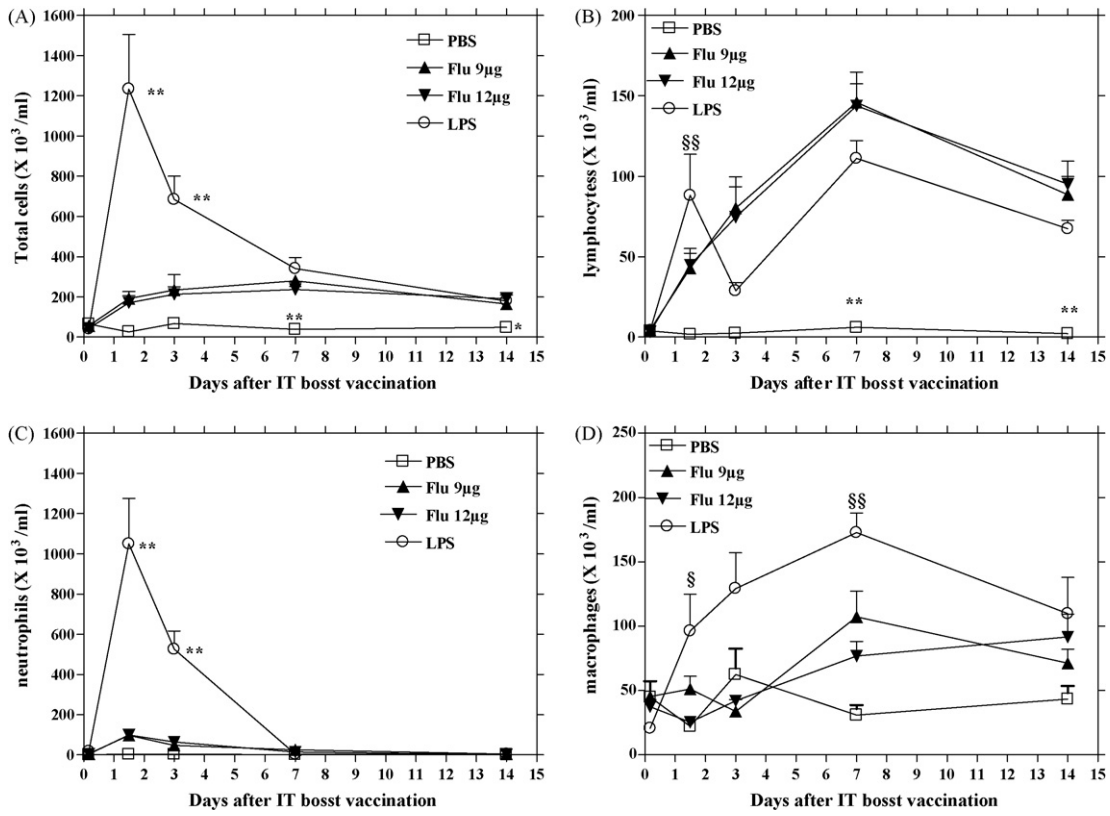

Figure 1 Mice were immunized as described in Table 1A. At different time points following the intratracheal boost, lung was lavaged, and the BAL cells differentiated and counted. Total cell counts (A), lymphocytes counts (B), neutrophils counts (C) and macrophages counts (D) are expressed as cells $\times 10^3$/ml. * indicates groups whose values are significantly different from those of the other groups. § indicates groups whose values are significantly different from those of the PBS mice. One symbol indicates significant differences ($P < 0.05$), two symbols indicate highly significant differences ($P < 0.01$).

monary influenza vaccination, pulmonary delivery of LPS as positive control generated a sharp increase in total cell numbers, which were maintained significantly above the negative control group from 36 h to 14 days after delivery (Fig. 1A). The early cellular influx was almost exclusively composed of neutrophils with a peak after 36 h and persisted for at least 72 h after LPS instillation (Fig. 1C).

The histological lung slides of influenza vaccinated mice at 36 h did not show a striking difference with the negative controls (Fig. 2). The flu 12 µg group showed some minor indications of a possible alveolar proteinosis (slightly reddish veil). This was further investigated.

BAL TNF-α levels

To address the question whether pulmonary vaccination might induce pro-inflammatory cytokines, we monitored the TNF-α level in BAL fluid as marker for local inflammation. Pulmonary influenza vaccination with either 9 µg or 12 µg HA did not affect BAL TNF-α level at any time point following i.t. vaccine administration, when compared to negative controls (PBS, Fig. 3). LPS instillation as positive control induced a sharp and highly significant increase of TNF-α in BAL 4 h after administration ($P < 0.01$ vs. other groups, Fig. 3). At later time points, LPS-induced TNF-α production was down-regulated and reached negative control levels by 36 h after pulmonary instillation.

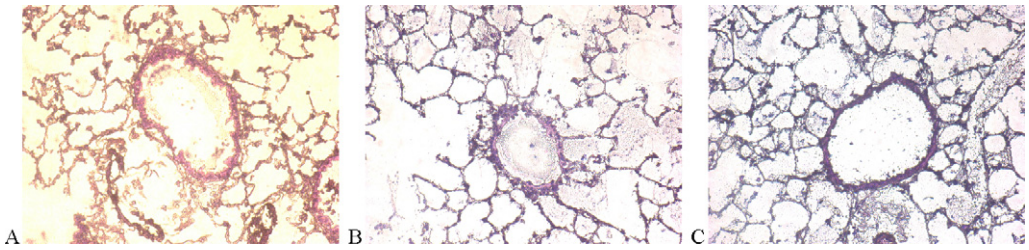

Figure 2 Effect of vaccination with whole and split influenza vaccine on lung tissue integrity. Mice were immunized as described in Table 1A. The lung was removed 36 h after the IT boost immunization for histology. (A) negative controls (PBS), (B) flu 9 µg and (C) flu 12 µg.

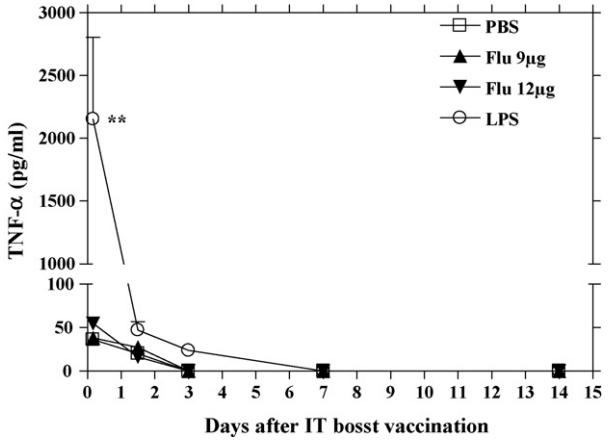

Figure 3 Effect of pulmonary influenza vaccination on TNF-α level in BAL. Mice were immunized as described in Table 1A. At different time points following the IT boost, the lung was lavaged, and BAL levels of TNF-α were measured by ELISA. ** $P < 0.01$ vs. all other groups.

BAL biochemical parameters

For further analysis of local effects caused by the influenza vaccination, we measured several additional biomarkers in the BAL fluid.

LDH activity. Lactate dehydrogenase being a strictly intracellular enzyme, LDH activity in BAL was measured as a marker of epithelial cell integrity. The LDH activity in BAL fluids of pulmonary influenza vaccinated mice and negative controls (PBS) were similar at 4 h and 14 days after i.t. administration. In between these two time points, influenza vaccination induced a slight increase in BAL LDH activity, peaking at 36 h and plateauing until 72 h. LPS treatment induced a significant increase of LDH activity in BAL fluids at 36 h and 72 h post-administration ($P < 0.01$ vs. other groups, Fig. 4A).

Protein content. Pulmonary influenza vaccination did not significantly interfere with BAL total protein levels compared to negative controls until 14 days post-administration (Fig. 4B). A small increase was observed at 36 h and 72 h, but total protein levels were decreased at later time points. The temporal kinetic of BAL total protein content in LPS-treated control animals was comparable to that observed for LDH activity in the same animals, i.e., a significant increase at 36 h and 72 h post-administration, which was downregulated at later time points.

Albumin content. The albumin content in BAL was used as a marker of the permeability of the pulmonary tissue, from the vascular bed to the airway epithelium. The albumin content in BALs of influenza i.t. vaccinated mice was not significantly different from that of negative controls. However,

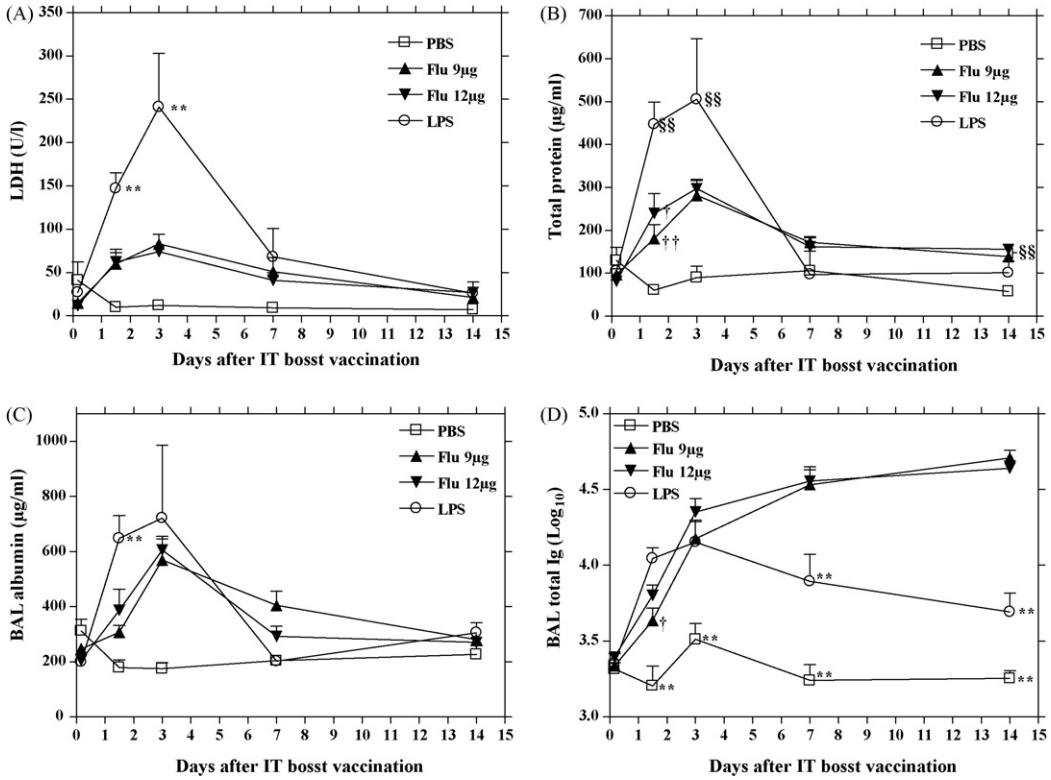

Figure 4 Effect of pulmonary influenza vaccination on BAL (A) lactate dehydrogenase (LDH) activity, (B) total protein content, (C) serum albumin content and (D) total immunoglobulin (Ig) content. Mice were immunized as described in Table 1A. At different time points following the intratracheal boost, the lung was lavaged and BAL levels of LDH, total proteins, serum albumin and total Ig were measured. * indicates groups whose values are significantly different from those of the other groups. § indicates groups whose values are significantly different from those of the PBS mice. † indicates groups whose values are significantly different from those of the LPS mice. One symbol indicates significant differences ($P < 0.05$), two symbols indicate highly significant differences ($P < 0.01$).

a clear trend was noticeable: the albumin content steadily increased until 72 h post-administration and was then down-regulated. The LPS-treated controls showed a significant increase in albumin BAL levels at 36 h post-administration, this was then decreased from 72 h on.

Immunoglobulin content. The total Ig content in BALs of influenza i.t. vaccinated mice increased progressively with time and was significantly higher than that of negative controls from 36 h post-administration on ($P < 0.01$, Fig. 4C). The total Ig content in BAL of animals treated i.t. with LPS increased to the same extent as that of influenza vaccinated mice until 7 days post-administration. At this time point the Ig content was significantly lower than in influenza vaccinated mice, but was still significantly superior to that of negative controls ($P < 0.01$, Fig. 4C).

Effect of pulmonary influenza vaccination in allergen-sensitized and challenged mice

Airway reactivity

OVA sensitization and allergen airway challenges caused development of in vivo airway hyperreactivity (AHR) in response to unspecific airway provocation with methacholine as shown by the increase in maximal Penh values compared to negative controls (Fig. 5). Influenza vaccination subsequent to OVA sensitization/challenge reduced the development of AHR, as demonstrated by the reduction in maximal Penh values when compared to PBS/OVA/PBS mice. This effect was also observed with the pulmonary instillation of LPS following allergen-sensitization/challenge (Fig. 5).

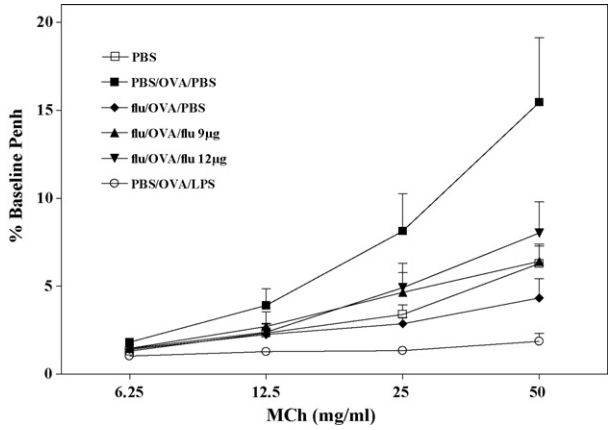

Figure 5 Mice were immunized and sensitized as described in Table 1B. On day 40, in vivo airway reactivity in response to increasing doses of aerosolized methacholine was measured by means of whole-body plethysmography.

BAL cell population

BAL cells of negative control animals (PBS, Fig. 6) were identified as mainly macrophages. OVA airway challenges of sensitized mice caused allergen-induced airway inflammation characterized by a highly significant influx of eosinophils, lymphocytes and macrophages into the lung (Fig. 6). Pulmonary influenza vaccination with 9 μg or 12 μg of hemagglutinin (HA) following flu i.n. priming and OVA sensitization/challenge did not significantly alter the cellular pattern of BAL fluids when compared to the flu/OVA/PBS group (Fig. 6). Only a slight, but not significant, increase of neutrophilic accumulation was observed. In contrast, the intratracheal instillation of LPS clearly affected BAL cell counts, inducing a highly significant influx of cells, mainly differentiated as neutrophils ($P < 0.001$ vs. other groups, Fig. 6).

BAL TNF-α level

Pulmonary influenza vaccination, with 9 μg or 12 μg HA, following i.n. priming and OVA sensitization and allergen airway challenges did not significantly affect the BAL TNF-α level as a marker of early airway inflammation. In contrast, the instillation of LPS to OVA-sensitized/challenged mice induced a significant increase of BAL TNF-α levels (Fig. 7).

Serum allergen-specific IgE

OVA sensitization induced a highly significant increase in serum levels of OVA-specific IgE compared to negative controls (PBS, Fig. 8). Pulmonary administration of influenza vaccine following OVA sensitization/challenge did not significantly affect OVA-specific IgE levels. However, i.n. flu priming induced a trend for decreased OVA-specific IgE levels, which was enhanced by subsequent administration of

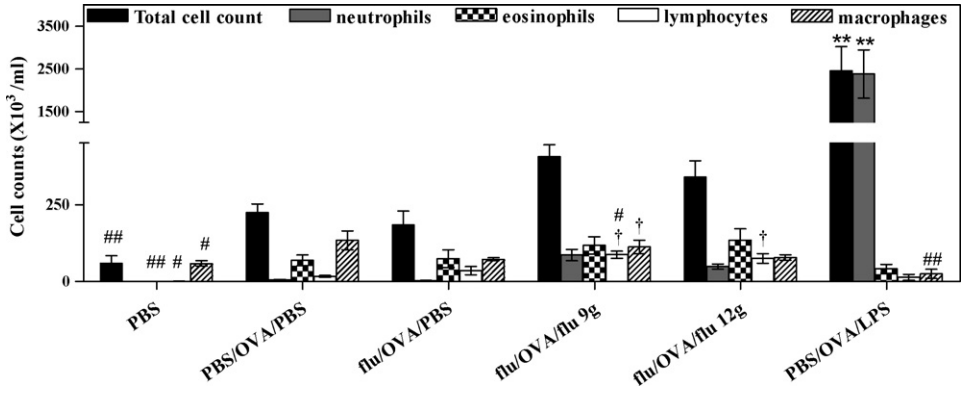

Figure 6 Mice were immunized as described in Table 1B. On day 41, the lung was lavaged and the cells recovered were stained and differentiated. Cell counts are expressed as cells $\times 10^3$/ml. # indicates groups whose values are significantly different from those of the PBS/OVA/PBS group. One symbol indicates significant differences ($P < 0.05$), two symbols indicate highly significant differences ($P < 0.01$). ** $P < 0.01$ vs. all other groups, † $P < 0.05$ vs. PBS/OVA/LPS.

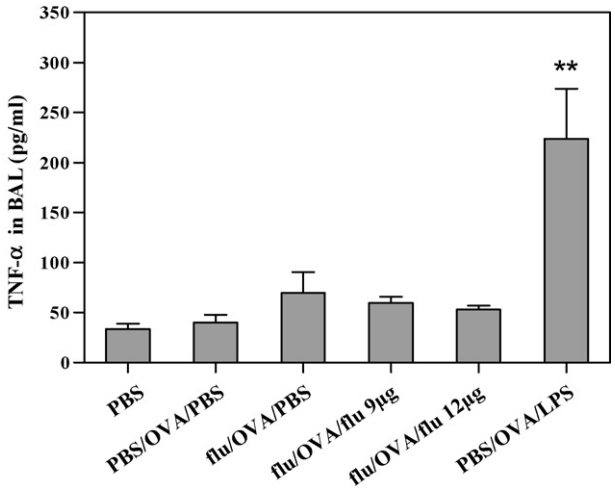

Figure 7 Mice were immunized and sensitized as described in Table 1B. On day 41, broncho-alveolar lavages were performed. BAL levels of TNF-α were measured by ELISA. ** $P < 0.01$ vs. all other groups.

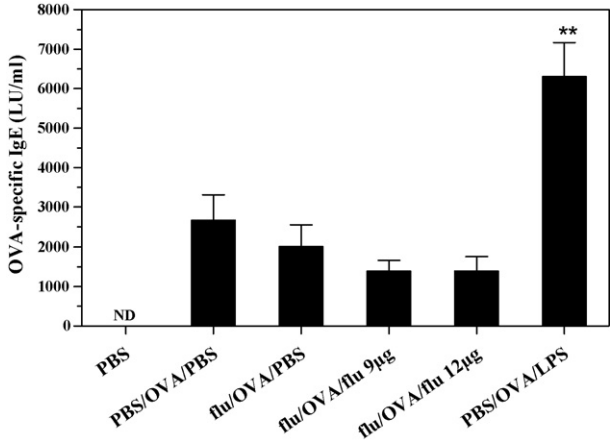

Figure 8 Mice were immunized and sensitized as described in Table 1B. On day 41, blood samples were collected. Serum levels of OVA-specific IgE were measured by means of ELISA. ** $P < 0.01$ vs. all other groups. ND: below detection limit (12 LU/ml).

influenza vaccine into the lung. On the contrary, intratracheal instillation of LPS to OVA-sensitized/challenged mice significantly increased serum OVA-specific IgE levels (Fig. 8).

Discussion

The aim of the present study was to evaluate the local effects of pulmonary vaccination with high doses of split virus influenza vaccine in non-asthmatic and asthmatic mice. The doses used in our experiments were twofold (3× 3 μg HA, trivalent) or eight fold (12 μg HA, monovalent), respectively, the usual dose administered to elicit significant immune responses in mice against seasonal influenza antigens (1.5 μg HA/strain = 1/10th of the human dose) [13].

First, we investigated the temporal kinetics of different lung inflammatory markers in normal mice over a period of 2 weeks following pulmonary influenza vaccination or LPS administration for the positive control. Our observations clearly confirmed the established cascade of the innate immune response to an antigenic viral fragment. This response is initiated by an early production of pro-inflammatory mediators, such as TNF-α, IL-1 and IL-8 (MIP-2 and KC in mice), causing upregulation of adhesion molecules (for leukocytes) on the vascular endothelium and on the distal airway epithelium, and resulting in the recruitment of neutrophils into the alveolar compartment and activation of lung macrophages [19]. Degranulation of neutrophils and macrophages results in the release of elastases that stimulate macrophages to produce LTB4 [20] cathepsin G, reactive oxygen species (ROS), and proteinases [21]. These proteinases and ROS damage the epithelium [22] and increase the permeability of the bronchial mucosa, resulting in protein exudation into the airways [23].

This temporal kinetic of lung inflammation markers was particularly evident in the LPS-treated mice, in which a strong production of TNF-α preceded a massive influx of neutrophils, which on its turn headed significant LDH and total protein activity in BAL (Figs. 1, 3 and 4A and B). This pattern was also observed in influenza vaccinated mice, but the response was significantly lower in its quantity and, most importantly, limited in time. The notion of reversibility of the inflammatory response caused by vaccination is most crucial. Due to its function as a defence mechanism, inflammation is a key effector process in any kind of innate immune response. This can be illustrated by the finding that soluble antigens and low doses of particulate antigens that do not induce pulmonary inflammation do not produce a primary immune response [24]. However, inflammation is a double-edged sword as it also may damage the host tissues if its activity is sustained. Our data demonstrated that, at a time point at which the immune response to the vaccine is still active (i.e., 14 days post-immunization), the non-specific inflammatory reaction has already been turned off.

The immunological efficacy of pulmonary influenza vaccination at 14 days post-immunization was suggested by the significant increase in levels of cells and total protein content in BALs of influenza vaccinated mice at this time point (Figs. 1A and 4B). The residual BAL cells were identified as lymphocytes (Fig. 1B), and the residual increase in total proteins in BAL 14 days after immunization consisted mostly of immunoglobulins (IgG and IgA, Fig. 4D). These total Igs steadily increased in BAL by time following pulmonary influenza vaccination and already reached highly significant levels when compared to negative controls (PBS) 36 h after vaccination and for the rest of the observation period. This corroborates previous data, obtained with smaller vaccine doses, in which we demonstrated that the pulmonary route induced local production of specific immunoglobulins [13]. This previous study demonstrated that the pulmonary route is an effective route of immunization to induce a broad immune response to an influenza vaccine [13].

Immunoglobulins are not the only sources of proteins in BAL. Proteins can also transudate from the vascular bed into the broncho-alveolar lumen when the epithelial permeability is increased. In the current study, the increase of LDH levels in BAL indicated epithelial damage, resulting in increased permeability as shown by a small concurrent peak in BAL total proteins at 72 h post-immunization. These proteins were identified to mainly originate from the plasma (Fig. 4C). Active resolution of inflammation is crucial if

injury to the lung is to be avoided. The plasma proteins that exit vessels are part of this self-limiting process since they include proteinase inhibitors which are transferred from the circulation to the site of inflammation [25]. This is not the only feature leading to resolution of the inflammatory reaction. Neutrophils have a short lifespan of a few hours, and the removal of apoptotic neutrophils by macrophages helps to minimize and prevent any permanent damage caused by neutrophilic inflammation [26]. Our data also supported this scavenger role of macrophages, as their numbers steadily increased in BAL over the first week period following immunization (Fig. 1D).

Taken together, the first part of our study clearly demonstrated that the production of pro-inflammatory cytokines and subsequent influx of inflammatory cells was limited in duration and magnitude following pulmonary influenza vaccination, while its immunogenic effect was maintained. However, another critical issue is the question if and how pulmonary vaccination may affect pre-existing inflammatory diseases in the lung, such as bronchial asthma, which we tried to answer in the second part of our study.

Utilizing a well established murine model for allergen-induced airway disease, we demonstrated that pulmonary influenza vaccination did not significantly exacerbate the cardinal features of asthma, i.e., the allergen-specific IgE formation, the development of AHR and eosinophilic airway inflammation [27]. AHR and OVA-specific IgE were even reduced when compared to unvaccinated allergen-sensitized/challenged mice (PBS/OVA/PBS) (Figs. 5 and 8), corroborating previous findings [28]. This was, for OVA-specific IgE, in strong contrast to the LPS-treated allergen-sensitized control animals that showed significantly increased OVA-specific IgE values. Studies by Tulic and co-workers [29] showed that LPS directly stimulated particularly those B cells which have been primed to produce IgE, if isotype switch has already occurred, i.e., at least 6 days following allergen-sensitization.

In contrast to its exacerbating effect on IgE production, LPS treatment completely abrogated the development of increased airway reactivity in allergen-sensitized/challenged animals. This has already been described [29] and has been shown to be associated with LPS stimulation of Th1 inhibitory cytokines IL-12 and/or IFN-γ [30–32]. Similarly, we previously demonstrated that the preventive effect of respiratory influenza vaccination on OVA-specific IgE production and development of AHR was also mediated through the induction of local Th1 production in response to the i.n. whole virion influenza priming [28].

This Th1 biased immune response following pulmonary influenza vaccination was further illustrated by the profile of the OVA-specific IgG subclasses in the serum (data not shown). Th1 polarization might also be causally related to the slight neutrophilic influx in the BAL fluid of flu vaccinated OVA-sensitized/challenged mice (Fig. 6). Such a causal relationship had already been described for LPS treatment [29], which exacerbated pulmonary neutrophil influx in OVA-sensitized/challenged mice similar to the present study. The slight neutrophilic influx in BAL of OVA-sensitized/challenged mice 36 h after influenza vaccination corroborated the infiltration observed in non-asthmatic vaccinated mice. The latter was shown to be reduced to levels of normal controls within 7 days post-immunization.

In conclusion, we demonstrated that the immune response induced by pulmonary influenza vaccination was not linked to a long-term upregulation of inflammatory responses or toxic side effects and did not exacerbate pre-existing allergic airway disease. Our data therefore further illustrate the promising potential of the pulmonary route for seasonal vaccination with inactivated split influenza virus. We strongly recommend further investigation to pursue this strategy, e.g., by GLP trials in primates and humans.

Acknowledgements

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