Comparison of different sugars for their immunostimulating capacities on influenza vaccines in vitro and in vivo

Master project conducted at the department of Medical Microbiology, Molecular Virology section, UMCG Groningen

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Contents

Abstract

In this study we assessed innate immune signaling and *in vivo* immune responses triggered by a spray freeze-dried whole inactivated influenza vaccine. Vaccines were prepared using the stabilizer sugars inulin, dextran or a dextran/trehalose mixture. Incubation of these formulations with RAW-blue cells and a panel of receptor-specific HEK-blue cell lines revealed that inulin was able to activate NFκB through the selective interaction with toll-like receptor 2, 4 and 5. However, only low responses were seen after the addition of an endotoxin inhibitor suggesting that the observed effects were mainly caused by contamination of our samples. Immune responses following pulmonary immunization of BALB/c mice were analysed by measuring different subtypes of immunoglobulins, amounts of antibody-secreting cells and amounts of cytokine-producing CD4+ cells. None of the formulations tested induced responses that were significantly higher than those induced by liquid WIV control indicating that the strong *in vitro* responses that were observed from inulin do not translate into *in vivo* adjuvanticity. In addition, immunization with any of the formulations resulted in immune responses with a strong Th2 bias as indicated by high amounts of IL-4 secreting cells and serum IgG1. Together, these data emphasize an underlying need for further research on adjuvanted pulmonary influenza vaccines in order to develop vaccines which are more potent and give more balanced responses.

Introduction

Yearly occurring influenza epidemics and occasional pandemics form a substantial public health burden. Infection with influenza virus causes respiratory disease with high morbidity and mortality. Especially individuals in risk groups like infants, elderly and pregnant women are at higher risk to get infected and often suffer from more severe disease and associated complications[1, 2]. Although exact numbers are uncertain, it is estimated that the yearly worldwide death toll from influenza epidemics lies around 500 000[3]. In the case of a pandemic with a highly virulent strain such as the H5N1 subtype these numbers may even be significantly higher.

Vaccination is regarded as the most cost-effective prophylactic measure to reduce morbidity and mortality for many infectious diseases including influenza[4]. In 2003 the World Health Organization set the objective to reach a vaccination coverage of the elderly population and other risk groups of at least 75%[5]. Presently most developed countries have vaccination programs for influenza. Still, coverage in many of these countries is below optimal[6-8]. Moreover, many countries in developing regions such as Africa and south-east Asia do not provide routine vaccination and coverage in these countries is poor. Based on a survey performed by the WHO it is estimated that during a pandemic outbreak only 13% of the global population would be targeted for vaccination[9]. Therefore the development of stable and affordable vaccine formulations that allow efficient production and global distribution is crucial.

Current available seasonal influenza vaccines can be divided into live attenuated influenza virus vaccines (LAIV) and trivalent inactivated vaccines (TIV). Both types have in common that they contain the expected antigenic variants of one influenza A/H1N1 strain, one influenza A/H3N2 strain, and one strain of influenza B[10]. Inactivated vaccines can be either composed as live whole inactivated virus (WIV), split virus, subunit or as the relatively novel virosomal vaccines[11]. In the last decades, subunit and split formulations have been the main focus of research due to a high incidence of side effects associated with WIV. However,

advances in production and purification technology led to the development of WIV vaccines with improved safety and efficacy[12, 13]. Recent studies comparing WIV and subunit formulations suggest that WIV can elicit superior immune responses in terms of magnitude and quality making it an attractive candidate for future vaccines[14-16]. In addition, WIV vaccines might confer a certain degree of protection against heterosubtypic infection by targeting conserved internal antigens[17].

To boost vaccine immunogenicity, many vaccines rely on stimuli of the innate immune system to mimic molecular danger signals associated with infection. This phenomenon is called pattern recognition and is mediated by a repertoire of pattern recognition receptors (PRRs) expressed on innate immune cells[18]. In this way, the recipient immune system is instructed to develop a pro-inflammatory response towards the vaccine antigens. Usually this is achieved by the addition of an adjuvant such as Freund's adjuvant or the more recently developed Toll-like receptor (TLR) ligands. These adjuvants exert an effect on innate immune cells by promoting factors like phagocytosis and microbial killing, antigen processing and presentation, cytokine secretion and expression of costimulatory molecules[18, 19]. This may eventually facilitate Th1/Th2 skewing and improve memory responses[20]. Some vaccine formulations also carry inherent stimuli of innate immunity and are therefore considered to be less dependent on the addition of an adjuvant. For instance, whole inactivated influenza vaccines contain viral RNA which may serve as intrinsic adjuvant by stimulation of TLR-7[15].

At present, most of the inactivated influenza vaccine formulations are administered by intramuscular (i.m.) injection. Because i.m. injection does not resemble the natural route of infection, these vaccines only induce systemic immune responses whereas local immune responses in the nose and lung mucosa are absent[21]. The requirement for administration by trained medical staff, risk of needle stick injuries and hepatitis infection, needle phobia and pain at the injection site are other drawbacks of i.m. administration[22]. Finally, these liquid dosage forms require storage at a narrow temperature range between 4-8°C. Stresses caused by freezing/thawing or elevated temperature can rapidly lead to loss of antigen integrity thereby reducing immunogenicity of the vaccine[23]. Since these storage conditions require elaborate transport and storage facilities (cold chain), this puts pressure on distribution in underdeveloped countries and vaccine stockpiling[24].

To overcome the limitations of i.m. immunization, a number of alternatives have been suggested including mucosal vaccine delivery. Targeting the host mucosa is one of the main goals in the design of novel vaccine strategies because of its high immunocompetence[21, 25]. This can be attributed to the large surface area which allows efficient antigen delivery and the presence of professional antigen presenting cells associated with the mucosal epithelium. In the context of influenza, mucosal immunization is of special interest because it may have the capacity to raise neutralizing antibodies at the port of entry for infection. This concept is exploited by the current intranasal LAIV vaccines[26]. However, the dosage forms used for this approach do not circumvent the problem of cold chain dependence. In addition, the application of these vaccines is restricted to the upper airways which have a relatively small surface area as opposed to the lower respiratory tract. Work in our group has therefore focussed on the development of heat stable dry powder vaccines for pulmonary administration[11]. These vaccines can be inhaled with relative ease with the use of simple devises without the requirement of medical personnel. Moreover, they have the advantage of being cold chain independent and therefore easier to store and distribute.

Lyophilisation techniques to create dry powder vaccines include freeze-drying, spray drying and spray-freeze-drying which differ in production procedure, physical particle properties and costs. Especially spray-freeze-drying (SFD) conveys considerable potential for influenza immunization due to formation of highly porous particles with favourable aerodynamic sizes, good deposition properties, and excellent antigen conservation under a wide temperature range[27, 28]. These properties allow delivery of structurally intact antigens to the proper sites of the respiratory tract, a feature which is believed to be essential for the efficacy of pulmonary vaccines[29]. Consequently, pulmonary SFD influenza vaccines have been found to induce a broad spectrum of immune responses including mucosal IgA secretion, even when these vaccines are stored at elevated temperatures for long periods of time[28].

During SFD, sugars are used as cryoprotectant to prevent loss of virus antigenicity from freezing and drying stress during lyophilisation and to provide stability to the dry product. By the rapid freezing of liquid vaccine droplets containing these sugars, an amorphous glassy matrix is formed surrounding the viral antigens. This matrix provides a physical barrier and reduces molecular mobility thus preventing structural changes and degradation. In addition, the excipient sugars provide stability by replacing the hydrogen bonds from the solvent to the antigens with their own free OH-groups. Sugars that are commonly applied in SFD are selected for their glass transition temperature (Tg) which should be well above storage temperature. In this way, the glassy state of the particle matrix is maintained during storage. These sugars include flexible oligomers (e.g. inulin), more sturdy oligomers (e.g. dextran) or mixtures of sturdy oligomers with disaccharides (e.g. a dextran/trehalose mixture). Strikingly, the possible immunogenic properties of these different stabilizer sugars in the context of vaccination have scarcely been studied.

The aim of this study was to identify the ideal sugar for stabilization of influenza WIV for pulmonary vaccination. To achieve this, we evaluated the immunogenicity of unprocessed WIV and three different spray freeze-dried WIV vaccine formulations containing the stabilizer sugars inulin, dextran or a dextran/trehalose mixture. In addition, we addressed the question whether stabilizer sugars are able to trigger innate immune responses and could thereby act as an adjuvant. To determine the effect of the spray-freeze drying process and possible synergism with whole inactivated virus on these responses, we compared the effects of unprocessed sugars, spray-freeze dried sugars and spray-freeze dried WIV vaccines.

In order to characterize the interaction between the different formulations and PRRs on innate immune cells, studies were performed on RAW-blue cells and a panel of different HEK-blue cell lines. RAW-blue cells are murine derived RAW 264.7 macrophages that carry a secreted embryonic alkaline phosphatase (SEAP) reporter construct inducible by NF-κB and AP-1, two major transcription factors downstream of the PRRs expressed by these cells[30]. Because these cells naturally express a wide array of pattern recognition PRRs, binding of a ligand to any of these receptors will lead to SEAP secretion in the culture medium which can be measured by a substrate reaction. In contrast, HEK-blue cells are based on the human HEK293 kidney cell line which does not express pattern recognition receptors. To monitor the involvement of different receptors on an individual level, a panel of PRR specific HEK-blue cell lines was used. Each of these cell lines is co-transfected with the SEAP reporter construct and only one type of receptor. Finally, the immunogenic properties of the vaccines were further evaluated by pulmonary immunizations in mice. The quantity and quality of the immune responses raised against WIV were determined by measuring different subtypes of immunoglobulins, amounts of antibody-secreting cells and amounts of cytokine-producing CD4+ cells.

Materials and methods

Vaccines

H3N2 virus A/Hiroshima was provided by Solvay (Weesp, The Netherlands). Whole inactivated virus (WIV) was obtained by 24 hours of incubation with 0.1% β-propiolactone (BPL) in HEPES buffered saline (HBS, 2 mM Hepes, 150 mM NaCl, pH 7.4) under continuous rotation at room temperature. In order to remove residual BPL and sucrose from the solution, the virus was dialysed against HBS overnight at 4°C in a 10K MWCO Slide-A-Lyzer cassette (Thermo Scientific). Total protein content was determined by Micro-Lowry as described before[*]. The HA concentration of the solution was taken as one third of the total protein content.

Subunit vaccine was prepared by solubilizing inactivated virus for 3 h under continuous rotation in 0.3 mg/ml Tween 80, 1.5 mg/ml hexadecyltrimethylammonium bromide (CTAB) in phosphate-buffered saline (PBS, pH 7.4, Gibco)[*]. The viral nucleocapsid was removed from the preparation by ultracentrifugation at 50K RPM for 30 minutes. Detergents were removed by overnight absorption onto 0,5g Biobeads SM2 (Bio-Rad, Hercules, CA) per ml of solution. The composition of WIV and subunit material was validated by SDS-PAGE on a 12,5% gel. Bands were visualized by silver staining following standard protocol[*].

For spray-freeze drying, 5% sugar solutions were made in HBS from either inulin (4 Kd, Sensus, Roosendal, The Netherlands), dextran (6kD, Sigma-Aldrich, Zwijndrecht, The Netherlands) or a 1:1 mixture of dextran and trehalose (Cargill, Kerfeld, Germany). For preparation of the SFD WIV vaccines, whole inactivated A/Hiroshima was added in a 1:200 ratio (HA:sugar). Solutions were sprayed into a vessel of liquid nitrogen with a 0.5mm nozzle on a Büchi 190 mini spray dryer. The nozzle was placed approximately 5 cm above the surface of the liquid nitrogen and the solutions were atomized with an air flow of 700 ln/h. After spraying was completed the tanks containing the powders were placed in a Christ model Epsilon 2-4 freeze-dryer (Salm & Kipp, Breukelen, The Netherlands) pre-cooled at shelf temperature of -40°C. After liquid nitrogen was evaporated, drying was performed at a pressure of 0.220 mbar with a condenser temperature of -85°C while the shelf temperature was gradually increased from -40 to 5°C over a time period of 32 hours. Next, the pressure was reduced to 0.055 mbar and the shelf temperature was further gradually increased to 20°C over a time period of 11 hours. The obtained powders were stored at room temperature in a vacuum dessicator containing silica gel until further use.

Cell lines

Murine RAW 264.7 macrophages expressing all TLRs (with the exception of TLR5) as well as RIG-I, MDA-5, NOD1 and NOD2 and a panel of human embryonic kidney cell lines (HEK293) each transfected with a different human PRRs (TLR2, 3, 4, 5, 7, 8, 9, NOD-1, NOD-2) were purchased from InvivoGen. All cell lines expressed a secreted embryonic alkaline phosphatase (SEAP) reporter construct inducible by NF-κB and AP-1, the two major transcription factors downstream of PRRs. Hence, activation of the expressed PRRs results in

[*] Protocols are provided in appendix

secretion of SEAP into the culture medium which is readily detectable using a colorimetric assay. HEK cells expressing the reporter construct but lacking a pattern recognition receptor were used as control for receptor-independent activation (Null-1 and Null-2). Cells were cultured in Dubecco's Modified Eagle's Media (Gibco) supplemented with 2 mM L-glutamine, 4.5 g/L glucose, 50 units/ml penicillin, 50 µg/ml streptomycin and 10% FBS. Different selective antibiotics were used depending on the cell line as specified by the manufacturer. Cultures were kept in an incubator at 37 ^{0}C and 5% CO_2 and were passaged twice a week at a 1:4 split ratio when a confluency of ~70% was reached. Prior to stimulation, cells were counted using a Neubauer counting chamber and diluted to a final concentration of 10^6 cells/ml.

Determination of NFκB activion

For the determination of NFκB activation in RAW-blue and HEK-blue cells, incubation experiments were performed with non-spray freeze-dried sugars, spray freeze dried sugars, spray freeze dried WIV vaccines and liquid WIV. LPS, MDP, IeDAP or TNF-α (InvivoGen) were used as positive controls. To ensure proper dissolution of the sugars and SFD vaccines, 2% pre-dilutions were made in pre-warmed culture medium. Next, serial two-fold dilutions were made in 96-well plates (Greiner, Alphen aan de Rijn, The Netherlands) in triplicate in culture medium until a final concentration of 1/128th of the starting concentration was reached. Finally, 10^5 cells were added in 100ul to each well and cultures were incubated overnight in an incubator at 37 ^{0}C and 5% CO_2. Measurement of SEAP activity using the Quanti-blue substrate (InvivoGen) was carried out according to the protocol provided by the manufacturer. Briefly, 40µl of culture supernatant was transferred into new 96-well plates and 160µl of substrate solution was added to each well. After approximately 30 minutes of incubation at 37°C absorbance was read at 650 nm using an ELISA reader (Bio-tek instruments, Inc., Vermont, U.S.A.).

Endotoxin inhibition

To assess the potential effect of endotoxin contamination of the inulin samples, RAW-blue cells were cultured in the presence of polymyxin-B (PMX) (InvivoGen). PMX is endotoxin neutralizing substance which binds directly to the lipid A portion of bacterial lipopolysaccharides thus neutralizing their biological activities. In addition, ultrapure inulin (Sigma) was used which was guaranteed to be free of contaminants. First, non-spray freeze-dried sugars and LPS control were serially diluted in 96-well plates as described above. Next 50 µg of PMX was added to the proper wells and preïncubated for 30 minutes to achieve optimal neutralization. Finally 10^5 cells were added to each well and kept overnight and NFκB activation was determined using the Quanti-blue assay as before.

In vivo immunizations

Animal experiments were conducted according to the guidelines provided by the Dutch Animal Protection Act and were approved by the Committee for Animal Experimentation of the University of Groningen, The Netherlands. Female Balb/C mice were obtained from Harlan (Zeist, the Netherlands). Freshly prepared vaccines containing 5µg of HA were administered intrapulmonary into anaesthetized and intubated mice 0 and 21. Powder vaccines were administered using a Penn Insufflator (Penn-Century inc, Wyndmoor, USA) as

described by Amorij et. al.[28]. Liquid WIV and HBS control was given by aerosolization with the aid of a Penn Microsprayer (Penn-Century inc, Wyndmoor, USA).

One week after the second immunization (day 28), animals were sacrificed by heart puncture under isoflurane anaesthesia. During heart puncture, blood was collected for determination of serum antibody responses. Immediately after sacrifice, lungs were perfused and nasal and bronchial alveolar lavages (BAL) were performed using a 20G Insyte Autoguard catheter (Becton Dickinson BV, Alphen aan de Rijn , The Netherlands) connected to a 1 ml syringe. Nasal lavage fluids were obtained by flushing twice with the same 1 ml PBS through the nasopharynx. Lung lavages were performed by repeated flushing of the lungs with the same 1 ml of PBS. Next, the lungs and spleens were harvested and stored in ice-cold IMDM Glutamax medium (Gibco) supplemented with 50 units/ml penicillin, 50 µg/ml streptomycin and 10% FBS. All samples were kept on ice until further analysis.

Haemagglutination inhibition assay

The haemagglutination assay was performed as described previously*. In brief, test sera were inactivated at 56°C for 30 minutes. Subsequently, sera were mixed 1:3 with a 25% kaolin stock and kept at room temperature for 20 minutes. After incubation, sera were spun down at 1200x g for 2 minutes and supernatants were serially diluted in 50µl in 96-well plates. Fifty µl containing 4 haemagglutination units of influenza virus was added to each well and incubated for 40 minutes. Finally, 50µl 1% guinea pig erythrocytes was added to each well and haemagglutination was allowed to occur for 1-2 hours. The haemagglutination inhibition (HAI) titers are defined as the highest dilution capable of preventing haemagglutination.

ELISA

Influenza HA-specific antibody responses were determined by ELISA as described before*. Briefly, ELISA plates were coated overnight with 500 ng subunit antigen per well. Twofold serial dilutions of sera, and mucosal lavage fluids were made in duplicate in PBS containing 0.05% Tween 20 and incubated for 1.5 h. Sera were analysed for IgG, IgG1 and IgG2a while lavages were analysed for IgG and IgA. In the case of IgG1 and IgG2a, purified antibodies with a known concentration were used for the standard curves. Antigen-specific immunoglobulins were detected by the addition of horseradisch peroxidase-conjugated goat anti-mouse antibodies (1:5000, BD biosciences). The staining was performed with substrate buffer (50 mM phosphate buffer, pH 5.5, containing 0.04% o-phenylenediamine and 0.012% H2O2) and the optical densities were measured at 492 nm using an ELISA reader (Bio-tek instruments, Inc., Vermont, U.S.A.). ^{10}Log titres are calculated as the reciprocal of the calculated sample dilution corresponding to an absorbance of 0.2. For mucosal fluids, only absorbances are shown.

ELISPOT

For quantification of immunoglobulin secreting cells from lungs and spleens, ELISPOT assays were performed. 96-well nitrocellulose filter plates (Greiner, Alphen aan de Rijn, The Netherlands) were coated overnight at 4°C with coating buffer and WIV at a concentration of 500 ng HA per well (B-cell ELISPOT) or with 500 ng/ml anti-mouse IFN-γ or anti- mouse

* Protocols are provided in appendix

IL-4 (BD biosciences) (T-cell ELISPOT). Next, wells were washed three times with PBS containing 0.02% Tween80 and blocked with 5%FBS in PBS for 30 minutes at room temperature. Erythrocyte depleted cells suspended in IMDM medium containing 10% FCS were added at a concentration of 10^5 cells per well in six-fold. Cells were incubated overnight at 37°C with or without 1μg subunit antigen per well. After overnight incubation, cells were lysed by incubating them on ice for 15 minutes and washed extensively with PBS. Biotinilated anti-mouse IFN-γ, anti-mouse IL-4 (BD biosciences) (T-cell ELISPOT) or HRP-conjugated anti-IgA together with AP-conjugated anti-IgG (BD biosciences) (B-cell ELISPOT) was added to each well in a 1:5000 dilution. IFN-γ and IL-4 spots were detected by adding streptavidin-conjugated alkaline phosphatase followed by the addition of 100μl BCIP/NBT substrate solution (Roche). IgG and IgA specific antibody secreting cells (ASC) were detected in the same wells by dual staining. First, IgA specific spots were detected using 3-Amino-9-ethylcarbazole (AEC) substrate (Sigma). After approximately 30 minutes of incubation at room temperature, plates were washed and IgG specific spots were detected using BCIP/NBT substrate (Roche). Wells were scanned using an ELISPOT reader (A.EL.VIS Elispot reader) and analysed using ImageJ software (version 1.45s). The number of positive spots was obtained by subtracting the number of spots observed in wells containing cells not stimulated with influenza antigen from the number of spots after incubation with antigen.

Statistics

All statistical analyses were performed using Graphpad Prism software (version 5.0). For analyses of the *in vitro* data, a one-way ANOVA was performed followed by post-hoc analysis using Bonferroni multiple comparison. For analysis of the *in vivo* data, homogeneity of variances was not assumed and statistics were performed by Kruskal-Wallis followed by Dunn's multiple comparison test. In all cases, $p < 0.05$ was taken as the minimum level of significance.

Results

NFκB activation in RAW-blue cells

Imunostimulatory effects of non-SFD sugars, SFD-sugars, WIV and SFD-WIV vaccines were assessed on Rawblue macrophages. LPS and medium were used as positive and negative control respectively.

Figure 1 shows absorbance levels relative to the LPS control. Among the non-spray freeze-dried sugars (non-SFD, panel A) and spray freeze-dried sugars (SFD, panel B) inulin raised strong dose-dependent effects whereas dextran and the dextran/trehalose mixture had no effect on NFκB activation. In the vaccine groups (SFD + WIV, panel C), liquid WIV, WIV spray freeze-dried in the presence of dextran (WIV dextran) and WIV spray freeze-dried in the presence of a dextran-trehalose mixture (WIV dextran/trehalose) all elicited dose dependent activation at a similar level. WIV spray freeze-dried in the presence of inulin (WIV inulin) triggered NFκB activation at a much higher level as compared to the other vaccines. Statistical comparison of the non-SFD and SFD sugars (panel D) reveals that in non-SFD form only inulin triggered significant SEAP secretion (inulin versus control) and that its potency is slightly raised by spray freeze-drying (SFD inulin versus non-SFD inulin). In contrast, dextran was only able to induce relatively moderate responses in spray freeze-dried form. When comparing SFD vaccines to liquid WIV and to SFD sugars (panel E), it becomes

evident that in all formulations WIV conveys a stimulatory effect (SFD + WIV versus SFD and SFD + WIV versus control). However, only the inulin based vaccine was able to rise above both the response elicited by the SFD-sugar without WIV and the response elicited by a similar amount of liquid WIV without the sugar suggesting an additive interaction. Taken together, these data show that inulin and WIV elicit potent NFκB activation in RAW-blue cells and act in an additive fashion when applied together.

Determination of receptor specificity

Next, we investigated the selectivity and potency of the SFD sugars and SFD-WIV vaccines to act as agonist for various pattern recognition receptors by using a panel of HEK293 cell lines each transfected with a different human PRR (TLR2, 3, 4, 5, 7, 8, 9, NOD-1 and NOD-2). Control cell lines lacking any of these receptors but expressing the SEAP reporter construct were used to rule out any nonspecific effects. TNF-α and receptor-specific agonists were used as positive controls to assess the functionality of the reporter construct. Since some formulations showed nonspecific stimulation at the highest dose of SFD-sugars (appendix, additional figure 2), the second-highest dose was taken for comparisons between groups. Among the cell lines tested, cell lines harbouring TLR2, TLR4 or TLR5 showed detectable responses upon incubation with SFD-sugars (figure 2-5).

In HEK-TLR2 cells (figure 2), SFD-inulin was the only spray freeze-dried sugar that elicited strong dose-dependent activation (panel A). Although liquid WIV did not seem to affect HEK-TLR2 cells, increased reporter activity was observed for all SFD vaccines (panel B). In addition, activation by WIV-inulin was higher than by the other two SFD vaccines. Interestingly, dextran and the dextran/trehalose mixture did not evoke any effect when applied alone but give rise to measurable responses when applied together. A similar pattern of activation by our formulations was observed in HEK-TLR4 (figure 3) cells although in this case, WIV did not have an additive effect on top of inulin. However, because of the plateau in the titration curve of the positive control, we speculate that this might be due to saturation of the response. In HEK-TLR5 cells, liquid WIV did not activate NFκB whereas low levels of NFκB activity were seen in all other groups (figure 4). In contrast to earlier observations made by Geeraeddts et al.[15] that influenza WIV has the ability to trigger TLR7, neither liquid WIV nor any of the WIV vaccines showed any reporter activation in the corresponding cell line (figure 5). In the other cell lines tested, none of the formulations tested exerted any effect (appendix, additional figures 3 - 7). From this we concluded that the strong NFκB activation observed in Rawblue cells was selectively mediated by TLR2, TLR4 and TLR5.

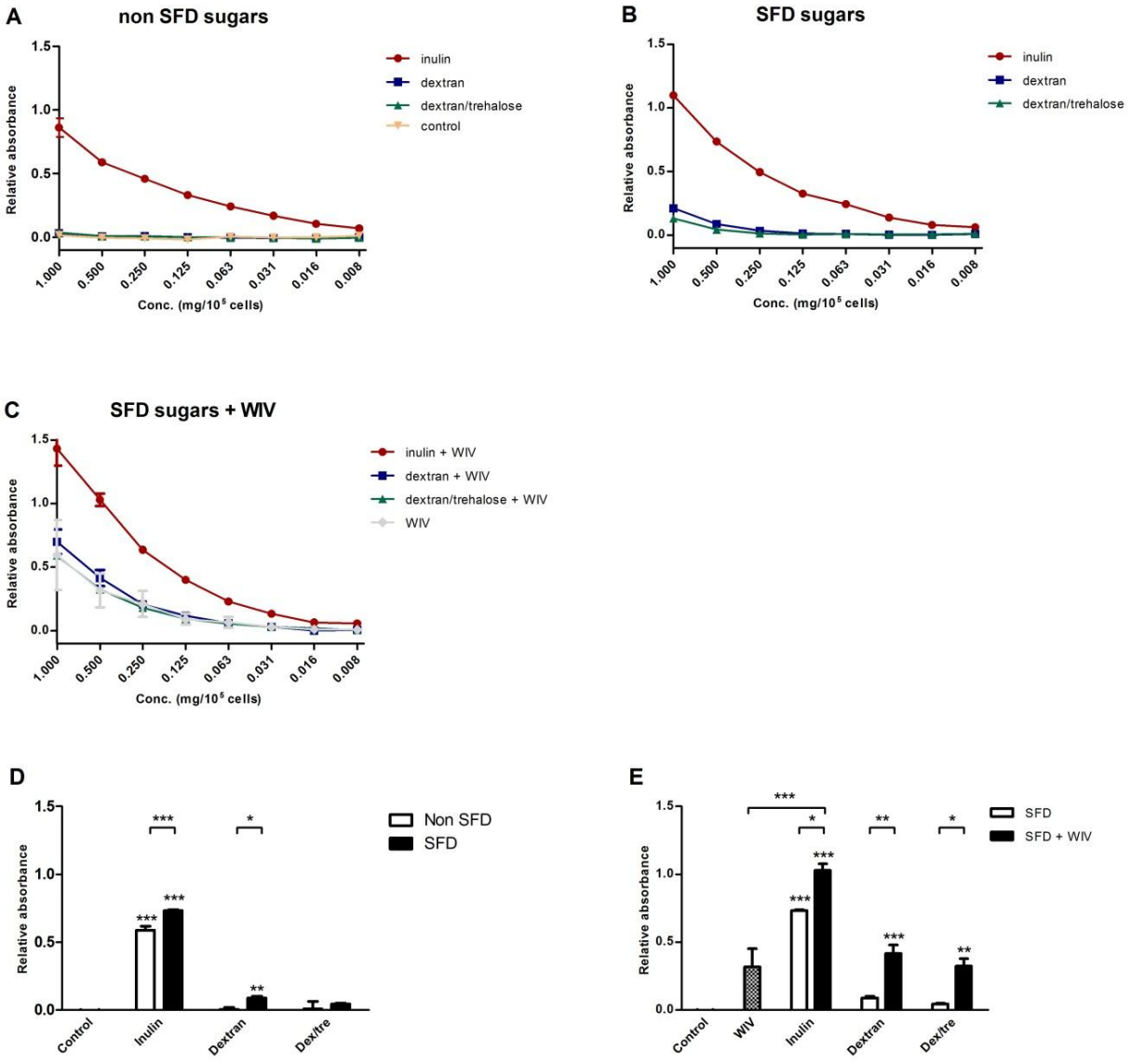

Figure 1 NFκB-activation in RAW-blue cells by different vaccine formulations. Mean absorbances relative to the LPS control are shown (n = 4 +/- SEM). Dose dependant responses to non-SFD sugars (A)**,** SFD sugars (B) and SFD vaccines (C). Panel D and E depict the effect of the spay freeze drying process and additive effects of WIV and sugars respectively by comparing responses in the second-highest concentration (0.5 mg/10^5 cells) (One way ANOVA followed by Bonferroni multiple comparison test, asterisks directly above bars indicate significance differences with medium control, * = $p < 0.05$, ** = $p < 0.01$, *** = $p > 0.001$).

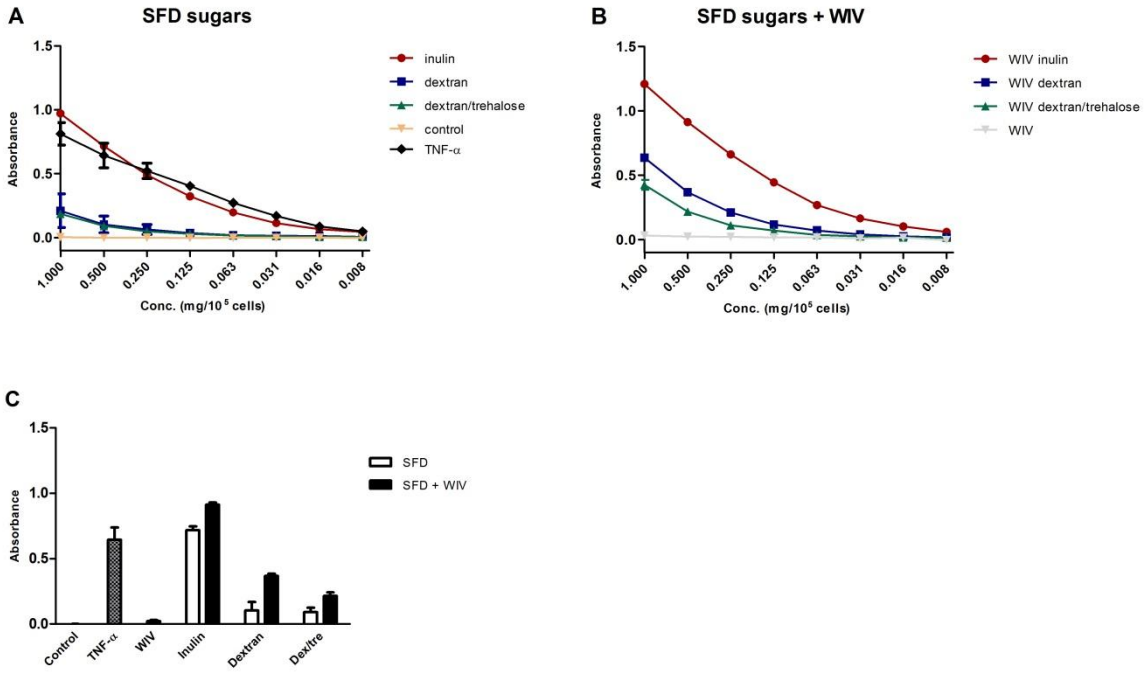

Figure 2 NFκB activation HEK-TLR2 cells. Absorbances are shown for SFD sugars (A)**,** SFD vaccines (B) and for 0.5mg/10^5 cells from all groups (D). (n = 2 +/- SEM)

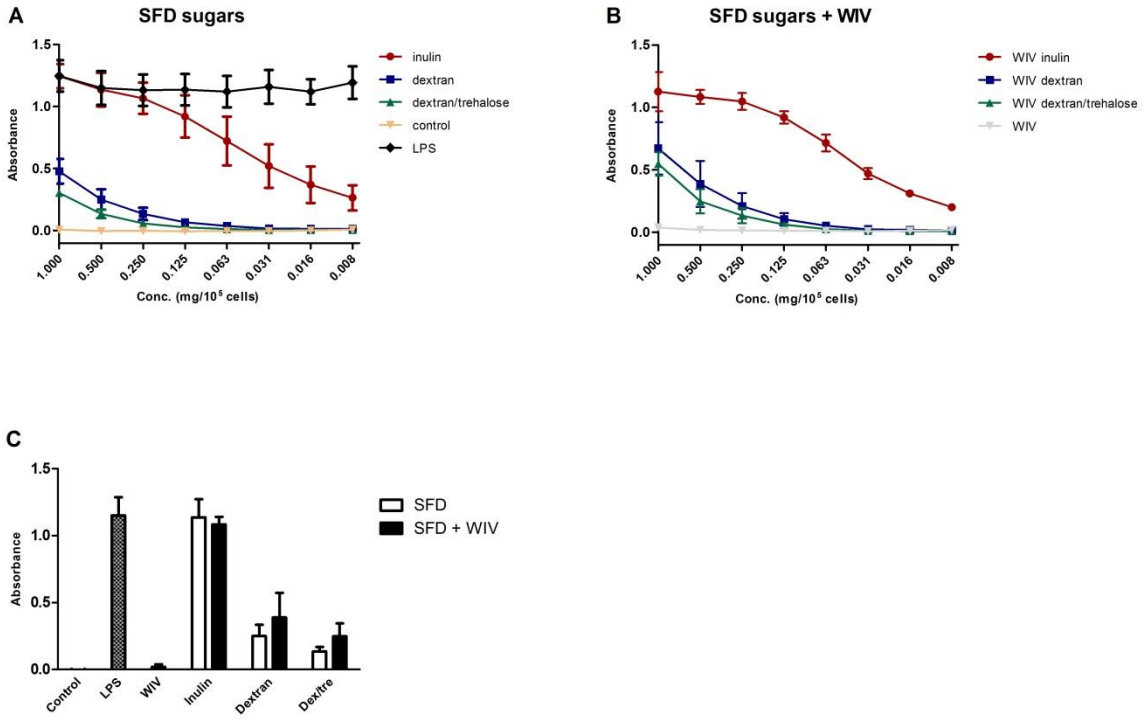

Figure 3 NFκB activation HEK-TLR4 cells. Absorbances are shown for SFD sugars (A)**,** SFD vaccines (B) and for 0.5mg/10^5 cells from all groups (D). (n = 2 +/- SEM)

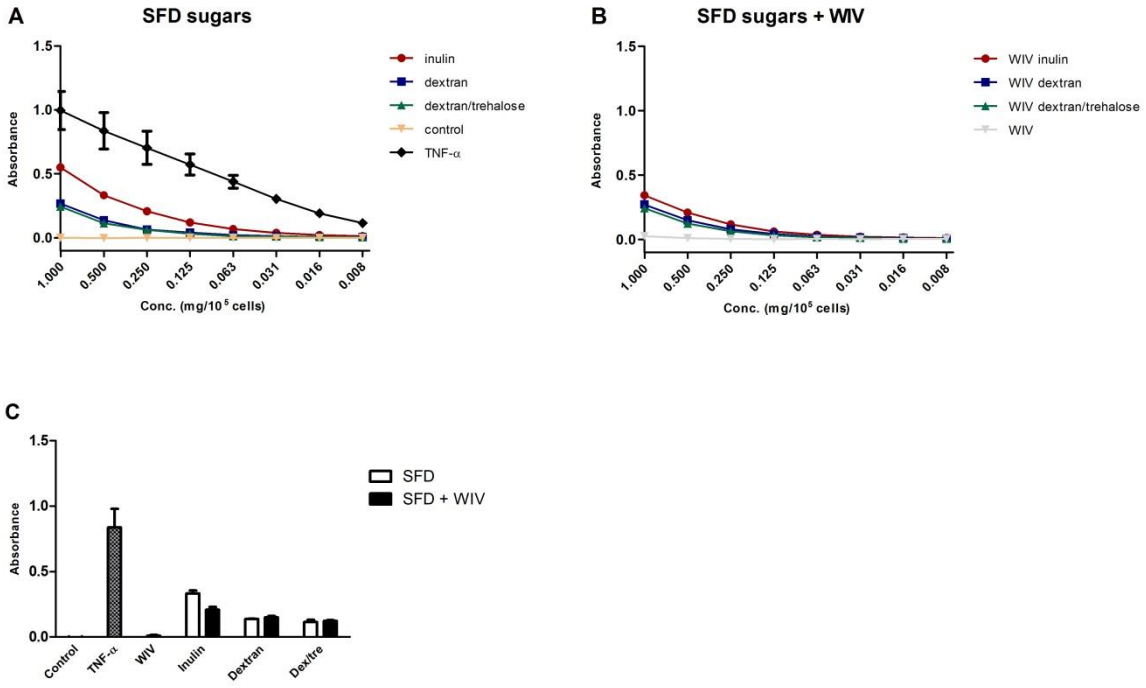

Figure 4 NFκB activation HEK-TLR5 cells. Absorbances are shown for SFD sugars (A)**,** SFD vaccines (B) and for 0.5mg/10^5 cells from all groups (D). (n = 2 +/- SEM)

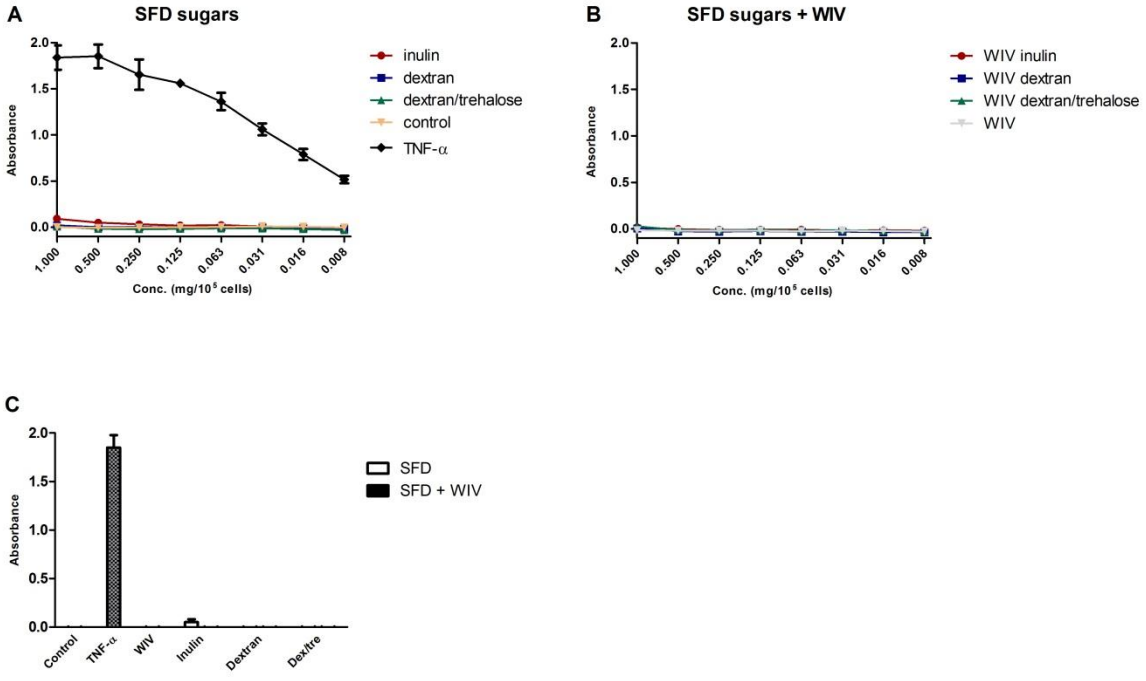

Figure 5 NFκB activation HEK-TLR7 cells. Absorbances are shown for SFD sugars (A)**,** SFD vaccines (B) and for 0.5mg/10^5 cells from all groups (D). (n = 2 +/- SEM)

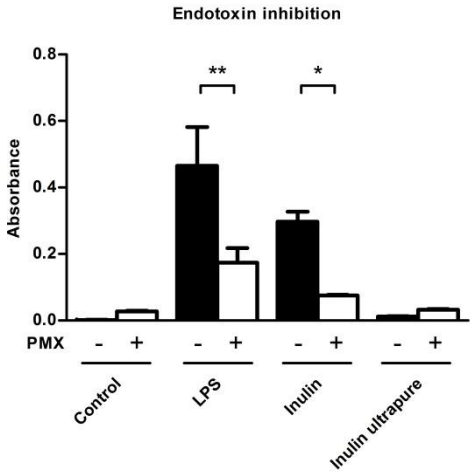

Figure 6 Effect of endotoxin inhibition on LPS stimulated and inulin stimulated RAW-blue cells. Bars represent the mean of n = 2 +/- SEM. (One way ANOVA followed by Bonferroni multiple comparison test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p > 0.001$)

Endotoxin inhibition

Next we addressed the question whether endotoxin contamination was responsible for NFκB activation by inulin in RAW-blue cells, the cell line expressing the majority of PRRs. For this purpose, inulin was tested in combination with the endotoxin inhibitor polymyxin B (PMX), a compound binding directly to the lipid A portion of bacterial lipopolysaccharides[31] (Figure 6). In addition, ultrapure inulin was used to validate the stimulatory effect of inulin observed previously. As shown by the absence of activation in the PMX positive control, PMX itself did not affect RAW-blue cells. After incubation of the cells with LPS, a high level of activation was observed which was strongly reduced when the LPS solution was pre-incubated with the endotoxin blocker thus confirming its ability to block LPS mediated activation. Likewise, inulin elicited a strong increase in NFκB activation which was significantly decreased by PMX. The endotoxin-free inulin did not elicit any response in either the PMX-free or PMX pre-incubated condition. Together these data suggest that the stimulatory effect on RAW-blue cells mediated by inulin observed earlier was mainly caused by an endotoxin component of the samples used in our experiments. The residual effect by PMX preïncubated inulin together with the observations made in TLR2 and TLR5 cells indicate that there also is a certain degree of endotoxin independent activation.

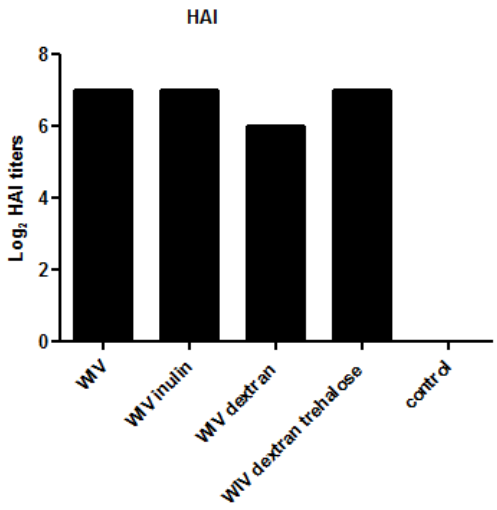

Figure 7 Log2 HAI titers induced by pulmonary immunization in mice. Spray freeze-dried whole inactivated influenza vaccines containing 5 µg of antigen were administered on day 0 and day 21. WIV and HBS controls received liquid immunizations. On day 28, mice were sacrificed and pooled sera were analysed for HAI. Data are displayed as the mean of 3 wells +/-SD..

In vivo immunogenicity of SFD vaccines

Systemic antibody responses

In order to assess immunogenicity of the different vaccine formulations and to elucidate potential *in vivo* adjuvant effects of SFD sugars, pulmonary immunizations were carried out in mice. Subjects were immunized by dry powder inhalation with SFD vaccine containing 5 µg of antigen on day 0 and 21. Positive and negative control animals received liquid WIV and HBS respectively. One week after the second immunization, animals were sacrificed and sera were analysed for HAI titer and influenza-specific immunoglobulins. As shown in figure 7, all vaccines elicited equivalent levels of HAI titers except WIV dextran which elicited a slightly lower titer.

Likewise, we observed an equal induction of antigen-specific serum IgG by WIV inulin and WIV dextran/trehalose. IgG induction by liquid WIV and WIV dextran did not differ significantly from the HBS control (figure 8). Serum IgG2a and IgG1, which are indicative for a Th1 and Th2 response respectively, were taken as a correlate for quality and balance of the immune responses raised against the vaccines. All vaccines were able to induce antigen-specific IgG2a and IgG1. However, the amount of serum IgG2a and IgG1 differed greatly. The relative abundance of antigen-specific IgG1 reveals that pulmonary vaccination with any of these vaccines resulted in an immune response which was very strongly skewed towards a Th2 phenotype. Put together, none of the SFD vaccines raised immune responses that were superior in terms of magnitude and/or quality to either control or other vaccines tested suggesting that none of the sugars had an adjuvant effect.

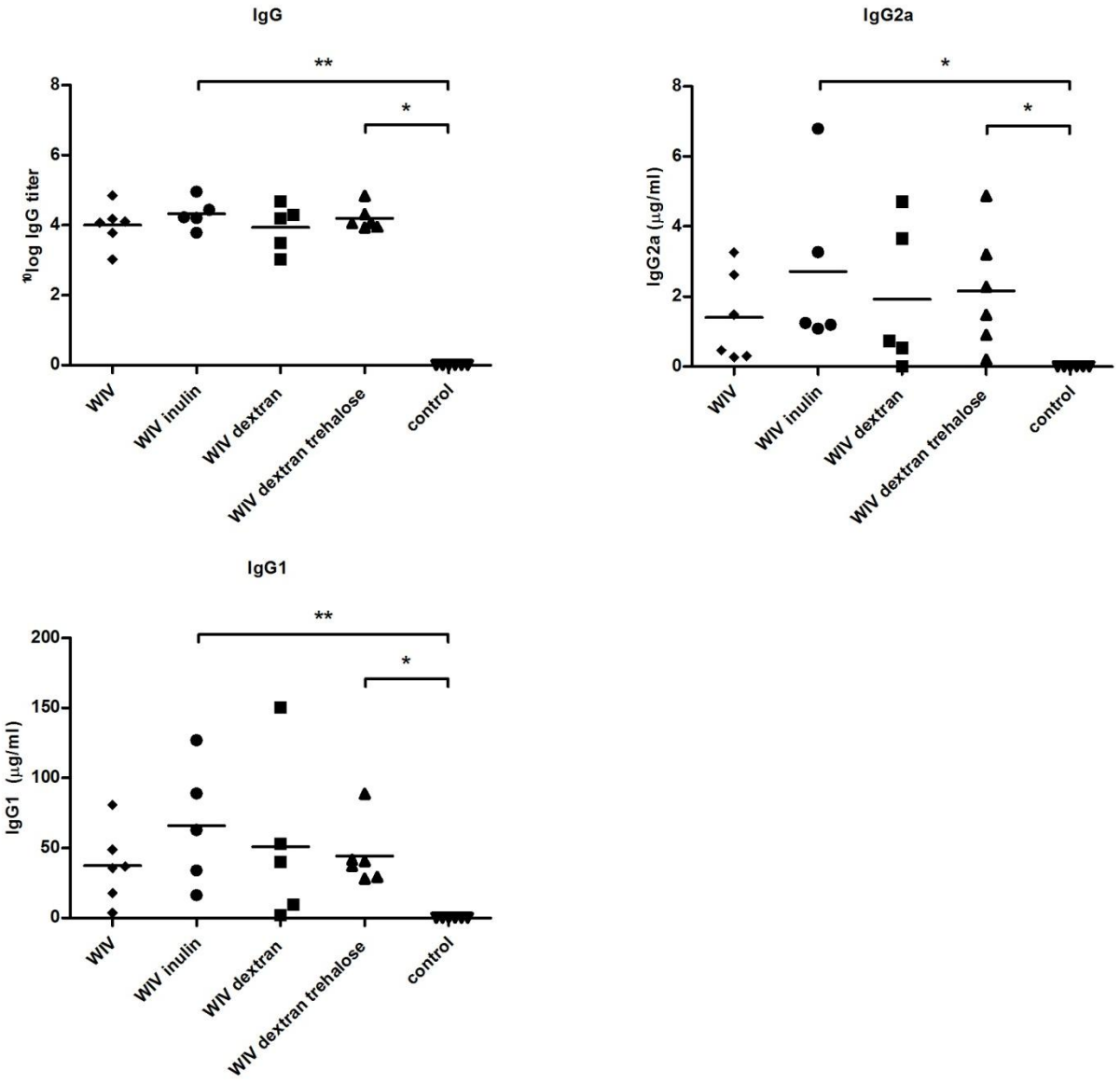

Figure 8 Serum immunoglobulin levels induced by pulmonary immunization in mice. (n = 6) (Kruskal-Wallis followed by Dunn's multiple comparison test, * = $p < 0.05$, ** = $p < 0.01$)

Mucosal immune responses

In order to compare mucosal immune responses induced by the different vaccine formulations, antigen-specific IgG and IgA ELISA's were performed on mucosal lavage samples taken from immunized mice. After immunization with WIV inulin or WIV dextran/trehalose, mice produced higher levels of lung (BAL) and nose IgA than mice which received HBS control (figure 9). In addition, the lung and nose IgA titers of mice immunized with liquid WIV or WIV dextran were not significantly higher than the HBS control group indicating inferior induction of mucosal IgA by these two vaccines. In general it seems that mucosal responses induced by the SFD vaccines were higher than those raised by liquid WIV although these differences are not significant and the method of administration (dry powder inhalation versus liquid aerosolization) might have affected this outcome. Observed differences were less pronounced in the IgG levels found in lung washes.

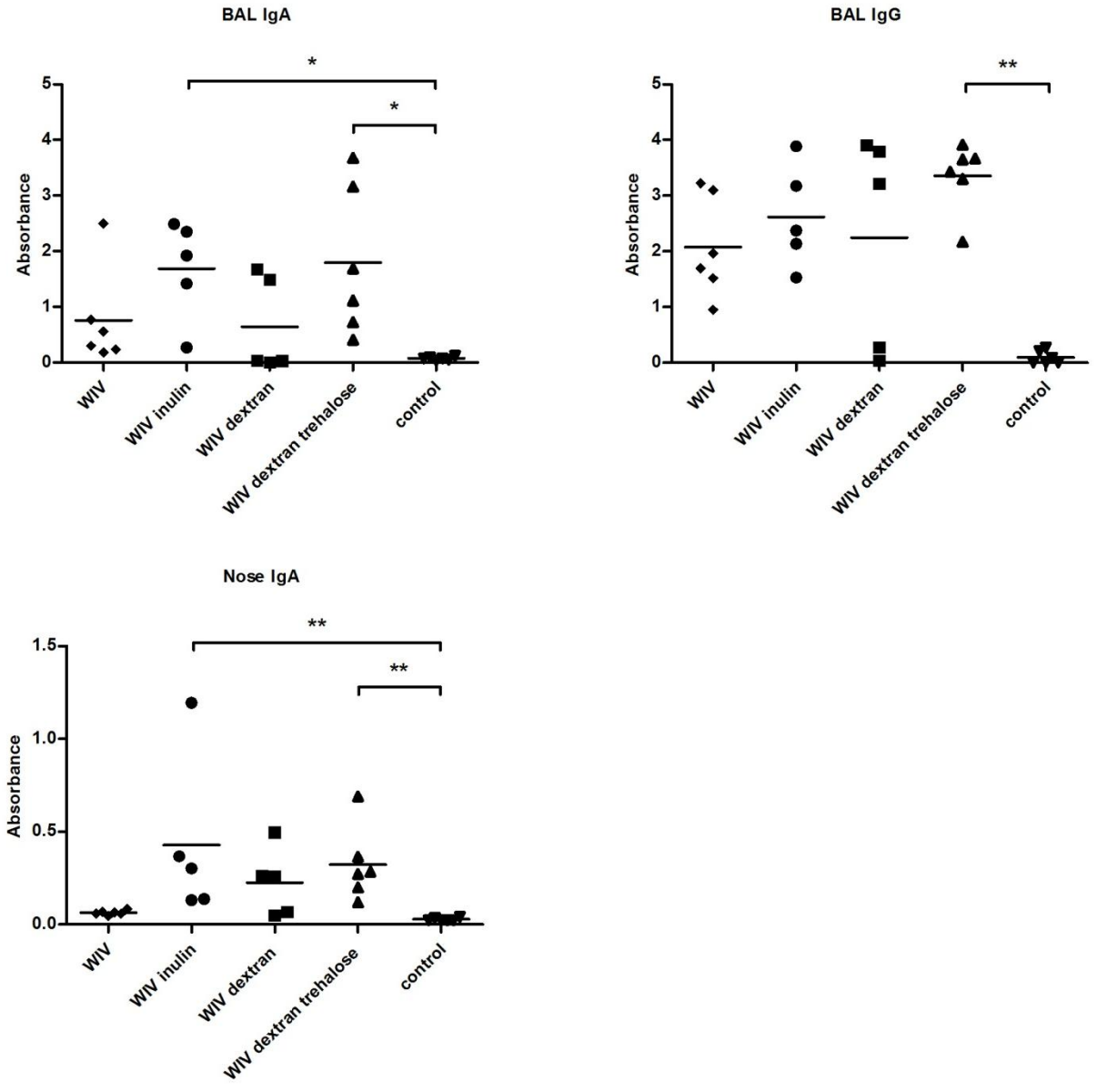

Figure 9 BAL and nose immunoglobulin levels induced by pulmonary immunization in mice. (n = 6) (Kruskal-Wallis followed by Dunn's multiple comparison test, * = $p < 0.05$, ** = $p < 0.01$)

Antibody-secreting cells

Next, the frequency of IgA and IgG secreting B-cells in the spleens and lungs of immunized mice was determined by ELISPOT. None of the immunizations resulted in formation of detectable amounts of antigen-specific IgA secreting cells within splenocytes (figure 10). In contrast, equal quantities of IgG secreting B-cells were found in spleens of mice immunized with any of the SFD vaccines whereas responses to liquid WIV did not differ significantly from the HBS control.

In the lungs, WIV inulin and WIV dextran/trehalose induced similar amounts of IgA secreting cells whereas WIV dextran failed to elicit such a response. High quantities of IgG secreting cells were found in mice immunized with either WIV inulin or WIV dextran/trehalose whereas responses in the WIV dextran group were again absent. Moreover, vaccination with liquid WIV did not result in formation of IgA and IgG producing cells at a statistically significant level matching our observations made in serum immunoglobulin levels.

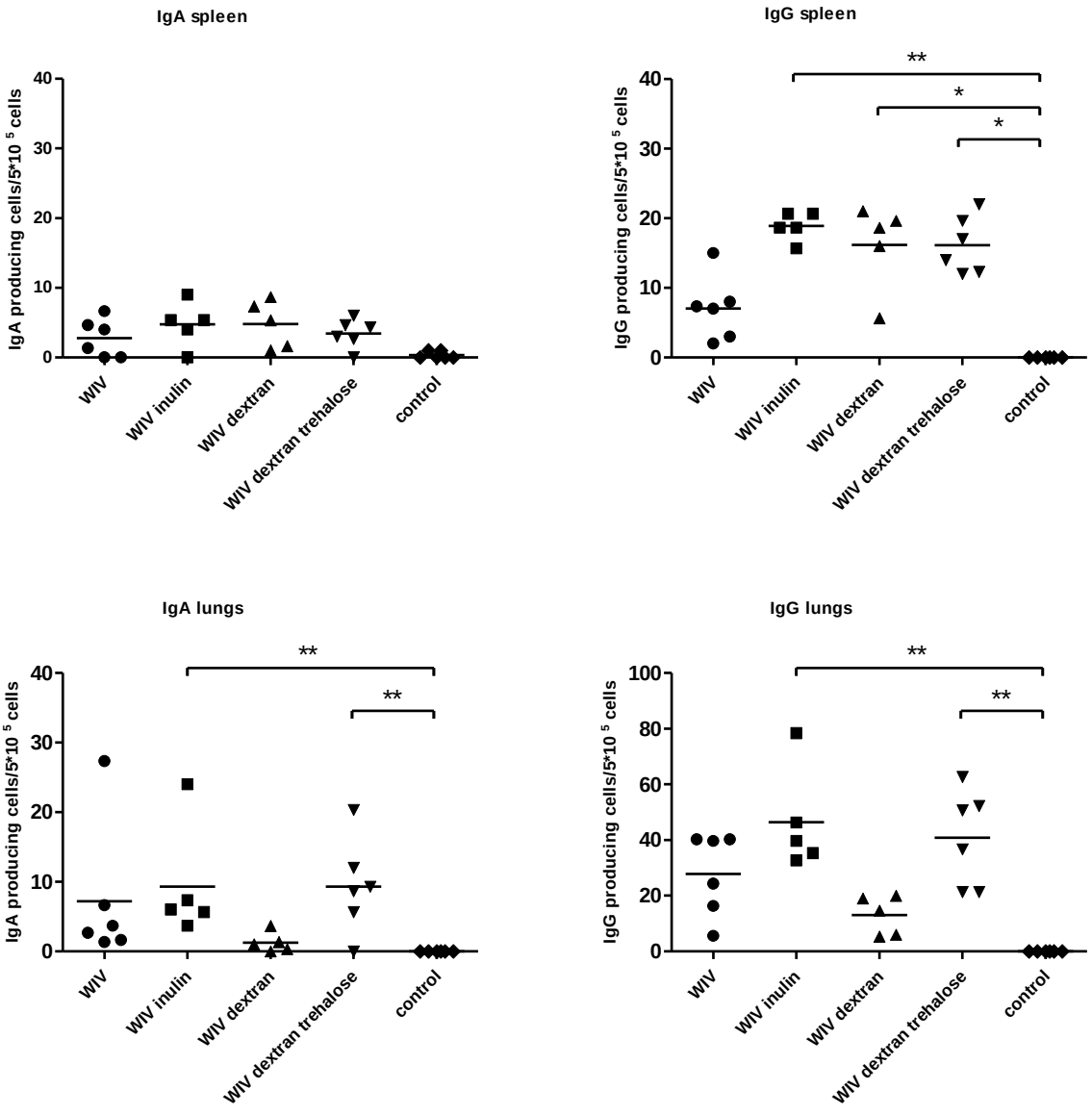

Figure 10 Enumeration of influenza-specific immunoglobulin secreting cells collected from spleen and lung homogenates of pulmonary immunized mice. (n = 6) (Kruskal-Wallis followed by Dunn's multiple comparison test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p > 0.001$).

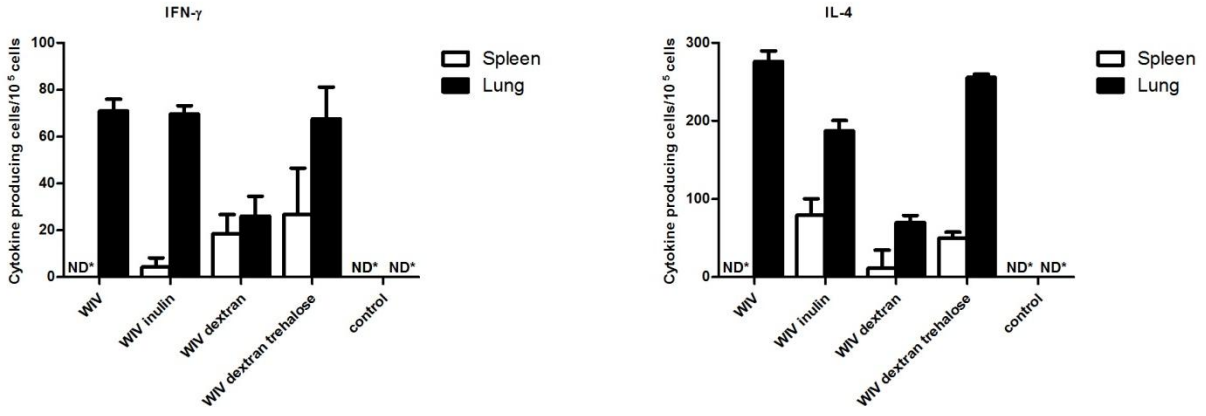

Figure 11 Enumeration of influenza-specific IL-4 (white bars) and IFN-γ (black bars) secreting cells in pooled spleen cells (left panel) or lung cells (right panel). Data are displayed as the mean of 4 wells +/-SD**.**

Cellular immune responses

Influenza-specific IL-4 and IFN-γ T-cells were enumerated in spleen and lungs using ELISPOT analysis. Because of insufficient material this assay was performed on pooled samples. Following immunization, intermediate amounts of IFN-γ producing spleen cells were found after immunization with WIV dextran or WIV dextran/trehalose (Figure 11). In contrast, WIV inulin and WIV dextran/trehalose triggered much higher formation of IL-4 secreting spleen cells. In the lungs of immunized mice, all vaccines except WIV dextran induced formation of IFN-γ producing cells as well as substantial amounts of IL-4 producing cells. In summary, our data from the ELISPOT assays indicate inferior induction of B-cell and T-cell immunity by WIV dextran and an overall tendency towards development of a Th2 dominant response as indicated by high amounts of IL-4 secreting cells and serum IgG1.

Discussion

In the present study we investigated the role of stabilizing sugars used for spray freeze-drying of the vaccines in the initiation of immunity. We found that in an in vitro system inulin could potently activate innate immunity through several pattern recognition receptors (PRR's).

In general, many carbohydrates are known to be stimulators of innate responses. Especially (but not exclusively) bacterial derived sugars and carbohydrate structures such as β-glucans and peptidoglycans strongly initiate signalling by PRR's in host immune cells[32, 33]. Receptors which have been described to recognize sugars include Dectin-1, a number of toll-like receptors (TLR's) and NOD-like receptors (NLR's)[18, 32, 34, 35]. The critical role of carbohydrates in immune system function together with their strong safety and tolerability record make them promising candidates for novel vaccine adjuvants[36].

Interestingly, there are a number of studies that implicate a role for inulin in immune induction. First, Koo et. al. reported that inulin is able to induce NFκB activation in interferon-γ primed RAW 264.7 cells[37]. Second, a number of inulin derivatives are being studied as potential vaccine adjuvants[38-40]. Recent studies show that inulin has the capacity to act on the alternative complement pathway and to boost acquired immune responses[41, 42]. Whether this effect on acquired immunity is indirectly mediated by complement deposition or whether direct interactions with innate immune cells also play a role is not known. Finally,

inulin has caught interest for its role in prebiotics where is has been described to act directly on immune cells in the Peyer's patches of the intestine by boosting the proportion and number of dendritic cells present[43].

Indeed, initial findings in this study implicated a direct interaction between inulin and NFκB signalling pathways through activation of TLR-2, TLR-4 and TLR-5. However, activation of three different TLR's by a single compound has never been described and seems rather unlikely. Especially interaction with TLR-5 is questionable since there is little resemblance between the oligosaccharide inulin and the natural ligands of TLR-5 which are conserved helical structures on bacterial flaggelins[44, 45]. Studies done so far point out that this receptor-ligand interaction is highly specific and independent of glycosylation[46]. To our knowledge no other agonists of TLR-5 have been described. Our scepticism was confirmed by the observation that NFκB signalling in RAWblue cells expressing all three receptors was largely abrogated when either an endotoxin inhibitor was added to the mixture or when ultrapure inulin was used. It is not unthinkable that the additional effect of the SFD process on NFκB activation is also related to contamination issues since this process is conducted in a non-sterile environment.

The *in vivo* experiments conducted in this study further strengthened the assumption that none of the sugars conveys additional immunostimulating capacity. Most importantly, the strong NFκB activation by inulin that was observed *in vitro* was not reflected by superior immune induction *in vivo.* This discrepancy raises questions about the predictive value of RAW-blue cells in terms of adjuvanticity. Apparently, the high sensitivity of this system causes it to react to trace amounts of contamination which do not seem to confer any adjuvant properties whatsoever. This strongly emphasizes the need to thoroughly asses the purity of agents used in such studies. In contrast to the observed RAW-blue activation by a non-adjuvanting compound, there are also established adjuvants that do not act on NFκB and therefore are unlikely to affect RAW-blue cells[47]. These adjuvants may for instance bypass the mechanism of PRR activation and rely on depot effects and/or inflammasome induction. For instance, it has been proposed by Li and colleagues that NLRP3 dependent inflammasome activation is a common mechanism for particulate adjuvants which comprise a substantial part of the commercially available products[48]. It could be worthwhile looking into such mechanisms when conducting future research on SFD vaccines, especially in relation to the particulate nature of these powders. In spite of the mentioned shortcomings, RAW-blue cells may still be a powerful tool in screening large amounts of potential PRR agonists, something that is not always possible using conventional techniques.

In the *in vivo* study, most of the observed responses to both WIV inulin and WIV dextran/trehalose were slightly stronger than those initiated by liquid WIV. However, earlier work done by our group already revealed that this may be an effect of method of administration (liquid aerosolization versus dry powder inhalation) rather than adjuvanticity[28]. This discrepancy makes the comparison of effects mediated by dry powder vaccines to liquid vaccine control less reliable. In order to obtain a more reliable assessment of *in vivo* effects mediated by these sugars, studies should therefore be done with reconstituted pulmonary vaccines which can be administered by liquid aerosolization. Moreover, structural analysis of WIV dextran (unpublished data) shows that dextran is insufficiently able to conserve the viral antigens during SFD and storage. This might explain why the immune induction by WIV

dextran is inferior in some cases. Finally, observed differences in terms of HAI titers, if any, were only very modest. HAI is considered one of most reliable correlates of protection in influenza immunization next to viral challenge models[49]. The absence of significant differences in HAI suggests that in terms of protection against infection all vaccines would perform equally.

In line with earlier findings[28], our data suggest a strong tendency of our formulations to promote Th2 skewed T-cell responses. This bias may be a result of the pulmonary environment which has been shown to promote preferential Th2-type differentiation[50]. The development of Th1 responses is considered to be of importance because they provide superior protection against infection and a certain degree of cross-protection[51-53]. In addition, Th1 responses support viral clearance by the induction of IFN-γ[54]. For pulmonary SFD vaccines to be successful, efforts should be made to direct the induced immune response towards a more Th1 phenotype, for instance by applying adjuvants known to be in favour of Th1 induction[47].

Taken together, the above study emphasizes the need of expanding our knowledge on adjuvanted pulmonary vaccines. This knowledge is essential for developing pulmonary vaccines that induce better immune responses both in terms of magnitude and quality. A deeper insight into mechanisms that play a role in the immunogenesis by these vaccines could be decisive.

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Appendix

List of abbrevations

- AEC 3-Amino-9-ethylcarbazole
- AP Alkaline Phosphatase
- AP-1 Activator protein 1
- BCIP 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
- BPL β-propiolactone
- CDC Centers for Disease Control
- CTAB Hexadecyltrimethylammonium bromide
- ELISA Enzyme-linked immunosorbent assay
- ELISPOT Enzyme-linked immunosorbent spot
- FBS Fetal bovine serum
- HA Haemagglutinin
- HAI Haemagglutination inhibition
- HBS HEPES buffered saline
- HRP Horseradish peroxidase
- IFN-γ Interferon-γ
- IL-4 Interleukin-4
- LAIV Live attenuated influenza virus vaccines
- LPS Lipopolysaccharide
- MDA-5 Melanoma Differentiation-Associated protein 5
- MDP Muramyl dipeptide
- MWCO Molecular weight cut-off
- NBT Nitro-blue tetrazolium chloride
- NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells
- NOD Nucleotide-binding oligomerization domain-containing protein
- PBS Phosphate buffered saline
- PMX Polymyxin-B
- PRR Pattern recognition receptor
- RIG-I Retinoic acid-inducible gene 1
- SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
- SEAP Secreted alkaline phosphatase
- SFD Spray freeze dried
- TIV Trivalent inactivated vaccines
- TLR Toll-like receptor
- TNF-α Tumor necrosis factor α
- WHO World Health Onganization
- WIV Whole inactivated virus

Protocols

Subunit Influenza virus

Wouter ter Veer 05/11/2008, changed 5/01/2011 (CTAB/Tween concentration)

Material/ miscellaneous

- Biohazard cabinet in ML-II lab
- Rotating device
- Inactivated virus
- Ultracentrifuge + TLA100.3 rotor

Solutions

- HBS: 145 mM NaCl, 5 mM Hepes, pH 7.4, sterilized by filtering.
- 0.4 % Tween 80 in PBS
- 0.8% CTAB in PBS
- Biobeads (activated in methanol)

Procedure

Day 0

- Inactivate the virus and dialyse it against PBS overnight

Day 2

- Add 600 µg/ml tween 80 and 3000 µg/ml CTAB to 800 µg/ml virus
- Incubate 3 hours at 4ºC (rotate)
- Centrifuge the virus at 50000 RPM TLA100.3 for 0.5 hour at 4ºC, to remove the nucleocapsid
- Add 634 mg/ml Biobeads to the supernatant. Incubate overnight at 4ºC (rotating or shaking)

Comments:

Tween 80: density= 1.064 (g/ml)

Protein determination Micro-Lowry

Anke Huckriede, 12-5-1997

Literature:

- Peterson, G.L., Anal. Biochem. 83, 346-56, 1977

Material:

Miscellaneous
- Eppendorf tubes
- Vortex
- Eppendorf centrifuge (4°C)
- 1 ml plastic cuvettes
- spectrophotometer

Solutions
- 0.3% Na-deoxycholate (w/v)
- 72% trichloracetic acid (TCA, (w/v))
- solution A (1 l): 4 g NaOH
  20 g Na_2CO_3
  0.2 g Na-tartrate (or 0.234 g Na-tartrate dihydrate)
  10 g SDS
- solution B (100 ml): 0.5 g $CuSO_4$x5 H_2O (or 0.32 g pure $CuSO_4$)
- Folin reagent
- BSA standard 0.4 mg/ml

Procedure:

Protein precipitation
- pipette 0 to 10 μg protein standard into Eppendorf tubes (in duplicate)
- pipette unknown sample (amount containing 5 - 10 μg protein) into tubes
- adjust volume to 0.5 ml with H_2O
- add 50 μl Na-deoxycholate stock, vortex
- incubate for 10 min
- add 50 μl TCA stock, vortex
- centrifuge 15 min in Eppendorf centrifuge in the cold room, or at 10000 rpm in JA18.1 in Sorvall centrifuge
- carefully remove supernatant

Protein detection
- prepare Lowry detection reagent by mixing 49 parts solution A and 1 part solution B
- resuspend pellets from protein precipitation in 1 ml Lowry reagent, vortex
- incubate for 10 min
- prepare Folin solution by diluting Folin reagent 1:1 with H_2O
- add 100 μl Folin solution, vortex
- incubate for 30 min
- measure extinction at 750 nm

Comment:
If solution looks hazy after incubation with Folin solution, spin down and use supernatant.

SDS-PAGE

Anke Huckriede, 6-5-1997

Literature:

- Laemmli, U.K., Favre, M. (1973): Maturation of the head of bacteriophage T4. J. Mol. Biol. **80**, 575-599.
- Hames, B.D. and Rickwood, D. (1990): Gel electrophoresis of proteins. A practical approach.

Material:

Miscellaneous
- elecrophoresis equipment (BioRad)

Solutions
- 40% acrylamide stock, acrylamide:Bis 37.5:1 (Serva cat.no. 10681)
- 3 M Tris-Cl pH 8.8
- 1M Tris-Cl pH 6.8
- TEMED
- 10% ammonium persulfate (APS), not older than 1 week
- water-saturated isobutanol
- Bromophenol Blue stock in ethanol
- 5x sample buffer: 30% glycerol
  50% β-mercaptoethanol
  10% SDS
  0.32 M Tris-Cl pH 6.8
  20 mM EDTA
  0.01% Bromophenol Blue
- 5x running buffer (1l): 30.3 g Tris
  144 glycine
  5 g SDS

Procedure:

Acrylamide is toxic!!! Wear gloves whenever you handle acrylamide solution or gels!!

- assemble gel casettes
- prepare acrylamide solution for running gel according to the tabel (next page)
- pour gel (leave enough space for stacking gel!!)
- cover gel with water-saturated isobutanol, let polymerize (about 20 min)
- wash surface of gel with water, remove all water with help of a piece of filter paper
- pour stacking gel, place comb for forming of slots
- after polymerization remove comb, wash slots with water
- assemble casettes and electrodes, place in tray
- add running buffer (bottom of the gels has just to emerge into buffer, top has to be completely filled)
- apply samples and marker (if possible don't use the far left and far right slot)
- place lid in position, connect to power supply
- run gel at 150 V until Bromophenol blue front has leaked out
  (takes about 50 min for 7.5% AA gels and 1h15min for 12.5% gels)

per 2 gels [µl]	separating gel			stacking gel
	7.5%	10%	12.5%	
40% AA	1400	1900	2400	300
3 MTris pH8.8	940	940	940	-
1 M Tris pH 6.8	-	-	-	375
10% SDS	75	75	75	30
H_2O	5100	4600	4100	2300
TEMED	3.8	3.8	3.8	4
10% APS	38	38	38	19
Bromophenol bl				3

Silverstaining of polyacrylamide gels

Anke Huckriede, 23-07-1998

Literature: Blum, Beier, Gross Electrophoresis 8, 93-99, 1987

Material:

Solutions

- A: 200 ml methanol p.a.
  48 ml acetic acid
  152 ml H_2O
  use 50 ml/gel, add 25 µl 37% formaldehyde freshly
- B: 50 % ethanol p.a.
- C: **prepare freshly!!**
  10 mg sodiumthiosulfate in 50 ml H_2O
- D: **not older than 4 weeks!!**
  400 mg silver nitrate
  200 ml H_2O

- E: **prepare freshly!!**
  3 g Na_2CO_3
  1.25 ml solution C
  25 µl 37% formaldehyde
  adjust volume to 50 ml with H_2O
- F: 50 % methanol p.a.
  12 % acetic acid
- G: 30 % methanol

Procedure:

(use a clean tray, wear gloves, never touch the gel, perform all incubations under agitation!!)

- fix the gel for 1 h to overnight in solution A (don't forget to add formaldehyde)
- wash 3 x 20 min in sol. B
- incubate for precisely 1 min in sol. C
- wash 3 x briefly with H_2O
- incubate gel for 20 min in sol. D
- discard D in 'heavy metal waste'
- wash 3 x briefly with H_2O
- develop in sol. E, proceed as soon as first bands appear (development will carry on for some time!)
- wash 2 x with H_2O
- stop development with sol. F, incubate 10 min
- wash ca. 30 min in sol. G
- dry the gel directly or store in the refrigerator in sol. G

Determination of hemagglutination-inhibition titers in serum

M,Holtrop 140301

Purpose:
To quantitate serum antibody to a specific antigen, antibodies directed against these organisms will inhibit hemagglutination

HAU = haemagglutinating unit = the minimum amount of virus which will case haemagglutination of all the erythrocytes in a well

HAI = haemagglutination inhibition titre

- Prepare a stock solution of 10 % guinea pig erythrocytes as follows:
- Spin down (1200 rpm) whole guinea pig blood conserved with alsevar(or EDTA) to harvest erythrocytes,
- Wash the erythrocytes with PBS until a clear supernatant is obtained,
- Carefully remove all the supernatant, and roughly estimate the amount of erythrocytes (in ul or ml) obtained using a P-1000 Gilson pipet, The amount obtained equals 100% erythrocytes, Prepare a stock solution of 10 % erythrocytes and store the solution at 4 degrees, The stock can be used until hemolysis occurs (after 1-2 weeks)

Before carrying out the actual hemagglutination-inhibition assay first determine the amount of HAU(hemagglutination units) in the virus stock as follows,

- Fill a 96 wells round-bottom plate with 50 ul PBS per well, Add 50 ul of an appropriate dilution of the virus-stock (or use non diluted samples) in triplicate to the upper wells, Use the first lane as a negative control,
- Serially dilute the samples twofold, using the whole plate,
- Add 50 ul 1 % guinea pig erythrocytes to each well and mix gently
- Wait 1-2 hours until total hemagglutination has been reached,
- Score the highest dilution at which total hemagglutination is obtained; this is 1HAU ; use 4 HAU for the actual hemagglutination-inhibition assay
- For the actual determination of the hemagglutination-inhibition titer in mouse sera first inactivate the sera by incubating 75 ul aliquots 30 min, 56 degrees C,
- Add 3 parts, so 225 ul, of a 25 % kaolin stock solution to the sera and incubate 20 min, at room temperature,
- Centrifuge sera 2min 1700 rpm (6500 rpm MSE centr,)
- Fill (per series) two 96 well round bottom plates with 50 ul PBS per well; add 50 ul of the above obtained supernatants in duplicate to the upper wells of the first plate, using the first lane as a negative control, Serially dilute the samples twofold in PBS, Now use both plates next to each other to avoid missing the endpoint titer.
- Add 50 µl of the 4 HAU units per 50 µl containing virus dilution to each well and mix gently (from low to high concentrations) using the multi-channel pipet
- Incubate the plates 40 min, at room temp,
- Add 50 ul of a 1% guinea pig erythrocyte solution to each well,
- Allow hemagglutination to proceed for 1-2 hours and the score the highest dilution at which hemagglutination is obtained, and multiply the titer by 4 (taking into account the dilution of the sera)

ELISA

Material

- Coating buffer: 0,05 M carbonate-bicarbonate pH 9,6-9,8 (1,89 g Na_2CO_3 and 2,70 g $NaHCO_3$ per l)
- 10x PBS: 90 g NaCl, 16,2 g Na_2HPO_4 and 1,2 g KH_2PO_4 per liter
- PBS/Tween: PBS containing 0,05% Tween20
- Use a 1:5000 dilution for all SBA Ig-HRP conjugates
- Phosphate buffer: 0,1 M NaH_2PO_4, bring to pH 5,6 with 0,1 M Na_2HPO_4
- Staining solution: 20 mg OPD (o-phenylene-diamine-dihydrochloride, first dissolved in 2 ml methanol) per 100 ml phosphate buffer pH 5,6. Add 20 µl H_2O_2 to 100 ml staining solution just prior to application to the plate.

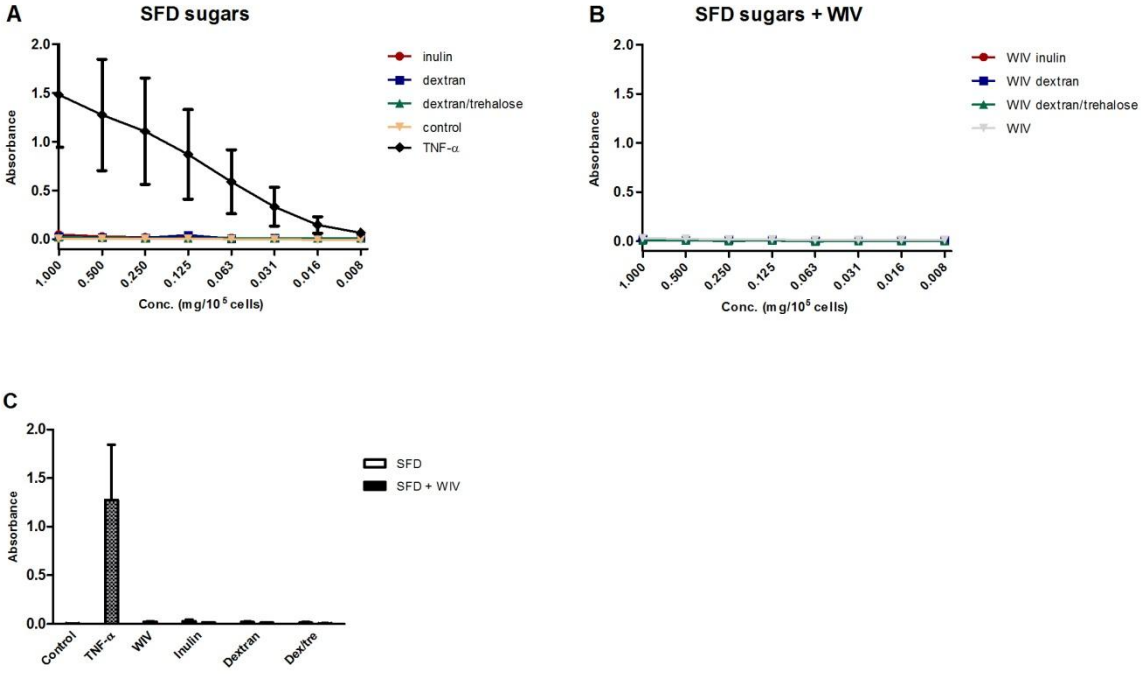
Van A/Panama virus (3,108 mg HA/ ml) dus per plaat 5 ml nodig met daarin 50 µg en dat is 16,1 µl van het virus.

Method

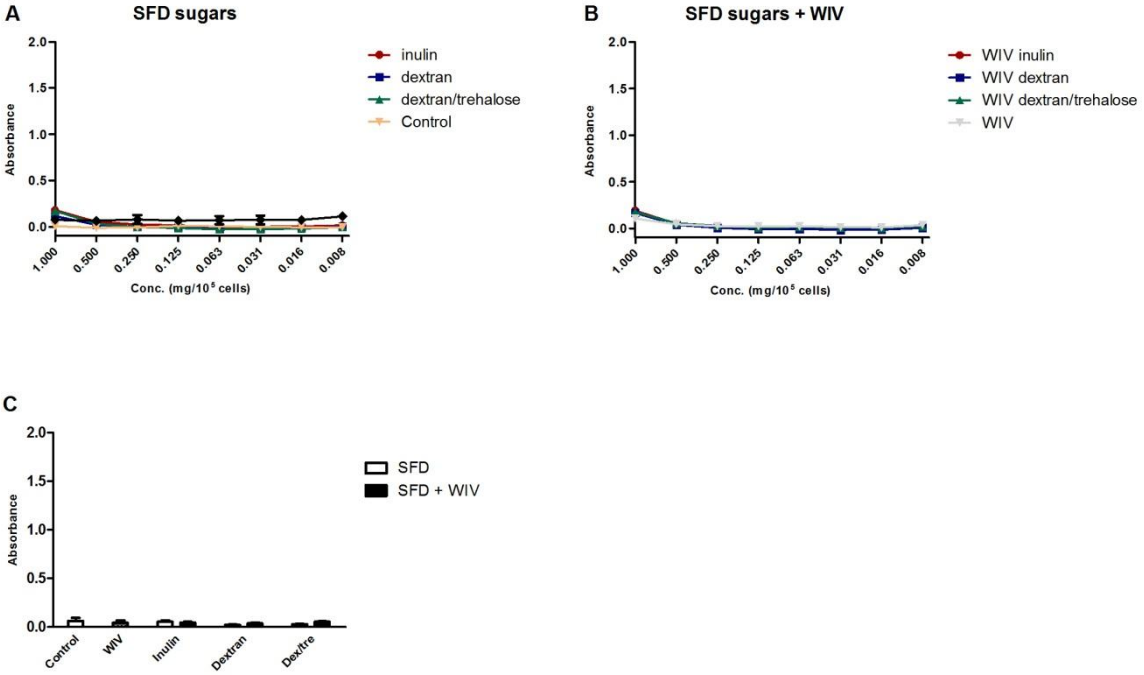
- Coat ELISA plate with high binding capacity (Greiner) overnight at 37°C with 200 ng influenza subunit vaccine (or 0,5 µg influenza virus protein per 50 µl per well) or 1 µg OVA per well in coating buffer in 100 µl.
- *If using influenza virus: pipet coating from wells with a multichannel pipet (put in chloride solution), add 200 µl of coating buffer, remove with multichannel (put in chloride solution) and again add 200 µl of coating buffer (remove with multichannel before putting blocking solution in.*
- Wash the plate once with coating buffer (spuitfles).
- Block the plate with a 2,5% milk powder solution in coating buffer for 45 minutes at 37°C (200 µl).
- Wash the plate once with coating buffer and twice with PBS/Tween (store the empty plates until use at -80°C)
- Apply samples to the plates: make sequential twofold dilutions using a multichannel pipette 100 µl/ well. Incubate 1,5 hrs at 37°C.
- Wash the plate 3 times with PBS/Tween
- Incubate the plate with the appropriate conjugate for 1 hr at 37°C
- Wash the plate 3 times with PBS/Tween and once with PBS
- For HRP conjugates: 100 µl staining solution, do not forget to add H_2O_2! Incubate 30 minutes, stop reaction by adding 50 µl 2 M H_2SO_4 per well. Read the absorbances in the ELISA reader at 492 nm.

- *For all AP conjugates:*
  incubate plate with 1 mg/ ml PNPP (p-nitro-phenylphosphate-disodiumsalt) in substrate buffer. Stop the reaction with 50 µl 0,1 M NaOH per well. Read absorbances in the ELISA reader at 405 nm.

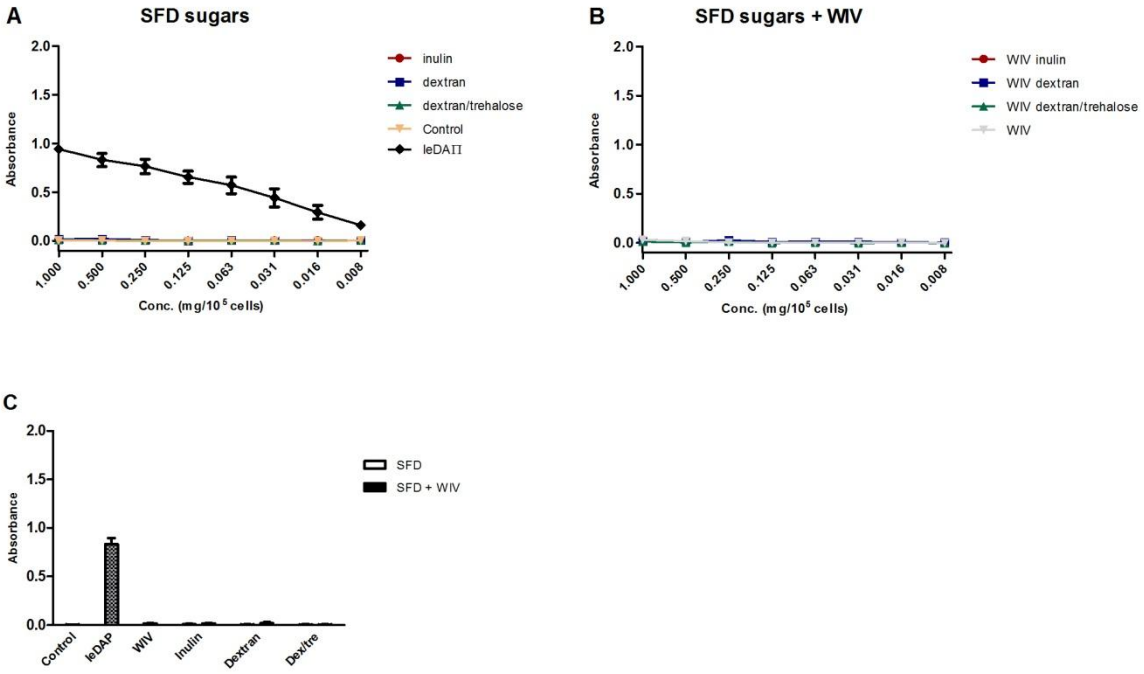
Additional figures



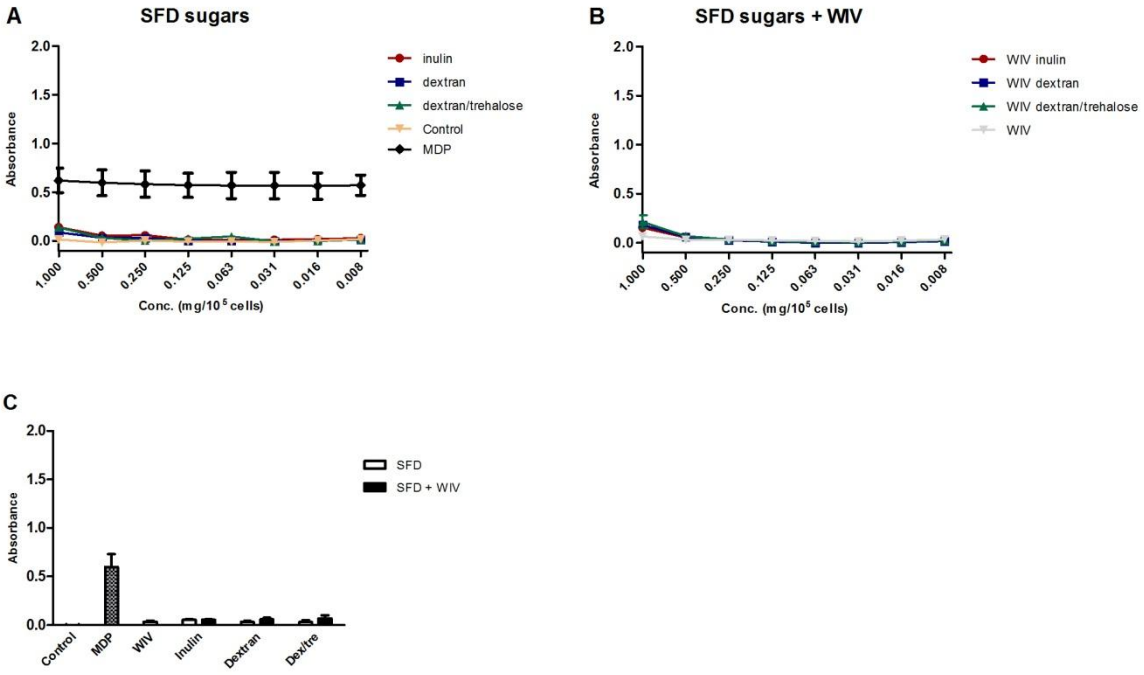
Additional figure 1 NFκB activation HEK-Null 1 cells. Absorbances are shown for SFD sugars (A)**,** SFD vaccines (B) and for 0.5mg/10^5 cells from all groups (D). (n = 2 +/- SEM)



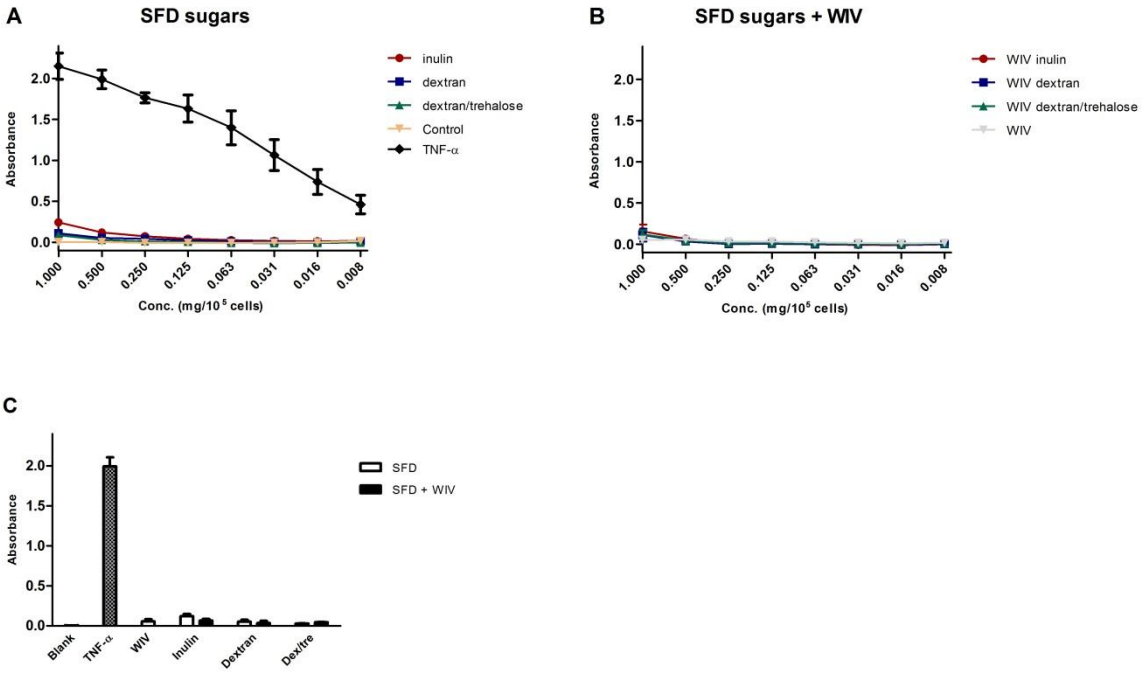
Additional figure 2 NFκB activation HEK-Null 2 cells. Absorbances are shown for SFD sugars (A)**,** SFD vaccines (B) and for 0.5mg/10^5 cells from all groups (D). (n = 2 +/- SEM)



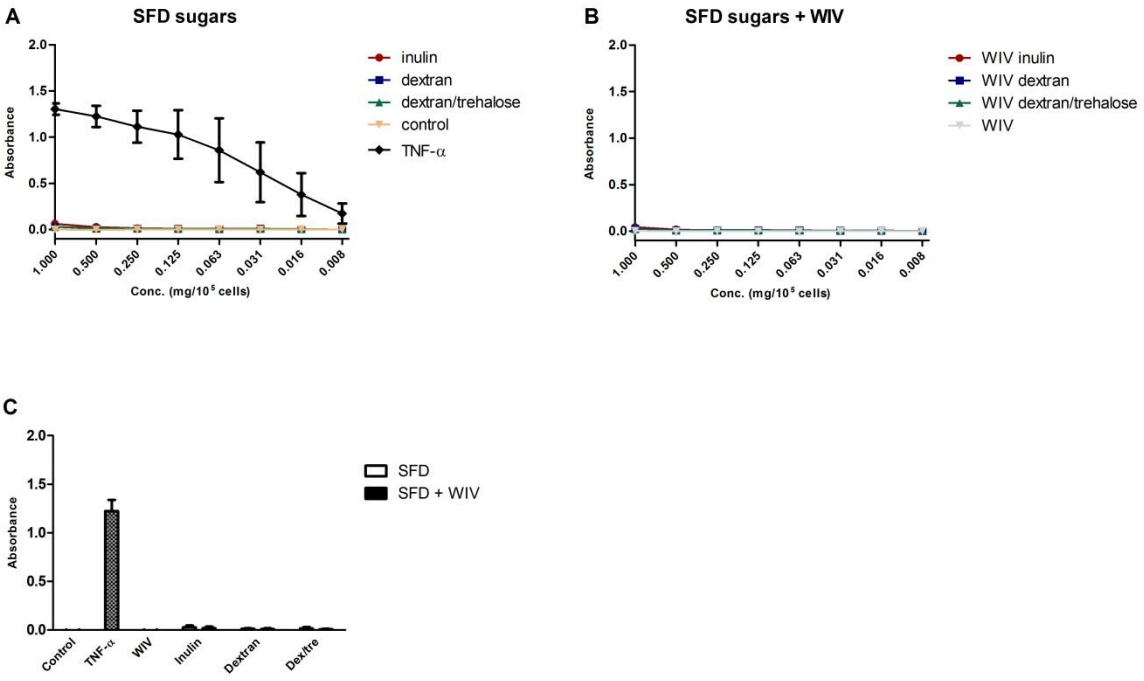
Additional figure 3 NFκB activation HEK-NOD 1 cells. Absorbances are shown for SFD sugars (A), SFD vaccines (B) and for 0.5mg/10^5 cells from all groups (D). (n = 2 +/- SEM)



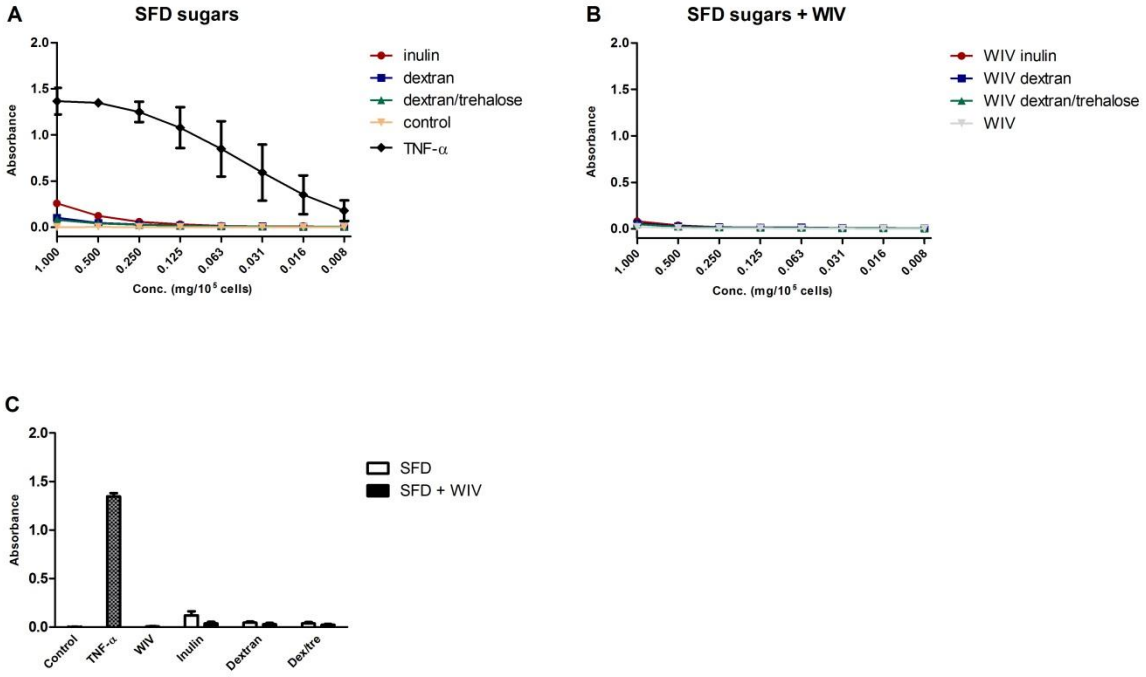
Additional figure 4 NFκB activation HEK-NOD 2 cells. Absorbances are shown for SFD sugars (A), SFD vaccines (B) and for 0.5mg/10^5 cells from all groups (D). (n = 2 +/- SEM)



Additional figure 5 NFκB activation HEK-TLR 3 cells. Absorbances are shown for SFD sugars (A), SFD vaccines (B) and for 0.5mg/10^5 cells from all groups (D). (n = 2 +/- SEM)



Additional figure 6 NFκB activation HEK-TLR 8 cells. Absorbances are shown for SFD sugars (A), SFD vaccines (B) and for 0.5mg/10^5 cells from all groups (D). (n = 2 +/- SEM)



Additional figure 7 NFκB activation HEK-TLR 9 cells. Absorbances are shown for SFD sugars (A)**,** SFD vaccines (B) and for 0.5mg/10^5 cells from all groups (D). (n = 2 +/- SEM)