Enhancement of Th1 immune responses to recombinant influenza nucleoprotein by Ribi adjuvant

Diego E. Cargnelutti[1], María V. Sanchez[1], Paula Alvarez[2], Lorena Boado[2], Nora Mattion[2], Eduardo A. Scodeller[1]

[1]Institute of Experimental Medicine and Biology of Cuyo (IMBECU) CCT-Mendoza-CONICET, Mendoza, Argentina; [2]Animal Virology Center (CEVAN), Institute of Science and Technology Dr. César Milstein, CONICET, Buenos Aires, Argentina

SUMMARY

A broad coverage influenza vaccine against multiple viral strains based on the viral nucleoprotein (NP) is a goal pursued by many laboratories. If the goal is to formulate the vaccine with recombinant NP it is essential to count on adjuvants capable of inducing cellular immunity. This work have studied the effect of the monophosphoryl lipid A and trehalose dimycolate, known as the Ribi Adjuvant System (RAS), in the immune response induced in mice immunized with recombinant NP. The NP was formulated with RAS and used to immunize BALB/c mice. Immunizations with NP-RAS increased the humoral and cellular immune responses compared to unadjuvanted NP. The predominant antibody isotype was IgG2a, suggesting the development of a Th1 response. Analysis of the cytokines from mice immunized with NP-RAS showed a significant increase in the production of IFN-γ and a decreased production of IL-10 and IL-4 compared to controls without RAS. These results are similar to those usually obtained using Freund's adjuvant, known to induce Th1 and CTL responses when co-administered with purified proteins, and suggest that a similar approach may be possible to enhance the performance of a T-cell vaccine containing NP.

KEY WORDS: Influenza virus, Recombinant nucleoprotein, Ribi Adjuvant System, Th1 immune responses.

Received November 19, 2012 *Accepted* February 15, 2013

INTRODUCTION

There is a great deal of interest in the development of influenza vaccines with a broader protective spectrum. The best strategy for this purpose may be the development of vaccines capable of inducing a Th1 immune response with specific CTLs. There is convincing evidence that influenza specific CTLs directed against epitopes shared by many strains are able to confer protection in murine models (Rimmelzwaan *et al.*, 2007), and possibly the same may be true for humans (Epstein, 2006; McMichael *et al.*, 1986).
Several approaches have been reported following this strategy. Systems based on genetic immunization or on the use of viral vectors have been remarkably efficient (Berthoud *et al.*, 2011; Price *et al.*, 2010; Zhou *et al.*, 2010). However, these methods involve sophisticated production technology, and their safety has not yet been fully demonstrated, casting doubts on the availability of this type of vaccines in the market in the short term.
A simpler alternative to produce a T-cell vaccine against influenza would be to formulate the purified proteins with adjuvants capable of inducing a Th1 immune response. There are several examples that indicate that this strategy works with conserved purified proteins of influenza (Guo *et al.*, 2010; Jelinek *et al.*, 2011; Thueng-in *et al.*, 2010).
Based on this background, we decided to study the immune responses induced by the recombinant nucleoprotein (rNP) formulated with the Ribi Adjuvant System (RAS) known for its ability to induce CTLs with purified proteins (Chaitra

Corresponding author
Diego Esteban Cargnelutti
IMBECU - CONICET, CCT-Mendoza, Argentina
Av. Ruiz Leal s/n - 5500 Mendoza, Argentina
E-mail: diegocargnelutti@hotmail.com

et al., 2007). The classical adjuvant system involving complete/incomplete Freund's adjuvant (CFA/IFA), known to induce a Th1 type immune response (Yip *et al*., 1999) was selected as the gold standard for comparison.

The RAS is a stable oil-in-water emulsion containing a non-toxic derivative of the lipopolysaccharide (LPS) of *Salmonella minnesota,* monophosphoryl lipid A (MPLA) and synthetic trehalose dicorynomycolate (TDcM).

MPLA is a safe and well-tolerated adjuvant approved for human use. TDcM is a low toxicity derivative of the mycobacterial cord factor trehalose-6,6-dimycolate (TDM) (Watanabe *et al*., 1999). TDM has been shown to be a potent adjuvant that induces the Th1 and Th17 arms of the immune response through the activation of the Syk-CARD9 signaling pathway in APCs (Agger *et al*., 2008; Davidsen *et al*., 2005; Khader *et al*., 2007; Schoenen *et al*., 2010).

Due to its significant toxicity, TDM is not applicable for practical use in humans. However, the synthetic analog trehalose-6,6-dibehenate (TDB) is non-toxic, has the same adjuvant properties as TDM and is currently been assayed in clinical trials (Milicic *et al*., 2012).

The effects of RAS on the immunogenicity of the hemagglutinin (HA) of influenza virus, either from viral or recombinant DNA source, has been studied or compared with several other adjuvants (Robuccio *et al*., 1995; Vanlandschoot *et al*., 1993). However, to the best of our knowledge, there are no reports on the use of MPLA, TDM or TDcM as single adjuvants to stimulate the immune response induced by purified NP. The results presented in this work indicate that the combination of MPLA and a Syk-CARD9 ligand is capable of inducing a Th1 type immunity of similar magnitude to that obtained with CFA/IFA. Therefore this combination should be taken into account in the search for adjuvants able to induce a Th1 response with purified rNP.

METHODS

Production and purification of rNP

The influenza strain A/PR/8/34 (H1N1) was provided by the World Health Organization and grown in the allantoic cavity of embryonated hen eggs according to standard procedures. The cloning of the full length NP gene of A/PR/8/34 (H1N1) into the pET30a plasmid vector (Novagen), and its expression in *Escherichia coli* BL21 (DE3), as well as the purification of the rNP by a three step chromatographic procedure have already been described (Cargnelutti *et al*., 2012). The purified rNP had an acceptable level of LPS contamination (50 endotoxin units per 50 µg of protein), as determined by the *Limulus amebocyte* lysate assay.

Formulation of experimental antigens and immunization schedule

RAS was purchased from Sigma-Aldrich and contains monophosphoryl lipid A (MPLA) 0.5 mg, plus trehalose dicorynomycolate (TDcM) 0.5 mg in 2% squalene-Tween 80-water. Each vial was reconstituted with 1 ml saline and mixed at 1:1 ratio with NP in PBS and administered in mice subcutaneously (1 dose =50 µl of emulsion =25 µg of MPLA and 25 µg of TDcM).

Freund's complete adjuvant (CFA) was purchased from Sigma Aldrich. Each ml contains 1 mg of *Mycobacterium tuberculosis* (H37Ra, ATCC 25177); heat-killed and dried, 0.85 ml paraffin oil and 0.15 ml mannide monooleate.

NP in saline was mixed with an equal volume of each adjuvant to form an emulsion. CFA was used for initial injections and IFA for subsequent boost.

Eight-to-nine-week-old inbred female BALB/c mice were used in immunization experiments. Three independent experiments were carried out with 5 mice per group. All experiments involving animals were approved by the Animal Care Committee of the Medicine School from the National University of Cuyo (IACUC number 0020398/2011).

For priming, groups of 5 mice were subcutaneously vaccinated with 10 µg of NP formulated in PBS, 10 µg of NP emulsionated with CFA or with 10 µg of NP formulated with 50 µl of reconstituted RAS. Three weeks later, mice were boosted with the same formulations except that CFA was switched to IFA. On days 0 (preimmune; PI) and 36 post prime immunization, blood samples were collected from each mouse to evaluate the presence of NP specific antibodies in serum. On day 36, mice were sacrificed by cervical dislocation and their spleens removed and processed to recover spleen cells to prepare cultures for in vitro determination of cytokine production.

Analysis of humoral immune responses
The humoral immune responses induced by the three NP formulations were evaluated by measuring total specific IgG, and the specific subtypes (IgG1 and IgG2a) by ELISA. Briefly, 96-well plates (MaxiSorp, Nalge-Nunc International) were coated with 300 ng/well of NP in PBS and incubated overnight at 4°C. After blocking with 5% skim milk in PBS, plates were incubated for 1 h at 37°C with serially diluted serum samples, followed by three washes with the same buffer, and reacted with goat anti-mouse IgG, IgG1 or IgG2a antibodies conjugated with horse-radish peroxidase (BD Biosciences). Plates were developed by adding tetramethylbenzidine (TMB, Pierce-Endogen) and incubating the plates in the dark. The reaction was stopped using 1 N H_2SO_4, and optical densities (OD) were read at 450nm with an ELISA reader (Multiskan Ex, Thermo Scientific). The ELISA end-point titers were expressed as the reciprocal of the highest sample dilution that yielded an OD 2 times the mean value of control blank.

Splenocyte cultures
Spleens from vaccinated or non-vaccinated mice were aseptically excised and used to prepare single-cell suspensions (4 x 10^5 cells) in RPMI-1640 culture medium supplemented with 4 mM L-glutamine, 24 mM $NaHCO_3$, 100 units/ml of penicillin, and 10% fetal calf serum. Cell viability was >95% as determined by trypan blue exclusion. Spleens cells were cultured in flat bottomed 96-well microtitre plates (Greiner Bio One) and stimulated with 1 µg/well of NP at 37°C in 5% CO_2 for 48 h.

Cytokine ELISA assay
For detection of cytokines, culture supernatants of spleen cells were collected after 48 h of antigen stimulation and tested for the presence of the cytokines by antigen-capture ELISA using OptEIA Set Mouse IFN-γ, IL-10 and IL-4 kits (BD Biosciences). Negative controls were incubated in medium alone and positive controls with medium containing Concanavalin A (2.5 µg/ml). All assays were performed in triplicate. The concentration of cytokines in the culture supernatants was calculated by using a linear-regression equation obtained from the absorbance values of the standards.

Statistical analysis
Differences between groups were tested for significance by Student's unpaired t-test using GraphPad Prism v4.00 for Windows (GraphPad Software). A p-value less than 0,05 was considered statistically significant.

RESULTS

NP-RAS immunization elicits strong antibody responses
The analysis of sera from inoculated mice indicated that in all cases there was a specific seroconversion to NP. However, in the group of animals inoculated with NP formulated with RAS, there was a significant increase in the mean titer of total IgG anti-NP from 1,040 to 12,800 (Figure 1A). As expected, a remarkable increase in the antibody titers was also observed when mice were immunized with NP formulated with CFA/IFA, this latter formulation being the most effective at inducing NP-specific IgG antibodies (mean titer 46.080).
In the RAS and CFA/IFA animal groups there was a preferential increase in the IgG2a subtype, whereas in the control group the prevalent subtype was IgG1 (Figure 1B). The ratio IgG2a/IgG1 increased from 0.045 (control group) to 0.96 with RAS formulation and from 0.045 to 0.92 with CFA/IFA (Figure 1C). The relative values of IgG2a and IgG1 have been used as indicators of the induction of Th1 and Th2 responses, respectively (Coffman *et al.*, 1988). Thus, in this case, IgG2a/IgG1 ratios were used as indicators of Th1 or Th2-biased responses induced by immunization. The difference in the magnitude of the total IgG titer compared to the anti-NP values of the subtypes IgG1 and IgG2a is likely due to the use of different anti-mouse peroxidase conjugates (see Materials and Methods).

NP-RAS immunization induces a Th1-biased response
To gather further information on the types of immune responses induced by the different immunization protocols, we used a capture ELISA to investigate the cytokines profile secretion (IFN-γ IL-10 and IL-4) in spleen cells from NP-PBS, NP-RAS or NP-CFA/IFA immunized mice. Re-stimulation with NP induced a significantly higher pro-

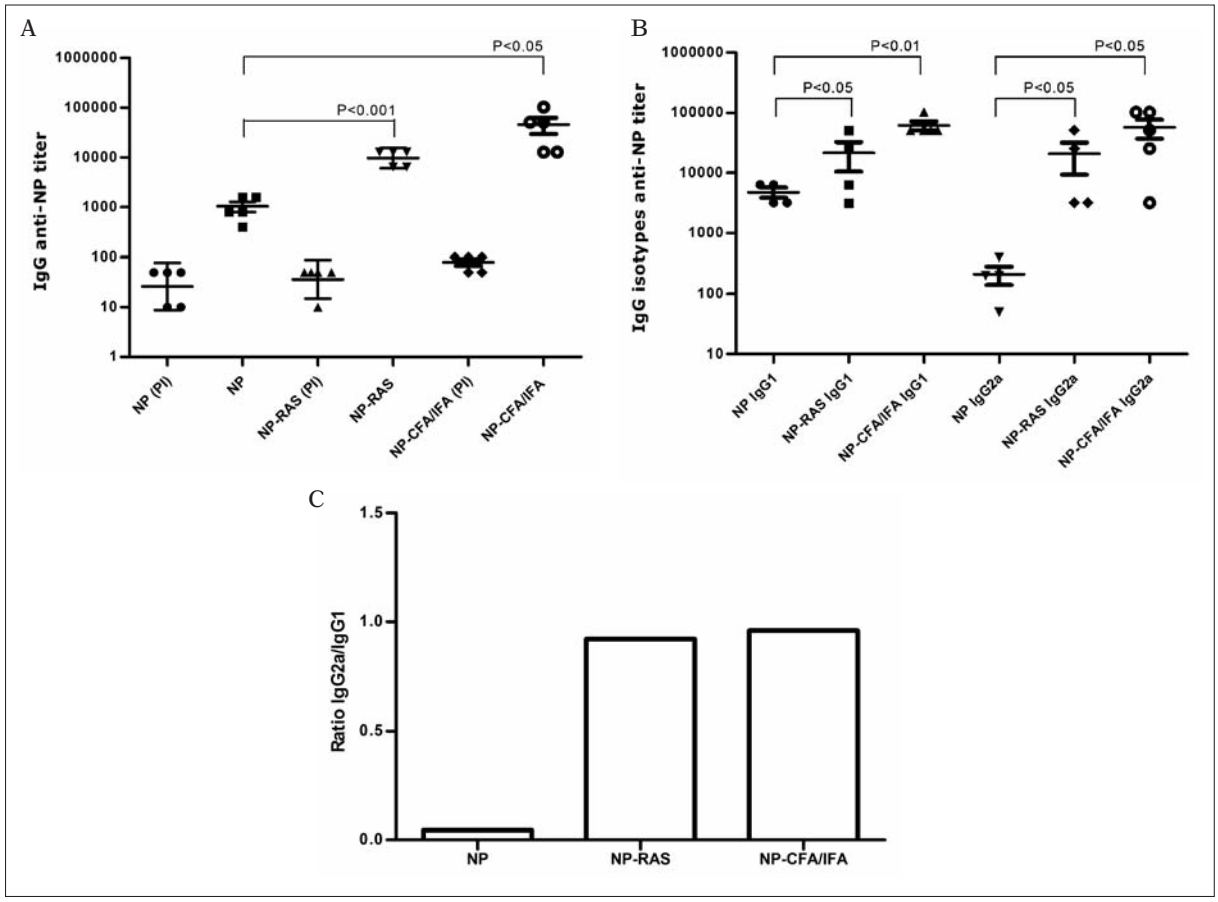

FIGURE 1 - *Analysis of the humoral immune responses (A). Total Specific IgG titers induced in mice immunized with NP-PBS, NP-RAS or NP-CFA/IFA. Values of individual animals are indicated. Horizontal lines indicate mean values (± SEM); PI = preimmune (B) Specific IgG isotypes. (C) IgG2a/IgG1 ratio corresponding to the same groups shown in panel A. Sera were evaluated by ELISA as indicated in the Materials and Methods section.*

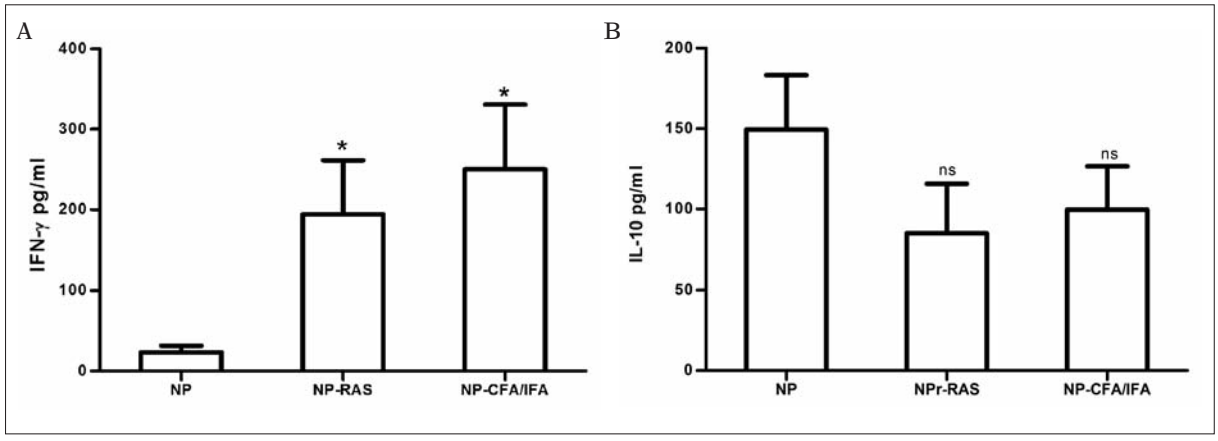

FIGURE 2 - *Cytokines profile. Quantitative ELISA analysis of IFN-γ (A) and IL-10 (B), secreted by splenocytes of mice immunized with NP-PBS, NP-RAS or NP-CFA/IFA upon in vitro stimulation with NP (1 μg/well). The results are presented as the mean value ± SEM. Asterisks over each bar indicate significant differences in comparison to control groups. *, P <0.05; ns = not significant.*

duction of IFN-γ in splenocytes from NP-RAS ($P<0.05$) and NP-CFA/IFA ($P<0.05$) immunized mice compared to the control group (Figure 2A), whereas no significant differences between the 3 groups were found regarding IL-10 production (Figure 2B). In addition, no IL-4 was detected in any of the culture supernatants of stimulated splenocytes (data not shown). Similar results were reported by other groups using antigens formulated with RAS (Coler *et al*., 2002; Ravindran *et al*., 2010). Thus, the NP-RAS formulation triggered a higher response of IFN-γ, but similar and low responses of IL-10 and IL-4. Taken together, these results suggest that immunization with NP-RAS induces a specific Th1-type immune response in mice. These results are consistent with the IgG2a/IgG1 ratios mentioned above.

DISCUSSION

The development of an effective immunity against intracellular pathogens is associated with a Th1 type immune response, dominated by the production of IFN-γ, IgG2a antibodies and CTL. Instead, the effective control of extracellular pathogens is associated with a Th2-type response with production of IgG1 and production of IL-4 and IL-5 (Constant and Bottomly, 1997). One of the major challenges in vaccinology is the development of vaccine formulations that will induce immune responses for the particular pathogen. Thus, adjuvants can be a valuable tool for tailoring the desired immune responses.

Recent results have indicated that the NP of influenza is an excellent candidate for developing a broad spectrum vaccine based on induction of cellular immunity. This has been achieved primarily using genetic vaccines or vectors (Berthoud *et al*., 2011; Price *et al*., 2010; Zhou *et al*., 2010), although there have also been meaningful results using purified recombinant protein formulated with adjuvants (Guo *et al.,* 2010; Jelinek *et al.,* 2011; Thueng-in *et al*., 2010).

The results presented in this work indicate that NP formulated with RAS induces higher total antibody titers than the unadjuvanted protein, though lower than those induced by the protein formulated with CFA/IFA (Figure 1A). Analysis of IgG subtypes involved in the immune response showed that the subtypes induced by NP-PBS were predominantly IgG1 while NP-RAS and NP-CFA/IFA induced a higher IgG2a/IgG1 ratio (Figure 1B and Figure 1C), suggesting a Th1-biased response. This is in agreement with previously published works using the RAS system (Chaitra *et al*., 2007).

The analysis in the cytokine production pattern in cultured splenocytes indicated that both NP-RAS and NP-CFA/IFA induce higher amounts of IFN-γ, and low concentrations of IL-10 and IL-4, a hallmark of Th1-type response (Figure 2A and Figure 2B). This is consistent with the fact that Th1-type responses are characterized by the secretion of IFN-γ by T cells (Dredge *et al*., 2002), something that is known to enhance IgG2 and suppress IgG1 production (Jurado *et al*., 1989).

Although CTL induction was not measured, it is known that Th1-type cellular responses and $CD8^+$-mediated CTL responses are generally related in their immune induction as well as in protective immunity (Sin *et al*., 2000).

The high titer of antibodies induced by NP-RAS should also be taken into account. Very recently, LaMere *et al.* (LaMere *at al*., 2011a) showed that "systemic immunization with NP readily accelerated clearance of a 2009 pandemic H1N1 influenza virus isolate in an antibody-dependent manner". It was also demonstrated that anti-NP IgG specifically promoted influenza virus clearance in mice using a mechanism involving both FcRs and $CD8^+$ cells, and that anti-NP correlated with enhanced NP-specific $CD8^+$ T cell responses (LaMere *et al*., 2011b). Moreover, it was recently reported that an intracellular mechanism for influenza virus neutralization in polarized epithelial cells is dependent on the transport of an influenza neutralizing IgG by the rat neonatal Fc receptor (Bai *et al*., 2011). These works strongly suggest that the titer of antibodies induced by immunization with NP-RAS would also be an important attribute of a vaccine of this type.

The two adjuvants used in this work were chosen for their recognized ability to induce CTLs with purified protein antigens (Chaitra *et al*., 2007; Rao *et al*., 2004). It is known that immunization with purified protein without adjuvant results in the preferential presentation of the antigen via MCH class II. Therefore, in the development of strategies to induce CTL with a purified protein one must consider the use of adjuvant with recognized ability to induce CTLs. It has re-

cently been shown that RAS can enhance $CD8^+$ T cell immune response to selected antigens and that the $CD8^+$ T cell response elicited by a protein formulated with RAS is equivalent to that elicited by immunization with DNA encoding the same protein (Chaitra *et al*., 2007).

We stress the following:

1) the type of immunity induced by NP-RAS reported in this work;
2) the fact that MPLA is already being used in human vaccine formulations;
3) the existence of a non-toxic derivative of TDM, which has shown excellent performance in pre-clinical toxicity assays (Fomsgaard *et al*., 2011) and is being studied in a Phase I clinical trial (Milicic *et al*., 2012). Taken together, these three facts suggest that this combination of adjuvants should be seriously considered for future developments of a T-cell influenza vaccine containing NP.

ACKNOWLEDGEMENTS

The authors are grateful to Ms. S. Rojana for her excellent technical work. This work was supported by the National Research Council of Argentina (CONICET) and the Agencia Nacional de Promoción Científica y Tecnológica of Argentina (PID2004-23112).

REFERENCES

Agger E.M., Rosenkrands I., Hansen J., Brahimi K., Vandahl B.S., Aagaard C., et al. (2008). Cationic liposomes formulated with synthetic mycobacterial cordfactor (CAF01): a versatile adjuvant for vaccines with different immunological requirements. *PLoS One*. **8**, e3116.

Bai Y., Ye L., Tesar D.B., Song H., Zhao D., Björkman P.J., et al. (2011). Intracellular neutralization of viral infection in polarized epithelial cells by neonatal Fc receptor (FcRn)-mediated IgG transport. *Proc. Natl. Acad. Sci. USA*. **8**, 108.

Berthoud T.K., Hamill M., Lillie P.J., Hwenda L., Collins K.A., Ewer K.J., et al. (2011). Potent CD8+ T-cell immunogenicity in humans of a novel heterosubtypic influenza A vaccine, MVA-NP+M1. *Clin. Infect. Dis*. **52**, 1-7.

Cargnelutti D.E., Sanchez M.V., Alvarez P., Boado L., Glikmann G., Mattion N., Scodeller E.A. (2012). Improved immune response to recombinant influenza nucleoprotein formulated with ISCOMATRIX. *J. Microbiol. Biotechnol*. **22**, 416-421.

Chaitra M.G., Nayak R., Shaila M.S. (2007). Modulation of immune responses in mice to recombinant antigens from PE and PPE families of proteins of Mycobacterium tuberculosis by the Ribi adjuvant. *Vaccine*. **10**, 7168-7176.

Coffman R.L., Seymour B.W., Lebman D.A., Hiraki D.D., Christiansen J.A., Shrader B., et al. (1988). The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol. Rev*. **102**, 5-28.

Coler R.N., Skeiky Y.A., Bernards K., Greeson K., Carter D., Cornellison C.D., et al. (2002). Immunization with a polyprotein vaccine consisting of the T-Cell antigens thiol-specific antioxidant, Leishmania major stress-inducible protein 1, and Leishmania elongation initiation factor protects against leishmaniasis. *Infect. Immun*. **70**, 4215-4225.

Constant S.L. and Bottomly K. (1997). Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Annu. Rev. Immunol*. **15**, 297-322.

Davidsen J., Rosenkrands I., Christensen D., Vangala A., Kirby D., Perrie Y., et al. (2005). Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from M. tuberculosis (trehalose 6,6 -dibehenate)-a novel adjuvant inducing both strong CMI and antibody responses. *Biochim. Biophys. Acta*. **10**, 22-31.

Dredge K., Marriott J.B., Todryk S.M., Dalgleish A.G. (2002). Adjuvants and the promotion of Th1-type cytokines in tumour immunotherapy. *Cancer. Immunol. Immunother*. **51**, 521-531.

Epstein S.L. (2006). Prior H1N1 influenza infection and susceptibility of Cleveland family study participants during the H2N2 pandemic of 1957: an experiment of nature. *J. Infect. Dis*. **193**, 49-53.

Fomsgaard A., Karlsson I., Gram G., Schou C., Tang S., Bang P., et al. (2011). Development and preclinical safety evaluation of a new therapeutic HIV-1 vaccine based on 18 T-cell minimal epitope peptides applying a novel cationic adjuvant CAF01. *Vaccine*. **16**, 7067-7074.

Guo L., Zheng M., Ding Y., Li D., Yang Z., Wang H., et al. (2010). Protection against multiple influenza A virus subtypes by intranasal administration of recombinant nucleoprotein. *Arch. Virol*. **155**, 1765-1775.

Jelinek I., Leonard J.N., Price G.E., Brown N., Meyer-Manlapat A., Goldsmith P.K., et al. (2011). TLR3-specific double-stranded RNA oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection. *J. Immunol*. **15**, 2422-2429.

Jurado A., Carballido J., Griffel H., Hochkeppel H.K., Wetzel GD. (1989). The immunomodulatory effects of interferon-gamma on mature B-lymphocyte responses. *Experientia*. **15**, 521-526.

Khader S.A., Bell G.K., Pearl J.E., Fountain J.J.,

RANGEL-MORENO J., CILLEY G.E., ET AL. (2007). IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. *Nat. Immunol.* **8**, 369-377.

LAMERE M.W., MOQUIN A., LEE F.E., MISRA R.S., BLAIR P.J., HAYNES L., ET AL. (2011a). Regulation of antinucleoprotein IgG by systemic vaccination and its effect on influenza virus clearance. *J. Virol.* **85**, 5027-5035.

LAMERE M.W., LAM H.T., MOQUIN A., HAYNES L., LUND F.E., RANDALL T.D., ET AL. (2011b). Contributions of antinucleoprotein IgG to heterosubtypic immunity against influenza virus. *J. Immunol.* **1**, 4331-4339.

MCMICHAEL A.J., MICHIE C.A., GOTCH F.M., SMITH G.L., MOSS B. (1986). Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes. *J. Gen. Virol.* **67**, 719-726.

MILICIC A., KAUR R., REYES-SANDOVAL A., TANG C.K., HONEYCUTT J., PERRIE Y., HILL A.V. (2012). Small cationic DDA: TDB liposomes as protein vaccine adjuvants obviate the need for TLR agonists in inducing cellular and humoral responses. *PLoS One*. **7**, e34255.

PRICE G.E., SOBOLESKI M.R., LO C.Y., MISPLON J.A., QUIRION M.R., HOUSER K.V., ET AL. (2010). Single-dose mucosal immunization with a candidate universal influenza vaccine provides rapid protection from virulent H5N1, H3N2 and H1N1 viruses. *PLoS One*. **4**, e13162.

RAO M., MATYAS G.R., VANCOTT T.C., BIRX D.L., ALVING C.R. (2004). Immunostimulatory CpG motifs induce CTL responses to HIV gp140 oligomeric type I envelope protein. *Immunol. Cell. Biol.* **82**, 523-530.

RAVINDRAN R., BHOWMICK S., DAS A., ALI N. (2010). Comparison of BCG, MPL and cationic liposome adjuvant systems in leishmanial antigen vaccine formulations against murine visceral leishmaniasis. *BMC Microbiol.* **24**, 181.

RIMMELZWAAN G.F., FOUCHIER R.A., OSTERHAUS A.D. (2007). Influenza virus-specific cytotoxic T lymphocytes: a correlate of protection and a basis for vaccine development. *Curr. Opin. Biotechnol.* **18**, 529-536.

ROBUCCIO J.A., GRIFFITH J.W., CHROSCINSKI E.A., CROSS P.J., LIGHT T.E., LANG C.M. (1995). Comparison of the effects of five adjuvants on the antibody response to influenza virus antigen in guinea pigs. *Lab. Anim. Sci.* **45**, 420-426.

SCHOENEN H., BODENDORFER B., HITCHENS K., MANZANERO S., WERNINGHAUS K., NIMMERJAHN F., ET AL. (2010). Cutting edge: is essential for recognition mincle and adjuvanticity of the mycobacterial cord factor trehalose and its synthetic analog-dibehenate. *J. Immunol.* **15**, 2756-28760.

SIN J.I., KIM J., PACHUK C., WEINER D.B. (2000). Interleukin 7 Can Enhance Antigen-Specific Cytotoxic-T-Lymphocyte and/or Th2-Type Immune Responses In Vivo. *Clin. Diagn. Lab. Immunol.* **7**, 751-758.

THUENG-IN K., MANEEWATCH S., SRIMANOTE P., SONGSERM T., TAPCHAISRI P., SOOKRUNG N., ET AL. (2010). Heterosubtypic immunity to influenza mediated by liposome adjuvanted H5N1 recombinant protein vaccines. *Vaccine*. **24**, 6765-6777.

VANLANDSCHOOT P., MAERTENS G., JOU W.M., FIERS W. (1993). Recombinant secreted haemagglutinin protects mice against a lethal challenge of influenza virus. *Vaccine*. **11**, 1185-1187.

WATANABE R., YOO Y.C., HATA K., MITOBE M., KOIKE Y., NISHIZAWA M. ET AL. (1999). Inhibitory effect of trehalose dimycolate (TDM) and its stereoisometric derivatives, trehalose dicorynomycolates , with low toxicity on lung metastasis of tumour cells in mice. *Vaccine*. **17**, 1484-1492.

YIP H.C., KARULIN A.Y., TARY-LEHMANN M., HESSE M.D., RADEKE H., HEEGER PS. ET AL. (1999). Adjuvant-guided type-1 and type-2 immunity: infectious/non-infectious dichotomy defines the class of response. *J. Immunol.* **1**, 3942-3949.

ZHOU D., WU T.L., LASARO M.O., LATIMER B.P., PARZYCH E.M., BIAN A. ET AL. (2010). A universal influenza A vaccine based on adenovirus expressing matrix-2 ectodomain and nucleoprotein protects mice from lethal challenge. *Mol. Ther.* **18**, 2182-2189.