Immunology Letters 134 (2010) 26–34

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/

Adjuvant effect of *Bacillus firmus* on the expression of cytokines and toll-like receptors in mouse nasopharynx-associated lymphoid tissue (NALT) after intranasal immunization with inactivated influenza virus type A

Peter Zanvit[a,*], Aleš Tichopád[b], Martina Havlíčková[c], Olga Novotná[a], Marie Jirkovská[d], Katarína Kološtová[e], Dana Čechová[a], Jaroslav Julák[a], Ivan Šterzl[a], Ludmila Prokešová[a]

[a] Charles University in Prague, First Faculty of Medicine, Institute of Immunology and Microbiology, Studničkova 7, 128 00 Prague, Czech Republic
[b] Technical University of Munich, Physiology Weihenstephan, Weihenstephaner Berg 3, 85354 Freising, Germany
[c] National Institute of Public Health, Šrobárova 48, 100 42 Prague 10, Czech Republic
[d] Charles University in Prague, First Faculty of Medicine, Institute of Histology and Embryology, Albertov 4, 128 00 Prague, Czech Republic
[e] Charles University in Prague, Third Faculty of Medicine, Department of Tumor Biology, Ruská 87, 100 00 Prague, Czech Republic

ARTICLE INFO

Article history:
Received 21 May 2010
Received in revised form 15 July 2010
Accepted 8 August 2010
Available online 13 August 2010

Keywords:
Multimarker gene expression
Nasopharynx-associated lymphoid tissue (NALT)
Toll-like receptors (TLR)

ABSTRACT

Due to the persisting threat of development of new highly pathogenic influenza A subtypes, a mucosal vaccination which would induce a potent and cross-protective reaction is desirable. We succeeded in mucosal immunization of mice with an inactivated influenza A virus by using delipidated *Bacillus firmus* (DBF) as adjuvant.

The mechanism of adjuvant effect was followed in NALT by comparing the response after intranasal immunization by inactivated influenza virus type A (H1N1) alone, adjuvant alone (DBF), or by a mixture of virus + DBF. Expression of selected gene groups was tested via qPCR at 7 different time-points: cytokines (IL-2, IFN-γ, IL-4, IL-6, and IL-10), type I interferons (IFN-α4, IFN-α11, IFN-α12, and IFN-β), toll-like receptors (TLR2, TLR3, TLR7, and TLR9), iNOS and CCR7. Intranasally administered DBF and the mixture of virus + DBF induced an elevated expression of IFN-γ, IL-6 and IL-10 cytokines, type I interferons, iNOS, and pDC markers in NALT. Multimarker qPCR data was analyzed by relative quantification and by principal component analysis.

DBF has been shown to be a very efficient adjuvant for the stimulation of innate immunity after IN immunization. DBF accelerated, increased, and prolonged the antiviral response.

1. Introduction

The influenza virus is a globally important respiratory pathogen causing nearly annual epidemics and occasional pandemics. Vaccination against influenza is currently conducted by parenterally administered split or subunit vaccines. These vaccines most often induce satisfactory homotypical systemic immunity, particularly the production of IgG class serum antibodies, but they fail to induce mucosal immunity or cross-protection. The threat of a sudden pandemic outbreak necessitates the development of better vaccines capable of inducing both mucosal and heterosubtypical immunity. Natural infection is accompanied by the production of antibodies, especially of the IgA class, in the respiratory tract. They cause a cross-protection (usually only intrasubtypical) more efficiently than serum antibodies. This finding implies that efficient immunization should be performed by the mucosal route, which should induce the production of mucosal IgA class antibodies [1,2]. At present, anti-influenza vaccination is mostly performed parenterally using an inactivated vaccine. Mucosal immunization of humans is licensed only in some countries and is used to a limited extent. Because of the presence of live viruses (cold adapted mutants) in this vaccine, its application is connected with a certain risk and its restricted indication range excludes persons with the highest risk – small children and old people [3]. Inactivated viruses are usually not sufficiently effective in mucosal immunization; and it is therefore necessary to use adjuvants capable of adequately strengthening the immune response. The use of adjuvants in vaccination is important for several reasons. Current modern subunit vaccines are better defined and often safer than the older whole-virion vaccines. These vaccines are less reactogenic but, on the other hand, also less immunogenic and the use of an adjuvant would therefore be desirable [4,5]. The immunization of mice with an inactivated influenza virus, as reported in our preceding studies, caused an increase in the immune response with the aid of a bacterial adjuvant, a delipidated form of the G+ bacterium *Bacillus firmus* (DBF). As we have previously described [6,7], adjuvant immune

* Corresponding author. Tel.: +420 22496 8509; fax: +420 22496 8455.
E-mail address: peter.zanvit@lf1.cuni.cz (P. Zanvit).

0165-2478/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.imlet.2010.08.006

response induced protection of immunized mice *in vivo* in an infectious influenza model not only against the homologous virus used for immunization but also against viruses of a different subtype (heterosubtypical protection).

The primary aim of the present study is to contribute to the elucidation of the mechanism of adjuvant effect of DBF, by studying the changes in the gene expression in the nasopharynx-associated lymphoid tissue (NALT) after intranasal immunization of mice. In rodents, NALT represents a paired lymphoid organ formed by an aggregation of lymphoid cells in the upper airway, and is considered to be the only well-organized lymphoid tissue of the respiratory tract [8,9]. NALT is also an induction site of MALT (mucosa-associated lymphoid tissue) and the site of a considerable isotype shift to the production of IgA class antibodies and clonal expansion of B lymphocytes [10]. NALT therefore plays an important role in inducing an immune response against pathogens in the upper airway. The first line of defense against influenza is innate immunity with its essential component, type I interferons, which are mainly produced by plasmacytoid dendritic cells (pDC). This function is closely connected with their ability to express TLR7 and TLR9 in early endosomes, enabling them to recognize foreign viral or bacterial nucleic acids.

To evaluate the effects of adjuvant immunization we studied via qPCR the expression of genes important for both the reaction of innate and adaptive immunity: toll-like receptors recognizing antigens of G+ bacteria and microbial nucleic acids (TLR2, TLR3, TLR7, and TLR9), type I interferons (IFN-α4, IFN-α11, IFN-α12, and IFN-β), type Th1 and Th2 cytokines (IL-2, IFN-γ, IL-4, IL-6, and IL-10), and some other genes (CCR7 and iNOS) at time-points of 3, 6, 12, 24, 48, 72, and 168 h post-immunization.

2. Materials and methods

2.1. Bacteria

The *B. firmus* strain CCM 2212 (Czech Collection of Microorganisms, Masaryk University, Faculty of Science, Tvrdého 14, Brno, Czech Republic) was aerobically cultivated in a liquid medium composed of peptone, beef extract, and urea (pH 7.2–7.4) at 37 °C to the late exponential phase. The cultures were washed with distilled water and inactivated by 0.4% aqueous formaldehyde at room temperature for 30 min. All bacteria were killed under these conditions, as proved by sterility tests. The inactivated biomass was washed three times with distilled water and lyophilized. Delipidated bacteria (DBF) were obtained from semi-dry biomass by overnight extraction with chloroform–methanol 2:1 (v/v). After extraction and drying at 50 °C, extraction was repeated. For the third extraction only chloroform was used. Liquid was filtrated out; and the mass dried [11,12].

2.2. Virus

Influenza virus A/PR/8/34 (H1N1) was proliferated by standard methods in the allantoic sac of chicken embryos. The resulting hemagglutination titer (HT) of the inoculated allantoid fluid used for immunization of mice was 1:256 and corresponded to 1.2×10^4 EID_{50}/ml. The virus was inactivated by formaldehyde (final concentration 0.025%) for 72 h at 4 °C; the efficacy of inactivation was tested by three blind transfers.

2.3. Animals

Adult BALB/c female mice (9–14 weeks old) were used for experimentation. The mice were obtained from AnLab Prague.

2.4. Immunization

The mice were immunized intranasally (IN) under halothane anesthesia by 5 μl of immunization mixture into each nostril (total volume 10 μl). Mice received a: 10 μl PBS, b: 10 μl virus alone (5 μl virus suspension + 5 μl PBS), c: 10 μl adjuvant alone (100 μg DBF in PBS) or d: 10 μl mixture of virus + DBF (5 μl virus suspension + 100 μg DBF in 5 μl of PBS). Mice were challenged with one dose and were killed in time intervals of 3 h, 6 h, 12 h, 1 d, 2 d, 3 d, and 7 d after immunization. In each experiment, groups of 3 mice were immunized.

2.5. Sample collection

The mice were exsanguinated from the abdominal aorta under ether anesthesia. NALT was separated from the upper jaw by peeling away the palate where NALT is localized bilaterally on the posterior side [13]. Samples from individual animals were placed in RNAlater stabilization reagent (Qiagen).

2.6. Histology

NALT samples fixed for a minimum of 24 h in 4% formaldehyde were dehydrated by graded ethanol and embedded in paraffin. 7 μm sections were stained with Haematoxylin–Eosin.

2.7. RNA isolation

Samples were homogenized with Ultra-Turrax T8 homogenizer (IKA) and total RNA was extracted using the RNeasy mini kit (Qiagen), following the manufacturer's instructions. RNA integrity was determined by gel electrophoresis in 1.5% agarose gel stained with ethidium bromide. The purity of the RNA was assessed by the ratio of absorbance at 260 and 280 nm. RNA purity was within a range of 2.0–2.1. The total RNA concentration was estimated by spectrophotometric measurements at 260 nm assuming that 44 μg of RNA per milliliter equal one absorbance unit. RNA was stored in aliquots at −70 °C until used for reverse transcription.

2.8. Real-time PCR

RNA was converted to cDNA using Taq-Man reverse transcription reagents (Applied Biosystems). A reaction mix for real-time PCR was made with Taq-Man Universal PCR master mix, water, and Assays on demand gene expression products for IL-2, IL-4, IL-6, IL-10, IFN-γ, TLR2, TLR3, TLR7, TLR9, IFN-α4, IFN-α11, IFN-α12, IFN-β1, CCR7, iNOS, and β-actin (all Applied Biosystems). 20 μl of reaction mix was aliquoted to the wells on a real-time PCR plate; and each sample was analyzed in duplicate. A volume of 5 μl of cDNA was added to each well. The PCR reaction was run on a 7300 real-time PCR System (Applied Biosystems) using standard conditions.

2.9. Data analysis and statistics

The threshold cycle (Ct) values obtained in qPCR were normalized to technical replicates and further with the reference gene (β-actin) as:

$$\Delta Ct = Ct_{goi} - Ct_{ref}$$

where the index *goi* indicates the gene of interest and index *ref* indicates the reference gene) The data was analyzed with Genex software (version 4.3.8) [14,15]. The RNA relative quantity (RQ) values are presented in Figure 2, having been calculated by the ΔΔCt method; whereas the Ct value for the control samples is

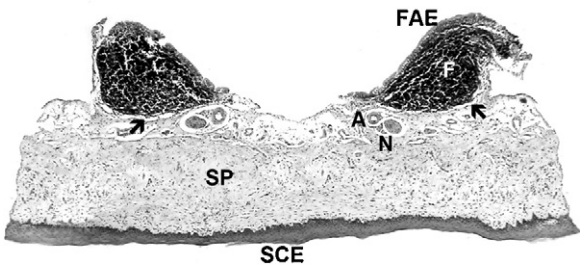

Fig. 1. Photomicrograph of the mice nasopharynx-associated lymphoid tissue (NALT): the follicle (F) is covered with the columnar follicle-associated epithelium (FAE); at the edge of the follicle high endothelium venules (arrows) occur; arteries (A) and nerves (N) are present in the upper part of the soft palate (SP) covered from the oral aspect with stratified squamous cells epithelium (SCE). Magnification = 40×.

represented by their mean, as well as the Ct value for the treated groups. To compare the effect of the immunization with the control at each time-point, an unpaired *t*-test was calculated for each RQ gene expression value. As this was an explorative study, we did not perform any adjustment for multiple comparisons. Comparisons that generated p-value ≤ 0.05 were considered potentially involved in the biological process (see Table 1).

The method of principal component analysis (PCA) was employed to disclose multivariate response to the treatment. PCA for the gene expression data, as well as statistical tests, were produced with SAS v. 9.1.3 for Windows. As $N = 3$ for each treatment group, no formal test of normality of the data could be performed; therefore the data was log-transformed to assure normal distribution. PCA involves a mathematical procedure that transforms a number of variables (here expression values of various genes) into a smaller number of uncorrelated variables called principal components, by which the dimensionality of the data is reduced to a number that can be represented in a scatter plot (here two dimensions). The first principal component accounts for as much of the variance in the data as possible; and each succeeding component accounts for as much of the remaining variance as possible. Normalized expression values of all responding genes were taken as the initial variables and reduced to only two principal components, thus facilitating resolution of treatment clusters in the scatter plot. Various functional groups of genes were composed; where treatment groups tended to cluster distinctly, an involvement of the group in the studied process was presumed.

3. Results

The change in gene expression in mice after IN immunization was followed in three separate experiments because of the large number of genes and time intervals. In each experiment the mice were separated into controls that received only PBS (ctrl virus, ctrl DBF or ctrl virus + DBF) and mice immunized either with the influenza virus alone (virus), with adjuvant only (DBF), or with the combination of the influenza virus plus adjuvant (virus + DBF). Expression was determined at time-points of 3 h, 6 h, 12 h, 1 d, 3 d, and 7 d after IN immunization in NALT (Fig. 1). Changes in gene expression after immunization were expressed by means of relative quantification (RQ), as well as by principal component analysis (PCA).

3.1. Relative quantification (RQ)

The method of relative quantification was used to study the gene expression in groups immunized with the virus, DBF or the combination virus + DBF, relative to the expression measured in mice immunized with PBS (ctrl virus, ctrl DBF, ctrl virus + DBF). Results are shown in Fig. 2 and their significance is given in Table 1. Indi-

Table 1
p-Values reflecting differences in relative RNA-amount (after qRT-PCR) between intranasally immunized mice (with virus alone, DBF alone and combination of virus + DBF) and their unimmunized controls in 7 time intervals.

	3 h			6 h			12 h			24 h			48 h			72 h			168 h		
	Virus	DBF	Virus + DBF	Virus	DBF	Virus + DBF	Virus	DBF	Virus + DBF	Virus	DBF	Virus + DBF	Virus	DBF	Virus + DBF	Virus	DBF	Virus + DBF	Virus	DBF	Virus + DBF
TLR2	0.681	**0.001**	**0.004**	0.866	**0.048**	0.119	0.244	0.669	0.725	0.243	0.147	**0.049**	0.641	0.925	0.051	0.718	0.829	0.180	0.627	0.217	0.108
TLR3	0.824	0.214	0.222	0.790	0.057	0.795	0.586	0.133	0.516	0.371	0.274	0.057	0.877	0.111	0.058	0.119	0.100	0.323	0.308	**0.045**	0.304
TLR7	0.607	0.994	**0.002**	0.140	0.333	0.067	0.236	0.883	0.506	**0.002**	0.367	0.182	0.172	0.671	0.111	0.175	0.265	0.203	0.855	0.299	0.537
TLR9	0.727	0.054	0.309	0.153	0.803	0.648	0.067	0.752	0.961	0.145	0.514	0.499	0.855	0.295	0.363	0.879	**0.036**	0.817	0.420	0.088	0.325
IL-2	0.651	0.217	0.069	0.179	0.167	**0.042**	0.252	0.493	0.148	0.198	0.585	0.545	0.405	0.998	0.090	0.466	0.080	0.066	0.074	0.271	0.522
IL-4	0.871	**0.007**	**0.034**	0.116	**0.004**	0.374	0.202	0.244	0.253	**0.017**	0.925	0.412	0.437	0.524	0.190	0.136	**0.034**	0.363	0.071	0.486	0.418
IL-6	0.961	**0.000**	**0.001**	0.749	**0.018**	**0.011**	0.294	0.669	0.344	0.408	**0.010**	**0.019**	0.406	0.770	0.899	0.999	0.190	0.120	0.163	0.848	0.766
IL-10	0.485	0.096	0.155	0.988	0.906	0.337	0.815	0.057	0.788	0.369	0.396	0.155	0.115	0.426	0.392	**0.039**	0.819	**0.042**	0.448	0.781	0.499
IFN-α4	0.154	0.848	0.254	0.976	0.888	0.582	**0.044**	0.904	0.302	0.968	0.194	**0.044**	0.316	0.665	0.526	0.516	0.685	0.546	0.802	0.330	0.976
IFN-α11	0.088	0.997	0.473	0.889	0.543	0.724	0.592	0.689	0.675	0.327	0.455	0.245	0.863	0.854	0.775	0.164	0.918	0.586	0.876	0.072	0.565
IFN-α12	0.309	0.902	**0.022**	0.540	0.075	0.408	0.116	0.672	0.281	0.334	0.922	0.075	0.293	0.841	0.696	0.587	0.800	0.202	0.193	0.195	0.490
IFN-β1	0.177	0.791	0.478	0.260	0.766	0.406	**0.018**	0.285	**0.018**	0.585	0.081	**0.034**	0.984	0.935	0.341	0.873	0.254	0.813	0.681	0.707	0.862
IFN-γ	0.494	**0.001**	**0.004**	0.498	0.073	**0.049**	0.058	0.372	**0.032**	**0.005**	0.654	0.910	0.867	0.383	0.135	0.477	0.102	0.208	0.258	0.586	0.697
iNOS	0.535	**0.001**	**0.012**	0.660	**0.014**	0.128	0.244	0.535	0.266	0.398	0.181	0.108	0.322	0.073	0.147	0.461	0.868	0.101	0.057	0.330	0.887
CCR7	0.310	**0.001**	**0.014**	0.248	0.103	0.108	0.291	0.495	0.588	0.995	0.301	**0.046**	0.979	0.734	0.361	0.515	0.438	**0.013**	0.773	0.375	0.789

As for each treatment group $N = 3$; no formal test of normality of the data could be performed. The data were therefore log-transformed to assure normal distribution. To compare the effect of the immunization with the control (IN application of PBS) at each time-point, an unpaired *t*-test was calculated for each gene. Comparisons that generated p-value ≤ 0.05 were considered significant and potentially involved in the biological process (bolded).

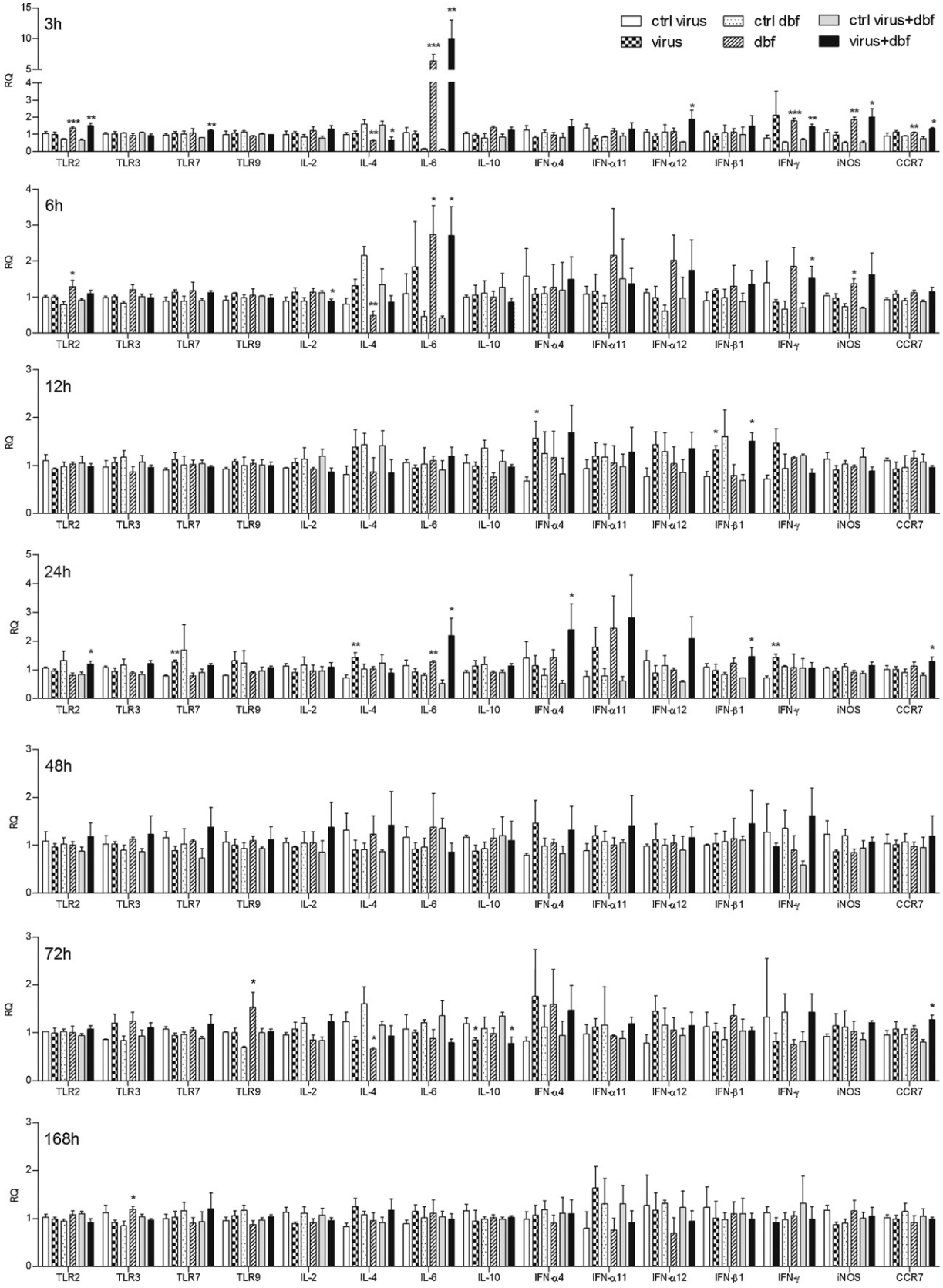

Fig. 2. mRNA relative quantity (RQ) in nasopharynx-associated lymphoid tissue (NALT) in 7 time intervals after immunization. Results were calculated from normalized gene expression data. The RQ values indicate the difference between the treated group and the controls. For each compared group the RQ average values ± S.E.M. are shown. Asterisks indicate significant differences between treated groups (with virus alone, DBF alone and mixture of virus + DBF) and their controls ($^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$).

vidual groups of immunized mice exhibited different dynamics of gene expression.

These results imply that the main changes in gene expression occur during the first 24 h after immunization. Groups immunized with DBF alone or with the combination of virus + DBF display very fast changes, peaking at 3 and 6 h post-immunization. Significant changes resulting from immunization with the virus alone were detected later – 12 and 24 h after treatment. Immunization with the virus alone mainly increases the expression of TLR7, IFN-γ, and type I interferons. DBF significantly increases the expression of TLR2, IL-6, iNOS, IFN-γ, and CCR7; and lowers the expression of IL-4. Relative to the virus alone, the combination virus + DBF accelerates the expression of TLR7 and type I interferons; when compared with DBF alone it increases the expression of TLR7, CCR7, and iNOS. Immunization with the combination virus + DBF has a faster and longer effects (3–72 h) on the increase in expression of type I interferons (particularly IFN-α4 and IFN-β1) than treatment with virus alone or DBF alone.

3.2. PCA analysis

The PCA method makes it possible to reduce a multidimensional space to two-dimensional one, thereby facilitating the graphical expression of differences between individual immunizations. To carry out PCA analysis, individual genes were divided into four functional groups. This enabled us to study the differences in gene expression between these functional groups of genes at individual time-points and in individual immunized groups. The functional groups were as follows: (1) TLR7, TLR9, and CCR7 – genes characteristic for pDC; (2) IFN-α4, IFN-α11, IFN-α12, and IFN-β1 – type I interferons; (3) IL-4, IL-6, and IL-10 – Th2 cytokines; (4) IL-2 and IFN-γ – Th1 cytokines. Individual groups of mice were characterized by ellipses, indicating their range of values. Light colored ellipses represent for non-immunized controls, dark colored ellipses illustrate the state after immunization (blue: immunization with virus alone, red: immunization with DBF alone, green: immunization with combination virus + DBF). The distance of control ellipses (ctrl) from the ellipses corresponding to groups of mice immunized with virus alone, DBF alone, or the combination virus + DBF, reflects the magnitude of difference between these groups. It should be noted that this plot does not provide information on whether the difference is due to a decrease or increase in expression relative to the expression in controls. Increased or decreased expression can be inferred from the results of relative quantification.

At 3 h post-immunization the difference in expression of genes characteristic for pDC in the group immunized with the virus alone were very low in contrast to the group immunized with DBF; with the largest change found in mice immunized with the combination virus + DBF. Expression of genes encoding type I interferons in this interval was most conspicuous in groups immunized with the virus alone and those immunized with virus + DBF. The largest difference in expression of groups of genes characteristic for Th1 or Th2 response was detected in groups immunized with DBF or with virus + DBF. At this time-point, immunization with the virus alone had a Th1 polarization (Fig. 3a). At 6 h post-immunization, the groups immunized with the virus alone and with DBF, exhibited alterations in expression of genes characteristic for pDC; and a still more conspicuous difference in expression in the group immunized with virus + DBF. No marked difference was found in the expression of genes typical for type I interferons. The group immunized with the virus alone exhibited a dominant expression of genes characteristic for Th1 as compared with Th2, in contrast to mice immunized with DBF or with virus + DBF, in which a marked difference in both Th1 and Th2 responses was detected (Fig. 3b). At the 12 h time-point, no pronounced change in the expression of genes typical for pDC was found between individual groups of mice. The largest difference in expression of genes encoding type I interferons was detected in the group immunized with virus + DBF, relative to groups immunized with the virus alone or with DBF. Groups immunized with the virus alone or with the virus + DBF showed a prevalence of Th1 over Th2 response, while mice immunized with DBF exhibited a marked difference in both Th1 and Th2 responses (Fig. 3c). At 24 h post-immunization the genes characteristic for pDC displayed the largest difference in groups immunized with virus alone or with virus + DBF, relative to the group immunized only with DBF. The biggest difference in expression of genes encoding type I interferons was found in the group immunized with the virus alone or with the virus + DBF. Mice immunized with DBF or virus + DBF displayed a difference mainly in the expression of genes characteristic for Th2 response. The group immunized with the virus alone showed a change in the expression of genes typical for both Th1 and Th2 responses (Fig. 3d).

The results of PCA imply that the most pronounced changes in the group of genes characteristic for pDC were detected in the group immunized with the virus + DBF. This group exhibits a similarity between the expression of genes of the functional groups for pDC and genes for type I interferons. Groups immunized with the virus alone evince a probable activation of pDC only later at 12–24 h post-immunization. In contrast, the group immunized with the virus + adjuvant exhibited a marked change in expression of genes typical for pDC occurring very early on 3 h post-immunization; and this increased expression persisted until 24 h. Immunization with DBF alone elicited a mild difference in the expression of genes characteristic for both pDC and type I interferons. Mice immunized with the virus alone showed a Th1 polarization of the immune response at 3–12 h post-immunization, however; 24 h after immunization the response was rather Th1/Th2 mixed. In groups immunized with DBF or virus + DBF, the type of immune response in the interval of 3–12 h post-immunization was mixed (Th1/Th2); at 12 h it was slightly polarized towards Th1; and at 24 h it was Th2-skewed.

The most efficient in terms of protection of the organism against virus infection appears to be immunization with the combination virus + DBF, which leads to a fast antiviral immune response based on pDC activation, the production of interferons, and the activation of a mixed Th1/Th2 immune response.

4. Discussion

The adjuvant used in our previous studies, the delipidated form of the nonpathogenic environmental G+ bacterium *B. firmus*, has previously been reported to have marked immunostimulatory properties in both *in vitro* and *in vivo* tests [16–19]. Even in high doses inactivated bacteria do not damage the viability of tissue culture cells; and mice tolerate high doses very well of not only inactivated but also live bacteria (intraperitoneal or mucosal application, Prokesova – not published data). The excellent adjuvant properties of this bacterium were first described for intratracheal immunization of mice in combination with the protein antigen ovalbumin [20,21], and later for intratracheal immunization with inactivated type B and also type A influenza viruses. Immunization with inactivated influenza viruses in combination with the adjuvant induced a high antibody response, both in serum (high production of class IgG antibodies) and in respiratory tract mucosa (high production of class IgA antibodies) and provided *in vivo* protection against influenza infection. The adjuvant was also demonstrated to have an effect on the induction of a marked intersubtypic cross-protection [6,7]. We are aware of differences between mouse and human immune systems and influenza virus is not natural pathogen for mice. Nevertheless, mice are used by absolute majority of researchers as model animal for influenza

studies. Strain A/PR/8/34 H1N1 was selected as a strain strongly pathogenic for mice and as a strain commonly used in experimental work. Moreover, the strain mentioned above is currently used for construction of vaccinal influenza virus reassortants.

The present study should contribute to clarifying the mechanism of adjuvant effect of DBF. The evaluation of the action of the adjuvant was based on changes in gene expression in NALT after intranasal immunization. In rodents, NALT is localized on both sides

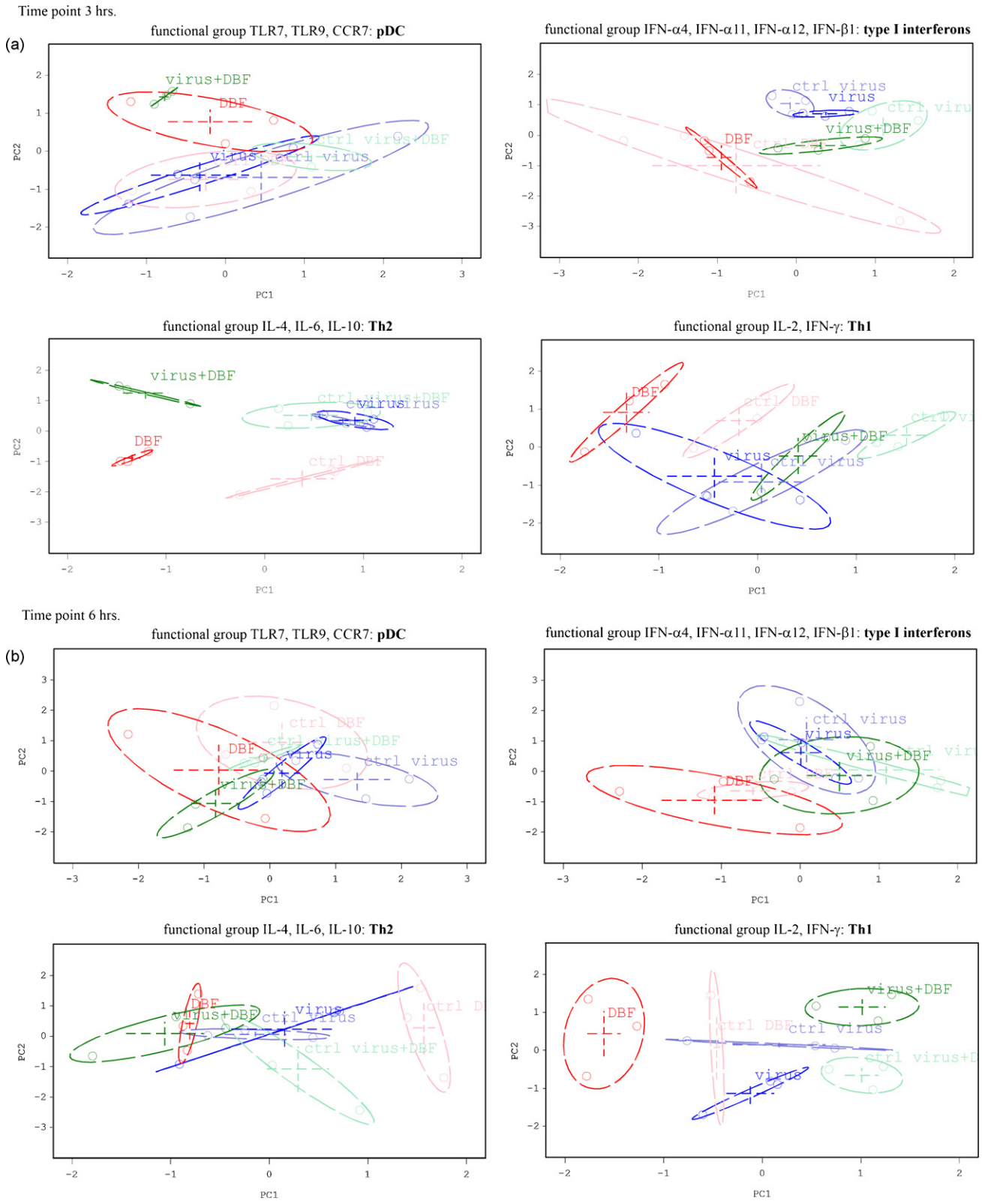

Fig. 3. (a–d) Principal component analysis (PCA). Results point out the differences in the gene expression patterns between the treated groups and their corresponding controls, for the 4 different gene functional groups (pDC, type I interferons and Th1/Th2 cytokines). The dark color has been used for imaging of the treated groups (dark blue – virus immunization, dark red – DBF, and dark green – combination of virus + DBF) and the light color for their corresponding controls.

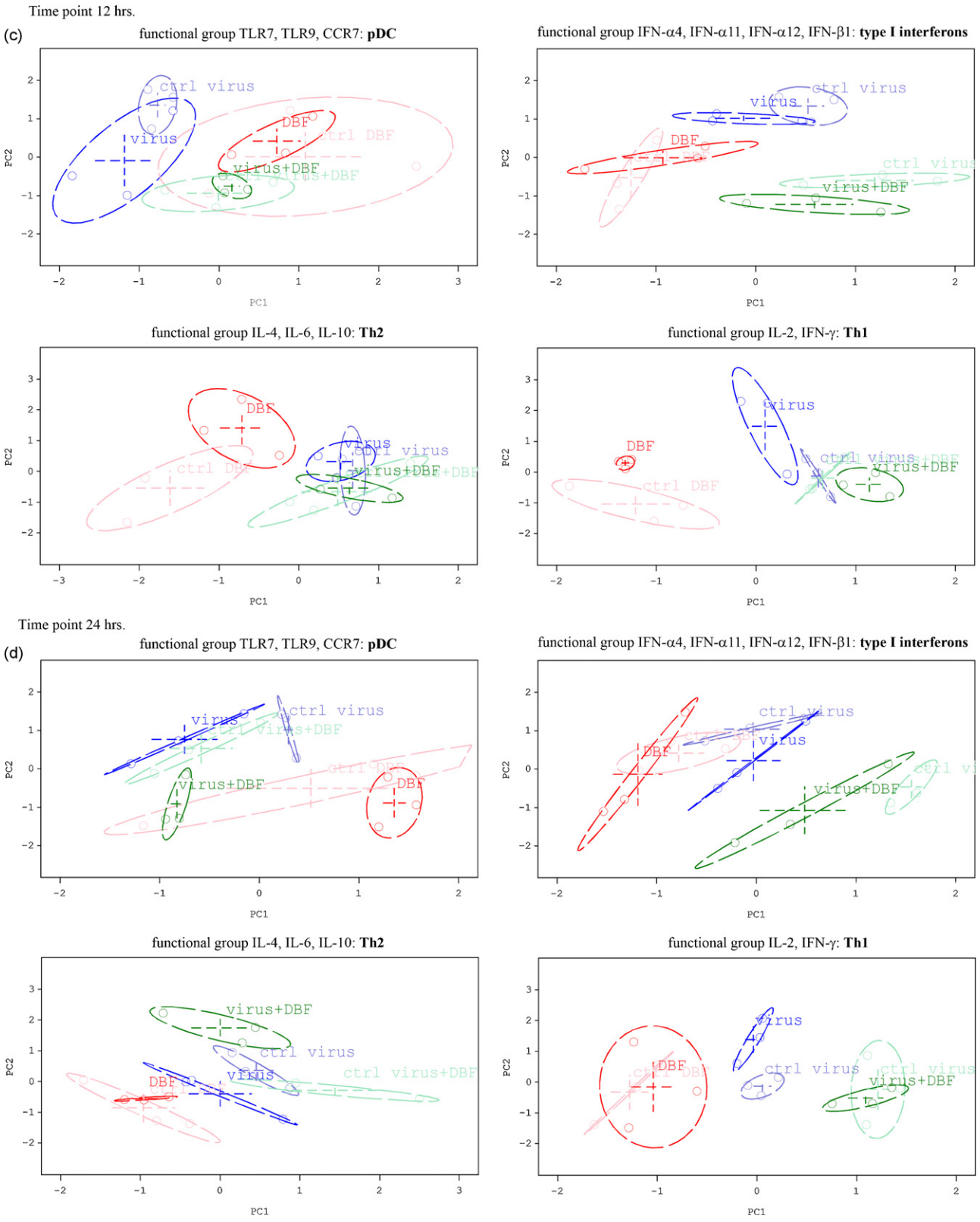

Fig. 3. (*Continued*)

of the nasopharyngeal duct on the upper side of the soft palate, and is considered an analogue of the Waldeyer's ring in humans. It consists of a number of follicles and includes regions rich in B and T lymphocytes, antigen presenting cells – dendritic cells and macrophages. The intrafollicular region contains high endothelium venules (HEVs), expressing a large amount of adhesive molecules, in which lymphocytes from blood circulation are captured and penetrate into the follicles. The epithelium covering NALT (so-called follicle-associated epithelium, FAE) contains a large number of antigen-transporting cells (M-cells). NALT is thus composed of

lymphoid cells that function in inducing and regulating the mucosal immune response [9,22]. Expression of mRNA of cytokines characteristic for Th1 and Th2 response in CD4+ cells in NALT was used to demonstrate a marked predominance of cytokines characteristic for Th0; thus indicating that these T lymphocytes are capable of inducing a very fast activation of immune response of both Th1 and Th2 types after recognition of the antigen on the nasal mucosa [23]. Intranasal administration of an antigen (e.g. bacterial cell wall or virus-associated antigens) in combination with cholera toxin as a mucosal adjuvant led to a marked activation of the Th2 immune response and the stimulation of IgA producing B lymphocytes in both upper and lower airways, as well as in the intestinal and urogenital tract [23,24].

Our results show that immunization with DBF alone has a very fast effect, affecting the expression of the genes under study very markedly already 3 h after immunization and decreases at later time-points. This is supported by the fact that bacterial adjuvants support mainly innate immunity. Affecting innate immunity then, influences adaptive immunity. DBF causes a highly significant early increase in expression of IFN-γ, which can considerably support the immune response of the Th1 type, important in the defense against viral infection. PCA analysis indicates that immunization with DBF brings about a marked change in type Th2 cytokines (IL-4, IL-10, and IL-6); however, this cannot be ascribed to stimulation of Th2 response because the expression of IL-4 is not significantly increased at any time-point – in fact at the first time-points of 3 and 6 h, the expression of IL-4 is significantly lowered. The large differences in PCA are apparently due to a markedly increased expression of IL-6, which along with the concomitantly increased expression of iNOS, is mainly caused by the inflammatory action of DBF. At the same time, DBF tends to also increase the expression of IL-10. An environment with increased concentrations of IL-6 and IL-10 is known to support the production of IgA, with the IgA class antibodies being an important component of mucosal protection against influenza virus. In contrast to the group immunized with DBF, mice immunized with the virus alone exhibited delayed and short-term changes in the followed genes expression. Only 24 h after immunization was a significant increase in expression of both IL-4 and IFN-γ demonstrated. Comparison of the results of RQ and PCA indicates that the onset of the Th1 type response occurs 12 h after immunization; therefore there appears to be a mixed Th1/Th2 response with a slight Th1 accent.

Adjuvant immunization with virus + DBF brings about a combination of the observed responses to the virus and to DBF individually. The response is fast, protracted, and has a mixed Th1/Th2 character: the Th1 response being the strongest after 3 and 6 h, while with Th2 after 12 and 24 h. At later time-points the changes in expression are small. Innate immunity represents the first defense line in protection against viruses. Innate immunity cells express on their surface "pattern recognition receptors" (PRR), which can affect the synthesis of type I interferons as well as the synthesis of pro-inflammatory cytokines after activation. A total of 13 types of toll-like receptors (TLR) that have so far been described in mice as capable of specific recognition of characteristic pathogen-associated molecular patterns (PAMP) on the surface of microbes. TLR2 recognizes different bacterial components such as lipoproteins, lipopeptides, and peptidoglycans and is the principal receptor for the recognition of G+ bacteria [25]. TLR2 is thus important for recognition of DBF obtained from G+ *B. firmus*. Our results point to a significantly increased expression of TLR2 at the time interval of 3–6 h after immunization in mouse groups that were immunized either with DBF alone or with the combination virus + DBF. TLR3 and TLR7 participate in the recognition of influenza virus in certain cell populations. After the recognition of the influenza virus by TLR7 present in early endosomes of pDC, a strong production of type I interferons sets in [26]. TLR9, which is also expressed in pDC, is able to recognize nonmethylated CpG regions in viral and bacterial ssDNA [27,28]; thus this receptor could also participate in the recognition of the bacterial adjuvant. Mature pDC are also characterized by an increased expression of the chemokine receptor CCR7, which plays a key role in the migration of pDC to lymph nodes. Our data demonstrate strong activation of genes characteristic for pDC (TLR7, TLR9, and CCR7), mainly in the group immunized with virus + DBF, at time-points soon after immunization. DBF alone does not cause any marked increase in TLR7 expression. The group immunized with the virus alone evinced only a non-significant increase in TLR7 expression 3 and 12 h after immunization, whereas the increase after 24 h was already significant. These data imply that immunization with the virus alone and with virus + DBF activates pDC, the immunization with virus + DBF causing a more marked and faster activation of genes typical for pDC. The increase in TLR3 expression was at the limit of significance, especially in the group immunized with virus + DBF in the interval of 24–72 h; whereas groups immunized with the virus alone or with DBF exhibited a relatively weaker increase in TLR3 expression.

Plasmacytoid dendritic cells, as the main producers of type I interferons, ensure an early innate protection against viral infection. The mouse genome contains 14 known IFN-α genes and 3 IFN-α pseudogenes. The highest anti-proliferation and antiviral activity relative to IFN-α1 is exhibited especially by IFN-α4, IFN-α11, INF-α12, and IFN-β [29]. For instance the activity of IFN-α4 is 5–10× higher than that of IFN-α1 [30,31]. A two-step mechanism of expression has been described in interferons [32,33]. Transcription of genes encoding IFN-α4 and IFN-β takes place very early after viral infection and is governed by the transcription factor IRF3. Transcription of further genes of interferons is then controlled by transcription factor IRF7. Viral infection first activates the expression of IFN-α4 and IFN-β; then followed by an increase in the expression of other interferon types. Our data indicate that in the group immunized with the virus + DBF, the increase in expression of interferons IFN-α4, IFN-α11, IFN-α12, and IFN-β begins early after immunization and can be detected at all time-points from 3 to 72 h, with a significant increase in IFN-α4 and IFN-β expression 24 h after immunization. The group immunized with the virus alone exhibited a significantly increased expression of IFN-α4 and IFN-β at 12 h post-immunization, while the group immunized with DBF showed only a non-significant increase in the expression of these genes 6 and 24 h after immunization. Type I interferons released by pDC not only prevent viral infection but also activate NK cells, myeloid dendritic cells (mDC), as well as B and T lymphocytes; and therefore participate in the regulation of both innate and adaptive elements of immunity. Adjuvant immunization extends this period during which the genes for type I interferons are activated.

Intranasal immunization with inactivated influenza virus in combination with our bacterial adjuvant increases the expression of a number of genes encoding the components of both innate and adaptive immunity. This results in an acceleration and extension of expression of genes for type I interferons, which is caused by the activation of pDC. Later mDC are also apparently activated, which are responsible for the expression of TLR3. Adjuvant immunization also supports the development of type Th1 immune response, which is indispensable for the development of cellular immunity, playing an important role in antiviral defense. The bacterial adjuvant supports the expression of inflammation mediators that can also positively affect antigen presentation and a specific immune response.

Our study represents a screening of the expression of a large number of genes at a number of time-points after IN immunization. The products of these genes participate in the activation of both innate and adaptive immunity.

Acknowledgements

This work was supported by the Ministry of Education, Youth, and Sports of the Czech Republic, grant MSM0021620806 and by the Grant Agency of the Czech Republic, grant 310/07/0675.

References

[1] Liew FY, Russell SM, Appleyard G, Brand CM, Beale J. Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity. Eur J Immunol 1984;14(April (4)):350–6.
[2] Waldman RH, Wigley FM, Small Jr PA. Specificity of respiratory secretion antibody against influenza virus. J Immunol 1970;105(December (6)):1477–83.
[3] Hoffmann E, Mahmood K, Chen Z, et al. Multiple gene segments control the temperature sensitivity and attenuation phenotypes of ca B/Ann Arbor/1/66. J Virol 2005;79(September (17)):11014–21.
[4] Hunter RL. Overview of vaccine adjuvants: present and future. Vaccine 2002;20(May (Suppl. 3)):S7–12.
[5] Ulmer JB, Valley U, Rappuoli R. Vaccine manufacturing: challenges and solutions. Nat Biotechnol 2006;24(November (11)):1377–83.
[6] Zanvit P, Havlickova M, Tacner J, Jirkovska M, Petraskova P, Novotna O, et al. Immune response after adjuvant mucosal immunization of mice with inactivated influenza virus. Immunol Lett 2005;97(March (2)):251–9.
[7] Zanvit P, Havlickova M, Tacner J, Novotna O, Jirkovska M, Cechova D, et al. Protective and cross-protective mucosal immunization of mice by influenza virus type A with bacterial adjuvant. Immunol Lett 2008;115(January (2)):144–52.
[8] Kraal Georg. Nasal-associated lymphoid tissue. Mucosal immunology. 3rd ed. Elsevier; 2005. p. 415–22.
[9] Kuper CF, Koornstra PJ, Hameleers DM, Biewenga J, Spit BJ, Duijvestijn AM, et al. The role of nasopharyngeal lymphoid tissue. Immunol Today 1992;13(June (6)):219–24.
[10] Cesta MF. Normal structure, function, and histology of mucosa-associated lymphoid tissue. Toxicol Pathol 2006;34(5):599–608.
[11] Carroll KK, Cutts JH, Murray EG. The lipids of Listeria monocytogenes. Can J Biochem 1968;46(August (8)):899–904.
[12] Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226(May (1)):497–509.
[13] Asanuma H, Thompson AH, Iwasaki T, Sato Y, Inaba Y, Aizawa C, et al. Isolation and characterization of mouse nasal-associated lymphoid tissue. J Immunol-Methods 1997;202(March (2)):123–31.
[14] Kubista M, Sindelka R, Tichopad A, Bergkvist A, Lindh D, Forootan A. The prime technique. Real-time PCR data analysis. GIT Lab J 2007;9/10(33–35).
[15] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 2001;25:402–8.
[16] Prokesova L, Novakova M, Julak J, Mara M. Effect of *Bacillus firmus* and other sporulating aerobic microorganisms on in vitro stimulation of human lymphocytes. A comparative study. Folia Microbiol (Praha) 1994;39(6): 501–4.
[17] Prokesova L, Mlckova P, Stankova I, Chloubova A, Novotna V, Ladmanova P, et al. Effect of Bacillus firmus on antibody formation after mucosal and parenteral immunization in mice. Immunol Lett 1998;64(December (2/3)):161–6.
[18] Prokesova L, Mlckova P, Stankova I, Ladmanova P, Jezkova J, Chalupna P, et al. Immunostimulatory effect of Bacillus firmus on mouse lymphocytes. Folia Microbiol (Praha) 2002;47(2):193–7.
[19] Zidek Z, Tuckova L, Mara M, Barot-Ciorbaru R, Prokesova L, Tlaskalova-Hogenova H. Stimulation of macrophages by *Bacillus firmus*: production of nitric oxide and cytokines. Int J Immunopharmacol 1998;20(July (7)):359–68.
[20] Mlckova P, Cechova D, Chalupna P, Novotna O, Prokesova L. Enhanced systemic and mucosal antibody responses to a model protein antigen after intranasal and intratracheal immunisation using *Bacillus firmus* as an adjuvant. Immunol Lett 2001;77(May (1)):39–45.
[21] Mlckova P, Polacek M, Cechova D, Maruskova L, Stankova I, Chalupna P, et al. Intratracheal and intranasal immunization with ovalbumin conjugated with Bacillus firmus as a carrier in mice. Folia Microbiol (Praha) 2005;50(3):247–53.
[22] Kuper CF, Hameleers DM, Bruijntjes JP, van der Ven I, Biewenga J, Sminia T. Lymphoid and non-lymphoid cells in nasal-associated lymphoid tissue (NALT) in the rat. An immuno- and enzyme-histochemical study. Cell Tissue Res 1990;259(February (2)):371–7.
[23] Hiroi T, Iwatani K, Iijima H, Kodama S, Yanagita M, Kiyono H. Nasal immune system: distinctive Th0 and Th1/Th2 type environments in murine nasal-associated lymphoid tissues and nasal passage, respectively. Eur J Immunol 1998;28(October (10)):3346–53.
[24] Yanagita M, Hiroi T, Kitagaki N, Hamada S, Ito HO, Shimauchi H, et al. Nasopharyngeal associated lymphoreticular tissue (NALT) immunity: fimbriae-specific Th1 and Th2 cell regulated IgA responses for the inhibition of bacterial attachment to epithelial cells and subsequent inflammatory cytokine production. J Immunol 1999;162(March (6)):3559–65.
[25] Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004;4(July (7)):499–511.
[26] Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 2004;303(March (5663)):1529–31.
[27] Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000;408(December (6813)):740–5.
[28] Uematsu S, Akira S. Toll-like receptors and Type I interferons. J Biol Chem 2007;282(May (21)):15319–23.
[29] Van Pesch V, Lanaya H, Renauld JC, Michiels T. Characterization of the murine alpha interferon gene family. J Virol 2004;78(August (15)):8219–28.
[30] Van HM, Bosveld IJ, Mooren AA, Trapman J, Zwarthoff EC. Properties of natural and hybrid murine alpha interferons. J Gen Virol 1986;67(October (Pt 10)):2215–22.
[31] Van HM, Bosveld IJ, Klaassen P, Zwarthoff EC, Trapman J. Structure-function analysis of murine interferon-alpha: antiviral properties of novel hybrid interferons. J Interferon Res 1988;8(February (1)):5–14.
[32] Marie I, Durbin JE, Levy DE. Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. EMBO J 1998;17(November (22)):6660–9.
[33] Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, et al. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. Immunity 2000;13(October (4)):539–48.