Cytokine 148 (2021) 155703

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Toward a universal influenza virus vaccine: Some cytokines may fulfill the request

Mojtaba Taheri [a], Mehran Nemattalab [b,c], Mohammad Mahjoob [b], Elham Hasan-alizadeh [d], Nina Zamani [e], Iraj Nikokar [f], Mehdi Evazalipour [g], Bahram Soltani Tehrani [h], Mohammad Shenagari [b,h,*]

[a] Department of Biotechnology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[b] Department of Microbiology, Faculty of Medicine, Guilan University of Medical Sciences, Rasht, Iran
[c] Department of Pharmacology, School of Pharmacy, Guilan University of Medical Sciences, Rasht, Iran
[d] Faculty of Medicine, Guilan University of Medical Sciences, Rasht, Iran
[e] Department of Microbiology, University of Tehran, Tehran, Iran
[f] Medical Biotechnology Research Center, School of Paramedicine, Guilan University of Medical Sciences, Rasht, Iran
[g] Department of Pharmaceutical Biotechnology, School of Pharmacy, Guilan University of Medical Sciences, Rasht, Iran
[h] Cellular and Molecular Research Center, Faculty of Medicine, Guilan University of Medical Sciences, Rasht, Iran

ARTICLE INFO

Keywords:
Universal influenza virus vaccine
Nucleoprotein
Molecular Adjuvant
Cross-reactive cellular immunity
Interleukin-18

ABSTRACT

The influenza virus annually causes widespread damages to the health and economy of the global community. Vaccination is currently the most crucial strategy in reducing the number of patients. Genetic variations, the high diversity of pandemic viruses, and zoonoses make it challenging to select suitable strains for annual vaccine production. If new pandemic viruses emerge, it will take a long time to produce a vaccine according to the new strains. In the present study, intending to develop a universal influenza vaccine, new bicistronic DNA vaccines were developed that expressed NP or NPm antigen with one of modified IL-18/ IL-17A/ IL-22 cytokine adjuvants. NPm is a mutant form of the antigen that has the ability for cytoplasmic accumulation. In order to investigate and differentiate the role of each of the components of Th1, Th2, Th17, and Treg cellular immune systems in the performance of vaccines, Treg competent and Treg suppressed mouse groups were used. Mice were vaccinated with Foxp3-FC immunogen to produce Treg suppressed mouse groups. The potential of the vaccines to stimulate the immune system was assessed by IFN-γ/IL-17A Dual FluoroSpot. The vaccine's ability to induce humoral immune response was determined by measuring IgG1, IgG2a, and IgA-specific antibodies against the antigen. Kinetics of Th1, Th2, and Th17 cellular immune responses after vaccination, were assessed by evaluating the expression changes of IL-17A, IFN-γ, IL-18, IL-22, IL-4, and IL-2 cytokines by semi-quantitative real-time RT-PCR. To assess the vaccines' ability to induce heterosubtypic immunity, challenge tests with homologous and heterologous viruses were performed and then the virus titer was measured in the lungs of animals. Evaluation of the data obtained from this study showed that the DNA-vaccines coding NPm have more ability to induces a potent cross-cellular immune response and protective immunity than DNA-vaccines coding NP. Although the use of IL-18/ IL-17A/ IL-22 genetic adjuvants enhanced immune responses and protective immunity, Administration of NPm in combination with modified IL-18 (Igk-mIL18-IgFC) induced the most effective immunity in Treg competent mice group.

1. Introduction

According to the World Health Organization (WHO), annual influenza virus epidemics worldwide affect one billion people, leading to approximately 3–5 million severe disease cases and 290,000–650,000 deaths [1]. Influenza pandemics can have more dangerous consequences. After four decades of using Amantadine in the prevention and treatment of influenza infections, resistance to the drug has increased. Neuraminidase inhibitors are currently used to treat and control influenza viruses, although growing reports of viruses are resistant to these drugs, especially Oseltamivir [2,3]. Given the growing resistance of influenza virus to antiviral drugs and the possibility of

* Corresponding author.

https://doi.org/10.1016/j.cyto.2021.155703
Received 30 January 2021; Received in revised form 6 September 2021; Accepted 7 September 2021
Available online 20 September 2021

pandemics and epidemics, vaccination seems to be the most effective way to control this viral disease.

Existing influenza virus vaccines mainly induce humoral immune response against the virus's surface glycoproteins, namely neuraminidase [NA] and hemagglutinin [HA] [4]. The humoral immune response to these vaccines is temporary and transient, so that antibody levels may reduce before the end of the flu season to the point where they cannot induce protection [5]. Most of the antibodies induced by these vaccines target the head domain of HA. Since most antigenic drifts occur in this domain, it is necessary to change the vaccine formulation and re-inject every year [6,7]. These vaccines' protective efficacy is limited to seasonal influenza viruses and does not cover emerging strains of pandemic influenza virus [8]. The performance of these vaccines may be as low as 10% and generally not as high as 60% [9,10]. Two approaches are possible to deal with major pandemics of influenza virus and eliminate defects in existing vaccines: 1] Increase the rate of vaccine production against the epidemic and pandemic strains. 2] Production of a vaccine covers all strains and does not need to change the annual formulation [11]. The biggest problem with producing a permanent vaccine against the flu is that the viral proteins against which the body produces a strong immune response are highly variable; instead, the body makes a weak immune response against the virus's fewer variable proteins. Heterosubtypic immunity phenomenon is the basis for the design of "universal influenza vaccines" which states that targeting host immune responses against highly conserved epitopes of different virus strains may provide comprehensive protection against infection. The development of universal influenza virus vaccines may be possible by directing immune responses against protected viral epitopes [1]. Although antibody responses are highly specific and susceptible to virus escape by antigenic changes in drift and shift, cellular immune responses are cross-reactive. Studies have shown that antigen-specific T cells play a critical role in controlling IAV infection [12–14]. Previous presence of cross-reactive TCD_4^+ cells can be effective in rapidly producing type-specific neutralizing antibodies [15]. Bystander-activated CD_8^+ T cells can play a protective role by secreting cytokines such as interferon-gamma [16]. The alignment of nucleoprotein genes of various virus strains reveals that the nucleoprotein is relatively conserved and has a maximum amino acid difference of 11% [17]. Due to the presence of sequences that stimulate the CTL response in the nucleoprotein, which is protected between different subtypes of type A influenza, the idea of designing a permanent vaccine based on nucleoprotein was formed in the minds of researchers [18]. The nucleoprotein has an NLS sequence in amino acids 327–347, which leads to the accumulation of this protein in the nucleus [19,20]. Some studies have shown that changes in amino acids 6–8 and their conversion to Ala-Ala-Ala, even without altering the NLS sequence, reduce nucleoprotein transport to the nucleus and were resulted its cytoplasmic accumulation [21,22]. Given that proteins accumulated in the cytoplasm can be presented more efficiently by the molecular machine responsible for antigen processing and presentation, NP mutants [NPm] is expected to be more capable of stimulating and inducing immune responses.

DNA vaccines can stimulate both the immune system's arms, are stable at ambient temperature, and can produce easily and cheaply on a large scale. The DNA sequence encoding the antigen clones or synthesizes in a short time in the expression vector, and the vaccine's performance can be improved by modifying the gene sequence or using adjuvant sequences in the expression vector [23–25]. IL-22 is a member of the IL-10 superfamily, which mainly secretes by T helper 17 cells, intrinsic lymphoid cells, innate natural killer cells [INK], and epithelial cells [26,27]. IL-22 limits premature apoptosis in epithelial cells and thus plays a role in maintaining mucosal surfaces' structural integrity and enables rapid regeneration of these cells [28]. The endogenous production of IL-22 during influenza infection reduces the risk of secondary bacterial infections [29]. Studies show that IL-22 plays an important role in host defense against mucosal viral infections [30,31]. IL-17A is one of the most important mediators in mucosal immunity that enhances the accumulation of inflammatory cells [32]. Studies illustrate that the main source of IL-17A production in the lungs during influenza infection is γδ T cells [33,34]. IL-18 is an important regulator of innate and acquired immune responses, especially in viral infections [35]. This cytokine is involved in inducing bystander activation of T cells [16]. This cytokine's prominent property in stimulating NK and CTL cells and subsequently increasing the lethal activity of these cells has led to the use of this cytokine in vaccine research and immunotherapy of tumors [36,37]. The critical role of regulatory T [Treg] cells in modulating immune responses against vaccines is clear. Several methods have been used for restricting Treg immune responses. Among them, stimulating immune responses against Treg cells expressing Foxp3 transcription factor is a Promising approach to decrease the frequency of Tregs. FoxP3 is the main transcription factor for Treg differentiation and function. In current study, we used the FoxP3-Fc fusion construct/protein to stimulating the immune responses against Treg cells. In the present study, to develop a universal influenza vaccine, bicistronic DNA vaccines capable of expressing the conserved antigen NP and NPm together with one of the modified cytokine adjuvants IL-18/ IL- 17/ IL-22 were designed and manufactured, and then the potential of these vaccines in stimulating the immune system and creating heterosubtypic immunity was evaluated in the Treg-competent and Treg-suppressed mouse models. The results showed that although the studied DNA vaccines could stimulate the cellular immune system and induce protective immunity, the NPm antigen-coding DNA vaccine with modified IL-18 [Igk-mIL18-IgFC] had greater potential for research in developing a universal flu vaccine. Regarding numerous articles have mentioned the role and plasticity of Th1, Th2 and Th17 responses to influenza candidate vaccines, the present study is intended to investigate these effects.

2. Materials and methods

2.1. Construction of bicistronic plasmids

In this study, pIRES2-eGFP bi-cistron vector [Clontech Laboratories Inc., Palo Alto, CA] used to make DNA vaccines. To enhance mouse IL-18 [mIL-18] secretion, the Immunoglobulin kappa [IgK] secretory signal sequence were inserted into the 5′ end of the mIL-18 gene coding sequence. In addition to increasing the half-life and effectiveness of biological adjuvants, the constant region of the heavy immunoglobulin chain [FCγ2a] was fused into 3′ end of the mice's coding sequence IL-18, IL-17A, IL-22 and Foxp3. The fabrication of $pIRES_2$-eGFP-IL-18 / IL-17A / IL-22 / $Foxp_3$ gene constructs and the process of purification and expression of FoxP3-Fc [IgG) fusion protein from BL_{21} has been described in previous studies [38,39]. For NP gene cloning, first the influenza A virus [strain A / Puerto Rico / 8/34 [H1N1]] in MDCK cell line was propagated and viral RNA were extracted by using QIAamp Viral RNA Mini Kit [QIAGEN] according to the manufacturer's instructions. cDNA synthesis was performed using an Applied Biosystems ™ High-Capacity cDNA Reverse Transcription Kit [Thermo]. The NP gene encoding sequence was amplified using Pfu enzyme [Thermo] and specific primers [Table 2. Primer sequences] by PCR and then cloned into pJET1.2 / blunt vector using CloneJET PCR Cloning kit [Thermo]. The NP mutant gene encoding sequence [NPm] was amplified by PCR using specific primers [Table 2. Primer sequences] and cloned into pJET1.2 / blunt vector. Plasmid pJET1.2-NP was used as a template to amplify the NPm sequence. To make the NP and NPm coding DNA-vaccines, we excised these two sequences from the pJET1.2 vector by digestion with BstxI / NotI enzymes, and we replace them with the eGFP sequence in the $pIRES_2$-EGFP-IL-18 / IL-17A / IL-22 vector. The accuracy of gene structures constructed by dual enzymatic digestion and sequencing was evaluated.

2.2. Immunofluorescence of modified NP protein

To evaluate NP and NPm genes' expression in eukaryotic cells, the

bicistronic DNA-vaccines that carries these two genes were transfected into HEK293T cell line using Lipofectamine 2000CD [Invitrogen] according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were washed with PBS and fixed with 4% paraformaldehyde. To increase permeability, cells treated with Triton X-100. The cells were then treated with an anti-NP murine monoclonal antibody [1/30, AbCAM] and fluorescein-isothiocyanate-conjugated goat anti-mouse IgG [1/50, AbCAM], respectively. Finally, the expression of NP and NPm proteins were evaluated by a fluorescent microscope.

2.3. Expression and biological activity assay of cytokines

Evaluation of expression and biological activity of mice cytokines IL-18, IL-22, IL-17A cloned in pIRES2-eGFP vector was described in a previous study [39,40]. In summary, DNA constructs were transfected into HEK293T cell line using Lipofectamine 2000CD [Invitrogen] according to the manufacturer's protocol, and the expression of cytokines by ELISA and Western blotting was investigated. To evaluate the biological activity of recombinant Igk / IL-18 / IgFc, IL-17A / IgFc and IL-22 / IgFc cytokines, DNA vaccines encoding the sequence of these cytokines using Lipofectamine 2000CD [Invitrogen] were transfected into the KG-1 macrophage cell line, according to the company's protocol. Then in the supernatant of cells transfected with Igk/ IL-18/ IgFc coding plasmids cytokine IFN-γ [U-CyTech Bioscience; Netherlands] and in the supernatant of cells transfected with DNA vaccines carrying IL-17A / IgFc and IL-22 / IgFc sequences, cytokine IL-6 concentration was assessed by ELISA [U-CyTech Bioscience, Netherlands]. Plasmid pIRES2-Foxp3/ IgFc was prepared as a gift from another research group. This DNA construct contained the mouse truncated foxp3 gene [lacks nuclear localization signal] fused to the IgG Fc fragment. The expression and purification process of recombinant Foxp3 has already been described [38,41].

2.4. Virus culture

Mouse Adapted Influenza H3N2 [A/ X/ 47] and mouse-adapted H1N1 [A/ PR /8 /34 or PR8] viruses were obtained along with MDCK cell line from Pasteur Institute of Iran. MDCK cell line was amplified in DMEM [Gibco] culture medium and standard eukaryotic cell culture conditions. The viral suspension was prepared with MOI = 0.001 from each of the viruses. The flask medium containing MDCK cells was drained, and the cells were washed with PBS. The T75 flask was inoculated with 2 ml and the T25 flask with 500 μl of viral suspension. The flasks were incubated for one hour at 37 °C, then the viral suspension was drained from the flask and the DMEM medium containing 10% TPCK was added to it. On the second and third days after inoculation, the virus titer was measured by HA test. After observing CPE due to Virus propagation, the culture medium contained virus were harvested. Virus titer was determined by HI and $TCID_{50}$ tests, and then the virus stock was kept at −80 °C.

2.5. Immunization schedule

The present study has ethics approval from the research ethics committee of Guilan University of Medical Sciences. We performed all its In Vivo tests following ethics guidelines and standard protocols. In this study, 5-week-old female inbred BALB/c mice purchased from the Laboratory Animal Center of the Pasteur Institute of Tehran [Karaj, Iran] and transferred to the Laboratory Animal Care Center Faculty of Pharmacy of the University of Guilan medical sciences. By the seven-week-old, the mice were randomly divided into 18 twenty-eight groups. For anti-Treg vaccination, nine groups out of 18 groups, in day zero, received 100 μg of pIRES-FoxP3 DNA-vaccine in 100 μl of endotoxin-free PBS, by IM injection in quadriceps. The groups then received 100 μl of recombinant protein [20 mg] twice [weeks 2 and 4] and 100 μl of IFA [Incomplete Freund's adjuvant] by injecting SC into the back of the neck. Influenza DNA vaccines were injected in different groups according to Table 1 three times with an interval of two weeks. Each mouse received 100 μg of endotoxin-free DNA vaccine extracted by Endo Free Plasmid Giga Kit [Qiagen] in 100 μl of endotoxin-free PBS using subcutaneously injection. Two weeks after the last injection, eight mice in each group were tested in virus challenging experiments, and another ten mice were used in immunoassays tests.

2.6. Antibody titration

After two weeks of the last transfusion, we took a blood sample from the tail vein of vaccinated mice to measure IgG1 and IgG2a concentrations by ELISA [Nunc GmbH, Germany]. First, the 96-well ELISA plate were coated with recombinant NP protein [Cat: 11675-V08B, Sino Biological]. For this purpose, 100 μl of borate-buffered saline containing 10 μg/ mL of recombinant antigen was added to each well, and then the plate was covered with foil and incubated overnight at 4 °C. PBS buffer containing 2% BSA added to each plate well for blocking, and were incubated at 37 °C for one hour. The wells then were washed with PBS containing 0.05% Tween 20. Then 50 μl of serially diluted mouse serum [1: 100 in PBS + BSA] was added to each well. The plate was incubated

Table 1
Immunization protocols.

Vaccination Groups		Day 0	Week 2	Week 4	Week 6	Week 8	Week 10
1	**NP**	**pIRES-eGFP**	**BSA**	**BSA**	**NP**	**NP**	**NP**
2	**NPm**	**pIRES-eGFP**	**BSA**	**BSA**	**NPm**	**NPm**	**NPm**
3	**Treg⁻ NP**	**pIRES-FoxP3-Fc**	**rFoxp3-IFA**	**rFoxp3-IFA**	**NP**	**NP**	**NP**
4	**Treg⁻ NPm**	**pIRES-FoxP3-Fc**	**rFoxp3-IFA**	**rFoxp3-IFA**	**NPm**	**NPm**	**NPm**
5	**IL-18/ NP**	**pIRES-eGFP**	**BSA**	**BSA**	**IL-18/ NP**	**IL-18/ NP**	**IL-18/ NP**
6	**IL-18/ NPm**	**pIRES-eGFP**	**BSA**	**BSA**	**IL-18/ NPm**	**IL-18/ NPm**	**IL-18/ NPm**
7	**Treg⁻ IL-18/ NP**	**pIRES-FoxP3-Fc**	**rFoxp3-IFA**	**rFoxp3-IFA**	**IL-18/ NP**	**IL-18/ NP**	**IL-18/ NP**
8	**Treg⁻ IL-18/ NPm**	**pIRES-FoxP3-Fc**	**rFoxp3-IFA**	**rFoxp3-IFA**	**IL-18/ NPm**	**IL-18/ NPm**	**IL-18/ NPm**
9	**IL-17/ NP**	**pIRES-eGFP**	**BSA**	**BSA**	**IL-17/ NP**	**IL-17/ NP**	**IL-17/ NP**
10	**IL-17/ NPm**	**pIRES-eGFP**	**BSA**	**BSA**	**IL-17/ NPm**	**IL-17/ NPm**	**IL-17/ NPm**
11	**Treg⁻ IL-17/ NP**	**pIRES-FoxP3-Fc**	**rFoxp3-IFA**	**rFoxp3-IFA**	**IL-17/ NP**	**IL-17/ NP**	**IL-17/ NP**
12	**Treg⁻ IL-17/ NPm**	**pIRES-FoxP3-Fc**	**rFoxp3-IFA**	**rFoxp3-IFA**	**IL-17/ NPm**	**IL-17/ NPm**	**IL-17/ NPm**
13	**IL-22/ NP**	**pIRES-eGFP**	**BSA**	**BSA**	**IL-22/ NP**	**IL-22/ NP**	**IL-22/ NP**
14	**IL-22/ NPm**	**pIRES-eGFP**	**BSA**	**BSA**	**IL-22/ NPm**	**IL-22/ NPm**	**IL-22/ NPm**
15	**Treg⁻ IL-22/ NP**	**pIRES-FoxP3-Fc**	**rFoxp3-IFA**	**rFoxp3-IFA**	**IL-22/ NP**	**IL-22/ NP**	**IL-22/ NP**
16	**Treg⁻ IL-22/ NPm**	**pIRES-FoxP3-Fc**	**rFoxp3-IFA**	**rFoxp3-IFA**	**IL-22/ NPm**	**IL-22/ NPm**	**IL-22/ NPm**
17	**Treg⁻**	**pIRES-FoxP3-Fc**	**rFoxp3-IFA**	**rFoxp3-IFA**	**BSA**	**BSA**	**BSA**
18	**mock**	**pIRES-eGFP**	**BSA**	**BSA**	**pIRES-eGFP**	**pIRES-eGFP**	**pIRES-eGFP**

BSA: Bovine SerumAlbumin, pIRES-eGFP: plasmid backbone, rFoxp3-IFA: recombinant Foxp3 + Incomplete Freund Adjuvant, Treg-: Treg suppressed.

Table 2
Primer sequences.

Target	Forward	Reverse
NP (Cloning Primers)	TAGTCACCACAACCATGGCGTCCCAAGGCACCAA (BstxI)	TAGTGTGCGGCCGCTTAATTGTCGTACTCCTCTG (NotI)
NPm (Cloning Primers)	TAGTCACCACAACCATGGCGTCCCAAGGCACCAA (BstxI)	TAGTGTGCGGCCGCTTAATTGTCGTACTCCTCTG (NotI)
IL-17a	GTGTCTCTGATGCTGTTG	AACGGTTGAGGTAGTCTG
IL-22	CGATTGGGGAACTGGACCTG	GGACGTTAGCTTCTCACTTT
IFN- γ	AGAAACATTCAGAGCTGCAG	TGTATAGGGAAGCACCAGGTG
IL-8	GTG CAG TTT TGC CAA GGA GT	TTA TGA ATT CTC AGC CCT CTT CAA AAA CTT CTC
IL-2	CCTGAGCAGGATGGAGAATTACA	TCCAGAACATGCCGCAGAG
IL-4	ACA GGA GAA GGG ACG CCA T	GAA GCC CTA CAG ACG AGC TCA
β-actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT

for one hour at 37 °C. The wells were washed four times with PBS. Horseradish Peroxidase [HRP] -conjugated anti-mouse IgG1 and IgG2a antibodies [SouthernBiotech] were added to each well and the plate was incubated for one hour at room temperature. The wells were drained and washed with PBS three times. After adding the substrate, the OD was measured at 405 nm by ELISA reader. To determine the Anti NP IgA titer, a quarter of lung tissue homogenized in PBS containing Protease Inhibitor Cocktail [Roche, Germany] and 0.05 M ethylenediaminetetraacetic acid. After centrifugation, a 1: 100 dilution of the supernatant was prepared and added to the ELISA plate coated with the recombinant NP protein. Finally, anti-mouse IgA [BD Pharmingen, Franklin Lakes, NJ, USA] was added to the plates, and the IgA titer similar to IgG determined after adding the substrate.

2.7. IL-17a/IFN-γ dual color FluoroSpot

Two weeks after the last injection, ten mice from each group were randomly selected and were sacrificed under ethical principles. After surgery, spleen of each animal was removed, and separately the spleen tissue was homogenized, and the cells released by a cell homogenizer. RBCs were lysed using ammonium chloride buffer [0.16 M NH4Cl, 0.17 M Tris]. The cells then were washed twice with RPMI-1640 [Gibco] medium and cultured on a 4-cell plate. Cells were cultured and maintained in for 40 h in RPMI medium with 10% FBS, 10 μg/ ml synthetic peptide [TYQRTRALV] and 1% Pen/strep at 37 °C with 5% CO2, 95% moisture. We used these cells for FluoroSpot and Real-time PCR tests. Evaluation of specific cellular immune responses was performed using IFN-γ / IL-17A Dual FluoroSpot assay kit [Mabtech, Sweden] according to the manufacturer's protocol. Briefly, 100 μl capture antibody was added to each well of 96-well plates, and plates were stored overnight at 4 °C. After washing, 2.5 × 105 stimulated spleen cells were counted and added to each well and incubated overnight in RPMI-1640 medium containing anti-CD28 [0.2 μg / ml] and synthetic peptide [10 μg / ml]. After 12 h, the wells were washed with PBS then 100 μl PBS-0.1% BSA containing diagnostic antibodies [R4-6A2-BAM diluted 1: 200 and 1 g / ml of IL17-II-biotin] were added to each well. After 2 h, the wells were washed with PBS. Anti-BAM-490 and SA-550 were diluted 1: 200 in PBS-0.1% BSA then 100 μl of it was added to each well. The plate was incubated for one hour in the dark at room temperature. After washing the plate with PBS, 50 μl of Fluorescence enhancer-II was added to each well. After 15 min of incubation in the dark at room temperature, the wells were drained, and the plate was dried in the dark. Fluorescence-forming cells [FFCs] were counted under a FluoroSpot Reader and Analyzer [Mabtech, Sweden]. The FluoroSpot results were represented as Fluorescence-Forming Units [FFU]/1 × 106 cells and were reported as fold of induction [FOI] compared to the mock control.

2.8. Homologous and heterologous lethal challenges

Mouse Adapted Influenza A / X / 47 [H3N2] and mouse-adapted H1N1 [A / PR / 8/34 or PR8] viruses were used for challenge test. To calculate CCID50 [50% cell culture infectious dose], a serial dilution of log 10 was prepared from each virus and inoculated into MDCK cell line. 5MLD50 [50% mouse lethal dose] were measured after intranasal inoculation with different amounts of each virus by the Reed-Muench method. Two weeks after the last immunization, the mice were anesthetized with ketamine/ xylazine, and challenged with 50 μl of PBS buffer containing 5MLD50 influenza virus intranasally. Two days after the challenge test, four animals from each group were randomly sacrificed and their lungs were isolated using aseptic techniques. After the lung homogenization of vaccinated animals, the cell-free supernatant of each lung was used to evaluate changes in virus titer by plaque assay. In other animals in each group, changes in weight and death rate were recorded up to 14 days after the challenge.

2.9. Cytokine assay by Real-time PCR

We evaluated the changes in the expression level of IL-17A, IFN-γ, IL-18, IL-22, IL-4 and IL-2 cytokines in response to vaccination by a semi-quantitative real-time RT-PCR test. First 10^5 stimulated spleen cells were counted, and their RNA was extracted using RNeasy Plus Mini Kit [Qiagen, Germany] according to the manufacturer's protocol. Then cDNA was synthesized using the QuantiTect Reverse Transcription Kit [Qiagen, Germany]. Real-time PCR performed using Maxima SYBR Green / ROX qPCR Master Mix [Thermo Fisher Scientific, USA] and StepOnePlus ™ Real-Time PCR System [ABI, USA]. β-actin was used as a housekeeping gene to normalize the data. After the normalization, changes in gene expression compared to the control group were calculated using the formula $2^{-\Delta\Delta CT}$. The specific primers used, listed in Table 2. All the experimental samples were reported as an n-fold change relative to the calibrator.

2.10. Statistical analysis

The statistics were analyzed using GraphPad InStat software. One-way ANOVA and the Student-Newman-Keuls test were performed using SigmaPlot (Systat Software, San Jose, CA, USA] to compare statistically significant differences between the groups. All the data were reported as the mean ± SD. The comparative analyses were done by one-way analysis of variance (ANOVA]. The Tukey-Kramer's correction was also applied when required for multiple comparisons. Survival rates were analyzed by Kaplan–Meier and log-rank tests. Differences were considered statistically significant when P value was less than 0.05.

3. Results

3.1. Confirmation of cloning procedures and biological activity of molecular adjuvants

The expression capability of pIRES-FoxP3-Fc gene construct was investigated in a previous study and confirmed by SDS PAGE and western blot tests. In addition, the presence of FoxP3-Fc fusion protein (IgG] after purification was confirmed using specific antibodies [38]. The ability of the Prime/ boost vaccination strategy against FoxP3 to reduce the number of FoxP3 Treg cells was also confirmed [41]. The process of constructing pIRES2-eGFP-IL-18/IL-17A/ IL-22 gene

constructs and the biological activity of the cytokines expressed by them were also investigated, and confirmed by ELISA [39]. Cloning of NP and NPm coding sequences in pIRES2-eGFP-IL-18/ IL-17A/IL-22 gene constructs were confirmed by enzymatic digestion and sequencing. Fig. 1.

3.2. Immunofluorescence

In order to evaluate the expression of NP and NPm antigens at the protein level, the gene constructs encoding the antigens were transfected into HEK-293 t cells. Transfected cells were stained by indirect immunofluorescence staining using specific antibodies against NP. The gene construct expressing the fluorescent protein eGFP [pIRES2-EGFP] was used as a transfection control. Fluorescent microscope images clearly show the expression of NP and NPm antigens in transfected HEK-293 t cells (Fig. 2).

3.3. Antibody responses in immunized groups

The results of the evaluation of IgG1 and IgG2a antibodies against NP (Fig. 3) show that the level of antibody in the groups receiving the bicistronic vaccines encoding NP and NPm compared to the mock and Treg groups was meaningfully higher ($P < 0.001$), which could indicate the ability of bicistronic vaccines to express antigen and thus induce a strong specific humoral immune response. IgG2a levels in groups receiving vaccines encoding IL-18 modified cytokine along with NP and NPm antigens were significantly higher than those in groups vaccinated with vaccine coding NP, and NPm antigens alone ($P < 0.001$). The highest IgG2a level was measured in the Treg group vaccinated with IL-18 / NPm, which could indicate the role of IL-18 in the shift of immune responses to Th1. The IgG2a/ IgG1 ratio was higher in Treg suppressed groups than in Treg competent groups with same treatment situation; this indicating the effectiveness of Treg cell suppression in boosting Th1 cellular immune responses. SIgA levels in the groups receiving NP and NPm coding vaccines were significantly higher than the mock and Treg groups ($P < 0.001$), which could be evidence of the ability of this antigen in inducing systemic and mucosal humoral immune responses. Among these groups, the level of SIgA in the groups receiving vaccines coding modified IL-22 and IL-17A cytokine along with NP and NPm antigens was significantly higher than groups, receiving the NP and NPm antigen-coding vaccines alone ($P < 0.001$), which indicated the ability of these cytokines to enhance the mucosal humoral immune response. $Treg^{-}$ IL-17/NPm group showed the highest level of SIgA.

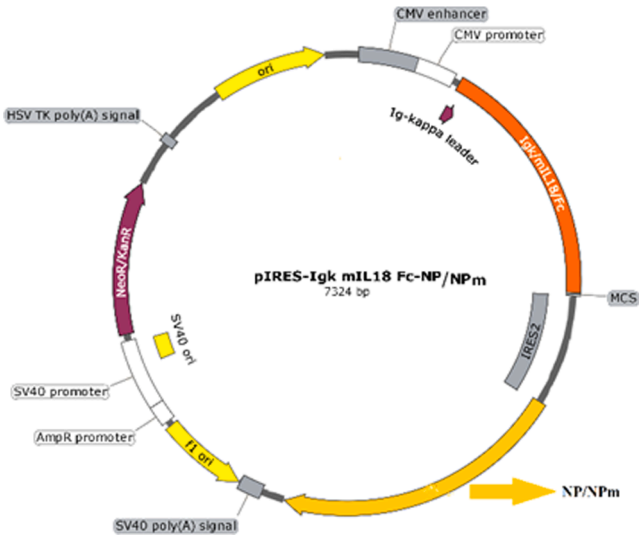

Fig. 1. The schematic map of the constructed IL-18/NP plasmid.

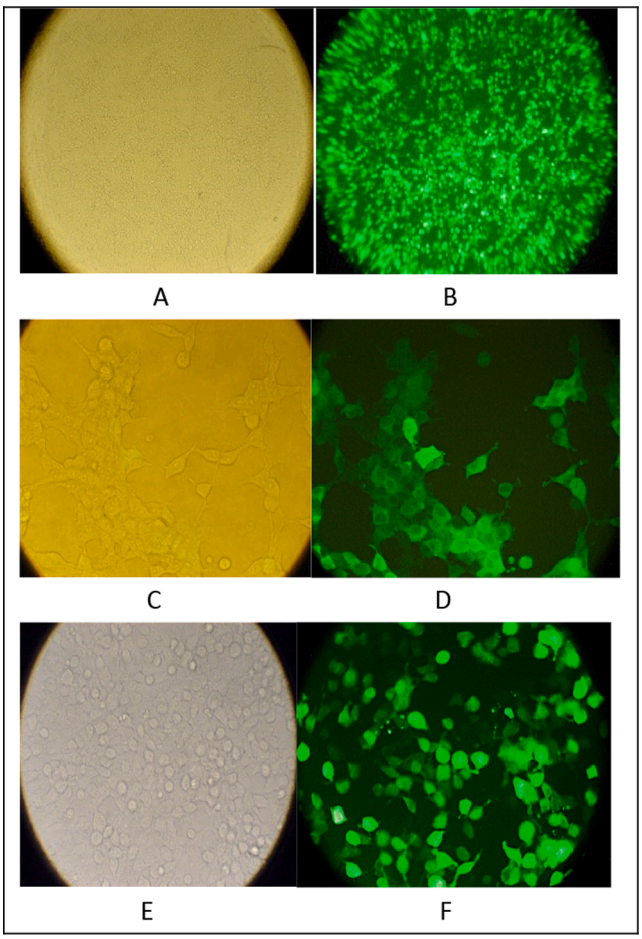

Fig. 2. Evaluate of expression and localization of NP and NPm antigens. HEK 293 cell line first were transfected with the gene constructs encoding NP and NPm proteins. In order to control the transfection process, the pIRES2-EGFP construct was transfected into HEK 293 cell line, the fluorescent images showed high-rate transfection (A and B). Then, in order to evaluate the expression and localization of the NP and NPm antigens, cells transfected with gene constructs encoding these proteins were indirectly immunostained with specific antibodies against NP. The result of indirect immunofluorescent staining showed preferential placement of NP antigen in the nucleus (C and D) and NPM in the cytoplasm (E and F).

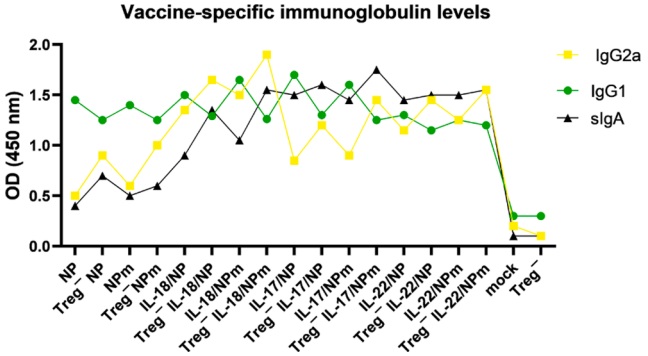

Fig. 3. ELISA tests for NP-specific serum IgG1, IgG2a and lung SIgA levels. The blood and lungs were sampled two weeks after the last immunization. The sera (1:100 diluted) and lungs samples were tested for NP-specific antibodies. This figure showed that the immunization with the IL-18/NPm in Treg suppressed situation compared with immunization with the NPm alone in Treg competent situation evoked significantly elevated lung IgG2a.

3.4. Protection against infection

The efficacy of vaccines in inducing protection against challenge with H1N1 and H3N2 viruses was examined by evaluation changes in pulmonary virus titer, survival rate, and weight changes. The results showed that the H1N1 and H3N2 virus titers in the lungs of animals which received vaccines encoding the NP and NPm antigen, were significantly lower than in the mock group ($p < 0.0001$), this indicates the ability of the immune response induced against NP antigen in creating protective immunity. In Treg IL-18/NPm and Treg IL-22/NPm groups, H3N2 and H1N1 virus titers were significantly lower than $Treg^-$ and $Treg^-$ NPm groups ($p < 0.0001$), which could be due to the effective action of modified IL-22 and IL-18 cytokines in enhancing protective immune responses. This decrease in H1N1 titer was observed in many Treg suppressed groups compared to the same Treg competent groups; however, a significant difference was observed only in the groups those received IL-22 / NPm and IL-22 / NP, ($p = 0.001–0.01$). The results of measuring the H3N2 virus titer have a similar pattern, so that, in general, the H3N2 titer in the Treg suppressed groups compared to same Treg competent groups was reduced. The difference is that in this case a significant reduce was observed in the groups receiving IL-18 / NPm and IL-22 / NPm ($p = 0.001–0.01$). This difference between Treg suppressed groups compared to same Treg competent groups could be a reason for the effectiveness of the Treg suppression method in enhancing the protective immune response (Fig. 4). Among the Treg competent groups, the IL-18/ NPm vaccinated group had the highest survival rate (100%) compared to the MOCK group when challenged with H1N1 (Fig. 5A) and H3N2 (Fig. 5C) ($P < 0.001$). Among Treg suppressed groups, IL-18 / NPm and IL-22 / NPm receiving groups with 80% survival rate had the best performance when challenged with a lethal dose of H1N1 (Fig. 5B). The IL-22 / NPm group also had the highest survival rate (80%) when challenged with the H3N2 virus (Fig. 5D). The results of recording the changes in animal weight show that among the Treg competent groups, the group receiving the IL-18 / NPm vaccine, and among the Treg suppressed group, the group receiving the IL-22 / NPm vaccine had the least number of changes compared to the MOCK group when challenged with homologous and heterologous viruses ($P < 0.001$) (Fig. 6).

3.5. Dual-color FluoroSpot results

The results of the fluorospot test showed that the number of IFN-γ-secreting cells in all vaccinated groups was significantly higher than the control groups ($p < 0.0001$). The number of IFN-γ-secreting cells in NPm group was considerably higher than NP ($P = 0.0048$). The number of IFN-γ-secreting cells was higher in Treg suppressed groups than in Treg competent groups. Among the Treg suppressed vaccinated groups, the IL-18 / NPm Treg group had the highest number of IFN-γ-secreting cells than the other groups ($p < 0.0001$) (Fig. 7. A). The $Treg^-$ IL-18/ NPm and $Treg^-$ IL-22/NPm groups had the highest number of dual IFN-γ / IL-17 secretory cells compared to the other groups ($p < 0.0001$). The number of dual IFN-γ / IL-17A secretory cells among Treg suppressed groups compared to the same Treg competent groups only for IL-18 / NPm and IL-18 / NP vaccine receiving groups was significantly increased. The number of dual IFN-γ / IL-17A cells was not significantly different between NP and NPm groups (Fig. 7. B). The number of IL-17 secreting cells in all immunized groups was significantly higher than mock and Treg control groups ($p < 0.0001$). The IL-17 / NP and IL-22 / NP groups had the highest number of IL-17A secretory cells (Fig. 7. C).

3.6. Th1 and Th2 and Th17 cytokine profile by Real-time PCR

The expression levels of Th1, Th2, and Th17 cytokines in all vaccinated groups show a significant increase compared to the control groups. The expression level of IFN-γ is higher in the Treg suppressed groups than in the Treg competent groups. The expression level of IFN-γ in the group receiving NPm coding vaccine was significantly higher than the group vaccinated with NP coding vaccine ($p < 0.0001$). The expression level of this cytokine was higher in the groups vaccinated with the modified IL-18 encoding vaccines than in the other groups. The highest level of IFN-γ expression observed in $Treg^-$ IL-18 / NPm group (Fig. 8. A). IL-4 (Fig. 8. B) and IL-8 (Fig. 8. C) expression levels were significantly higher in the groups receiving the IL-22 encoding bicistronic vaccines than in the other groups ($p < 0.0001$). IL-8 had a higher expression level in the Treg competent groups vaccinated with IL-22 / NPm and IL-22 / NP than the Treg suppressed groups immunized with the same vaccines ($p < 0.0001$) (Fig. 8. C). IL-12 expression levels were significantly higher in the groups receiving the IL-18 coding vaccines than in the other groups. The highest IL-12 expression level was observed in the $Treg^-$ group IL-18 / NPm ($p < 0.0001$) (Fig. 8. D). The Treg competent and Treg suppressed groups vaccinated with IL-17 / NP, and IL-17 / NPm had the highest expression levels of IL-17 (Fig. 8. E) and IL-22 (Fig. 8. F) in comparison to the control groups ($p < 0.0001$).

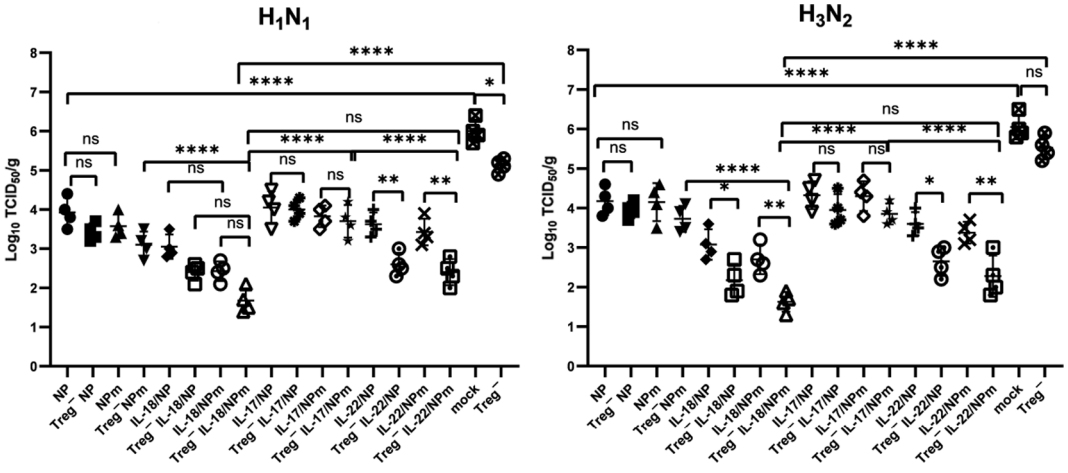

Fig. 4. Virus titers in the lungs of mice after challenge against H1N1 and H3N2 influenza viruses at day 2 post-infection. Animals were challenged with $5LD_{50}$ of homologous and heterologous viruses and the lungs were aseptically removed. The lungs were homogenized and cell free suspensions were collected and assessed for the lung virus titers by plaque assay. Data are depicted as the mean pfu per mg of lung homogenate + standard errors of the mean. ($p < 0.0001$: ****/ $p = 0.0001$ to 0.001: ***/ / $p = 0.001$ to 0.01: ** / $p = 0.01$ to 0.05: */ $p \geq 0.05$: Not significant).

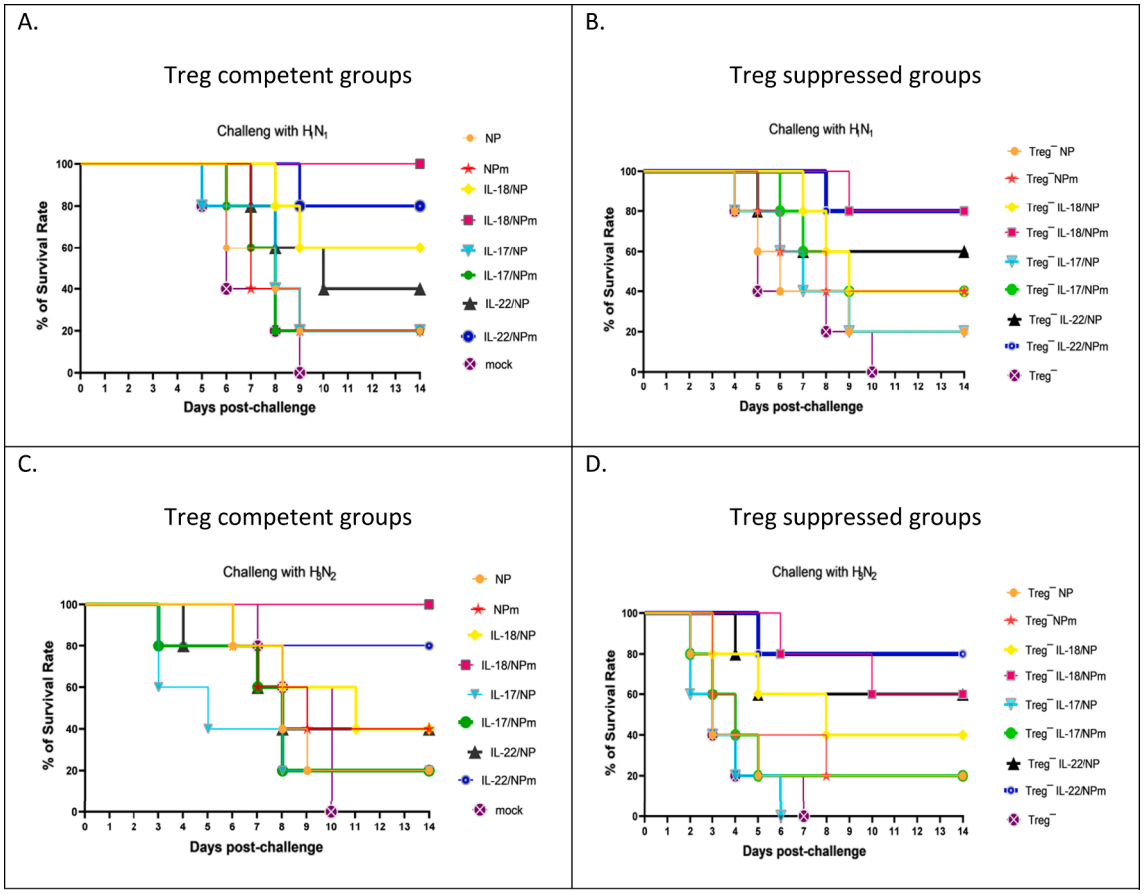

Fig. 5. The DNA vaccines were tested for their ability to provide protection against lethal challenge with two different influenza A viruses, and mice were monitored for survival for 14 days. Data have been analyzed by by log-rank test. As shown in the diagrams, Administration of IL-18/ NPm to Treg competent groups resulted in 100% protection against lethal challenge with both H_1N_1 (A) and H3N2 (C) viruses. In Treg suppressed groups, mice that vaccinated with IL-18/ NPm and IL-22/ NPm had the best survival rates after being challenged with the H1N1 virus (80%) (B) and the Treg suppressed mice that immunized with the IL-22/NPm DNA vaccine had a higher survival rate compared to other groups after the challenge with H3N2(D).

4. Discussion

The dominant influenza virus antigens, HA and NA, are highly variable and are constantly changing due to the high error rate of viral polymerase and the selective pressure of antibodies due to the human herd immunity mechanism [42]. Because vaccine production must begin months before the onset of seasonal flu to produce sufficient doses, the circulating virus may differ from the vaccine strain [8]. Numerous vaccination strategies to address the shortcomings of common influenza virus vaccines are being developed to produce a broad-spectrum cross-reactive universal vaccine. The current approach to the development of a universal vaccine for influenza virus has focused on shifting immune responses from the globular head domains to the immune subdominant domains such as the HA stem region or internal proteins such as nucleoprotein and matrix protein 1, which are highly conserved [43–45]. NP is one of the most important structural proteins of the virus that is highly conserved in different subtypes of each virus type [46,47]. This antigen has potent T-cell and CTL-stimulating epitopes that can induce protective immunity against the influenza virus [48]. The results of studies have shown that T cells that target these epitopes can be highly protective in mouse models [48–51]. Studies have shown that vaccination with DNA vaccine encoding NP induces potent cellular immune responses that can be boost by using cytokine adjuvants [52,53]. The fluorospot results, humoral immunity assessment, and relative quantitative real-time PCR in the present study also show that in the groups receiving the NP antigen-encoding vaccine alone compared to the control groups, indications for immune stimulation have increased significantly. NP has an nuclear localization signal at the N-terminus [17,54,55]. Studies have shown that the mutations in this sequence leads to the accumulation of NP in the cytoplasm [21,22]. Previous studies have shown that NP mutants, characterized by cytoplasmic accumulation, induce stronger immune responses, and provide greater protection against the influenza virus [22]. The present study results also show that NPm has more potential than NP for use as an immunogen in the vaccine composition and can induce a stronger cellular and humoral immune response. Since most of the organelles and molecular machines responsible for processing and presenting antigens are in the cytoplasm, proteins that have a cytoplasmic position are likely to be processed and presented more efficiently [22]. Therefore, the potential for further immunization of NPm is probably due to the increased supply of NPm epitopes by the antigen presentation pathways.

DNA vaccines against influenza infections are improving since the 1990s, but the initial excitement after success in mouse models declined due to poor performance in large animal models [25]. In many studies, various cytokines or their expression cassette have been used as biological adjuvants to increase the effectiveness of DNA vaccines [56]. The use of protein cytokines is not cost-effective because their expression and purification are expensive and require multiple injections [57]. Studies show that if the antigen and adjuvant genes expressed from one plasmid, the immune responses are much stronger. The simultaneous presentation of antigen and bioadjuvant by APC cells will lead to better stimulation of lymphocytes [58–60]. This feature could also increase the repeatability of the test and the reliability of the data. Therefore, to achieve this goal, the design of a bicistronic vector that can

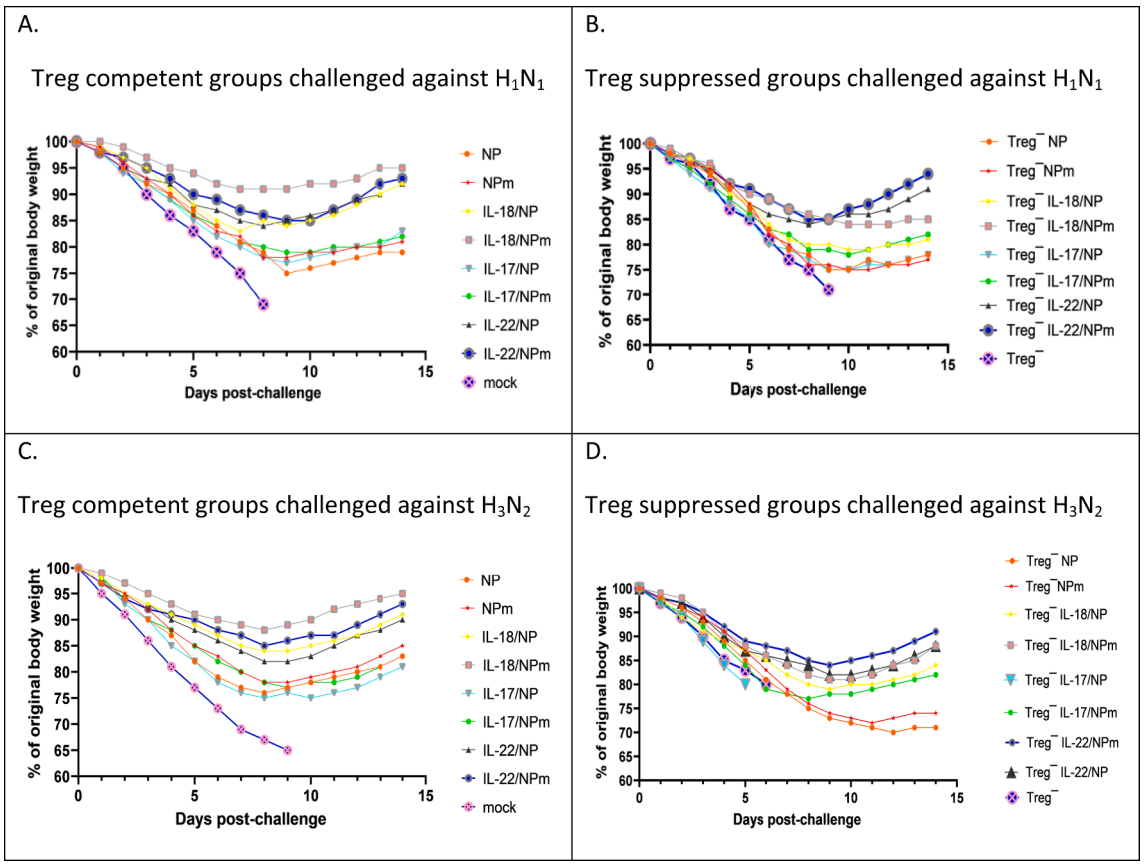

Fig. 6. In order to evaluate the effectiveness of DNA vaccines body weight of vaccinated mice was checked up to 14 days after challenge with H1N1 virus and H3N2. Among Treg competent groups, mice that received the IL-18/NPm DNA vaccine had the minimum weight loss after being challenged with the both homologous (A) and heterogeneous (C) influenza viruses but among the Treg suppressed groups, mice that vaccinated with IL-22/NPm had minimal weight loss after the challenge against H1N1 (B) and H3N2 (D) viruses.

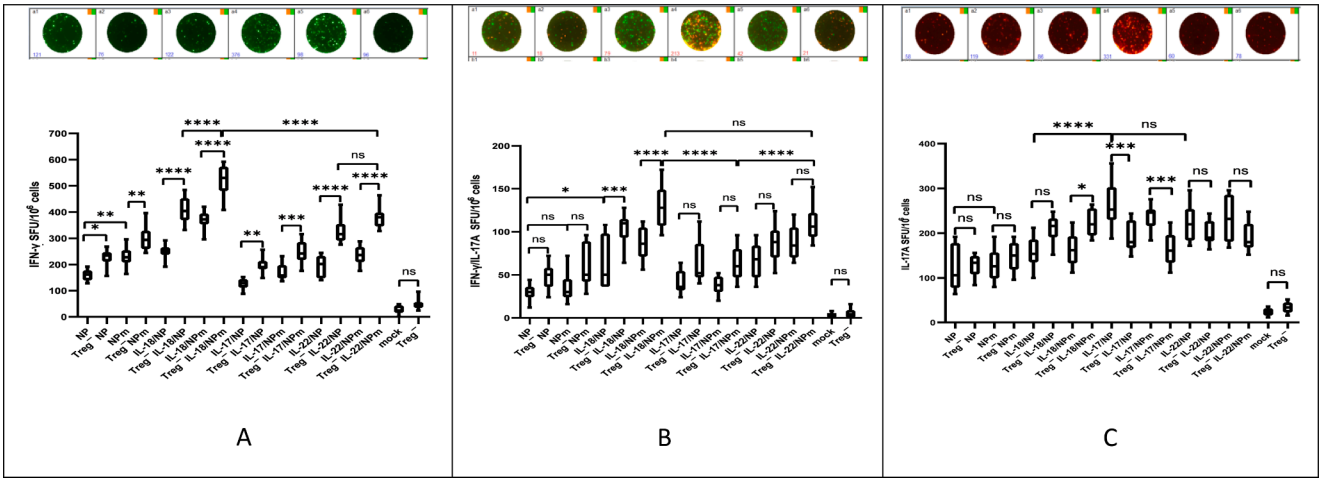

Fig. 7. Illustration of IFN-γ/IL-17A dual-color FluoroSpot assay images. Images were captured for IFNγ (*A*) (FITC filter) and used to generate the computerized overlay of the two filters showing double positive IFNγ + IL-17A cell responses (*central image*) and IL-17A (*right image*) (Cy3 filter). IFNγ, IFNγ + IL-17A, and IL-17A secreting cells are depicted as *green* (*left image*), *double* (*central image*), and *red* (*right image*) spots, respectively. The Treg suppressed IL-18/NPm administration has led to significantly higher frequencies of single IFN-γ-secreting T-cells in comparison to other groups (A). The higher proportions of dual IFN-γ/IL-17A secreting T cells were observed in Treg suppression groups that received IL18/NPm and IL22/NPm as compared to other groups (B). The strongest IL-17A level was seen in the mice that received IL-17A/NP (C). ($p < 0.0001$: ****/ $p = 0.0001–0.001$: ***/ $p = 0.001–0.01$: ** / $p = 0.01–0.05$: */ $p \geq 0.05$: Not significant).

simultaneously express antigen and adjuvant was considered in the present study. The vector used in this study was a bicistronic plasmid made by Clonetech called pIRES2-EGFP. In this study, MCS locus for cytokine gene cloning and the post-IRES locus for NP gene cloning were selected. The Kozak sequence was also used to enhance the NP and NPm genes expression in the design of primers used for cloning. The results of this study showed that this vector could express two genes simultaneously and on the other hand, the simultaneous expression of antigen

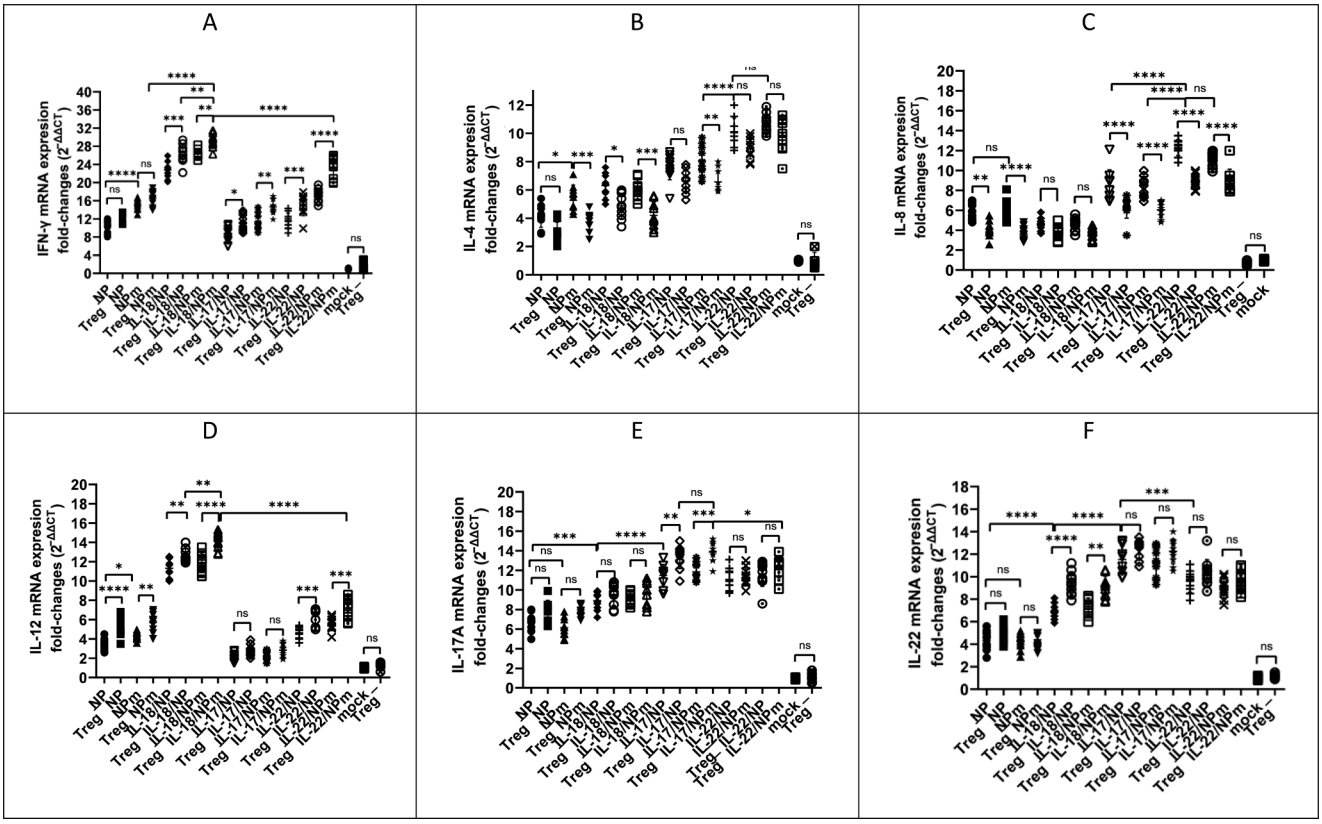

Fig. 8. Typical melting peaks obtained for each cytokine system applied to samples collected from mice #-1 at different points during the experiment. Comparison of the levels of cytokines as determined by SYBR-Green qRT-PCR. (A) Histograms representing the fold-changes ($2^{-\Delta\Delta CT}$) in the quantitation of cytokines in the vaccinated groups compared to the negative controls (samples collected before immunization). The level of IFN-γ in Treg suppressed group that received IL-18/ NPm were significantly higher than the other of groups. (B) IL-4 expression was higher in the groups that injected with IL-22 coding vaccines than in other groups. (C) IL-8 level of Treg competent groups that injected with IL-22/NP and IL22/NPm was significantly higher than the other groups. (D) IL-12 expression was increased in the groups that received modified IL-18 coding vaccine as adjuvant compared to other groups. (E) IL-17A cytokine expression was higher in the Treg suppressed animals vaccinated with IL-17/NP and IL-17/ NPm constructs than the Treg completed animals. (F) Groups that injected with IL-17/NP and IL-17/NPm could induce the highest levels of IL-22. ($p < 0.0001$: ****/ $p = 0.0001–0.001$: ***/ $p = 0.001–0.01$: ** / $p = 0.01–0.05$: */ $p \geq 0.05$: Not significant).

and adjuvant using a bicistronic vector can significantly increase the DNA vaccine's potential. One of the solutions that have been considered to increase the half-life and effectiveness of molecular adjuvants is the use of plasmids that express the desired adjuvant fused with the Immunoglobulin Heavy Chain Constant Region Gamma 2a [FCγ2a] [60,61]. Various hypotheses have been proposed to justify this increase in efficiency, the most plausible of which is that the presence of IgFc leads to bind recombinant molecules to Fc receptors at the surface of various cells involved in the immune response [such as B and T lymphocytes], stimulate the production and secretion of various cytokines/ chemokines by these cells [61]. In this condition the molecular adjuvants which were used also perform their biological activity independently. This study showed that cytokines IL-22, IL-17A, and IL-18 fused with FCγ2a have a biological activity after expression. Besides, the fluorospot and real-time PCR tests confirmed the high potential of these modified cytokines to exert adjuvant effects in vivo by augmentation of the immune responses.

IL-18 lacks a leader sequence. Incision induced by caspase-1 play an important role in facilitating the secretion of the mature and active form of IL-18 from the cytosol and cell membrane [62]. Although the use of the plasmid expressing mature form of IL-18 as a genetic adjuvant alongside the several DNA vaccines has been reported, the results show that without processing of Pro-IL-18 by caspase-1, the secretion of the mature IL-18 is highly inefficient, and limited to programmed cell death, cell apoptosis, or cell rupture [63]. In order to increase the secretion of IL-18 an accepted strategy is to bind the mature form of IL-18 to the secretion signal peptide of other proteins such as parathyroid hormone [64] or the kappa chain of immunoglobulin [65]. In this strategy, the plasmid expressing the mature form of IL-18 can be used as a molecular adjuvant while ensuring protein secretion. The present study results showed that IL-18 fused with kappa immunoglobulin chain after the successful expression is secreted from the cell and has biological activity. During natural viral infections, many T cells are activated in a cytokine-dependent manner and independent of T cell receptors. This phenomenon is known as "bystander activation". Recent studies have shown that mucosal-associated invariant T [MAIT] cells also can be activated by bystander effect during influenza infection and play a role in induction of protective immune responses in humans and mice [66,67]. Preliminary studies show that during IAV infections CD8+ memory T cells have been activated by bystander effect, attracted from the bloodstream to the airways of the lungs [68–70]. Despite not being specific for IAV, these memory CD8+ T cells express strong cytolytic capacity [69,70]. Bystander-activated T cells lack pathogen specificity but can nevertheless affect the immune response to infection. Modified IL-18 cytokine by secreting and stimulating IFN-γ expression can have a high potential in inducing the immune system's bystander activation mechanism and thereby enhancing immune responses. The present study results show that IFN-γ expression and secretion levels in the groups receiving the modified IL-18 coding vaccines were significantly increased compared to the other groups. The groups which received modified IL-18 coding vaccines had a higher survival rate [100%] not only when challenged with the lethal dose of the homologous virus, but also after challenge with the heterologous virus. In addition, homologous and heterologous virus titers in these animals' lungs were significantly lower than the

control group. Therefore, the potential of the modified IL-18 bio-adjuvant in inducing the immune system's bystander activation mechanism could play a vital role in the development of universal vaccines in future studies. IL-22 rapidly produces in lung tissue during IAV infection by natural killer cells [NK], non-conventional T cells [γδ T cells and natural killer T cells], and type 3 intrinsic lymphoid cells [type 3 innate lymphoid cells] [29,31,71,72]. Studies have shown that IL-22 has a protective effect on H3N2 and H1N1 influenza infections [31,72,73]. The ability of IL-22 to induce protective immune responses was also demonstrated in this study. Groups receiving the modified IL-22 coding vaccine showed high survival rates when challenged with the homologous and heterologous virus. The endogenous production of IL-22 during influenza infection reduces the susceptibility to secondary bacterial infections [29]. Its positive role in the respiratory system's fungal infections has also been reported [74,75]. IL-22 may also participate in airway epithelial regeneration and barrier repair [31,73]. These effects are exerted by inducing the expression of genes associated with epithelial cell proliferation and regeneration, antimicrobial peptides [Reg3β and Reg3γ], mucins, and chemotactic factors. Also, studies show that the protective role of IL-22 increases when combined with IL-18 or IFN-λ [30,76]. IL-17A can play both a protective and a detrimental role in viral infections. Some studies have shown that IL-17A attracted the CXCR5+ B cells into infected lungs [77]. Influenza virus infection naturally induces IL-17A expression and mice with IL-17A deficiency have lower survival rates and higher virus titers, indicating the protective role of IL-17A against the virus [77–79]. In contrast, another study showed that mice with IL-17RA deficiency after infection with the Influenza virus has a higher survival rate than wild mice [79]. These reports suggest that IL-17A produced in viral infections can increase tissue damage due to inflammation. In the present study, groups receiving IL-17A coding vaccines had a higher SIgA antibody levels and higher IgG1/ IgG2a ratio than other groups, which may indicate this cytokine's ability to improvement humoral response. These groups had the highest number of IL-17A secreting cells. Besides, the expression level of IL-22 and IL-17A genes in them had increased compared to other groups, which indicates the strengthening of Th17 immune responses. However, groups receiving IL-17A coding vaccines did not have the appropriate survival rate, virus titer, and weight change index after challenge with the homologous and heterologous virus. Therefore, it may conclude that although the induction of optimal levels of IL-17A may be beneficial, the high expression of this cytokine can induce tissue destruction during viral infection. In vaccine design strategies, maintaining homeostasis of the immune response to prevent the development of autoimmune and inflammatory diseases and, at the same time, enhance the effectiveness of the vaccine is of particular importance [80–82]. Although Tregs play a key role in reducing acute inflammatory viral infections by reducing tissue damage due to inflammation, they increase virus stability in chronic viral infections and suppress antiviral immune responses [83]. The results of a study show that immunization of BALB/ c mice with the PR8/ A/ 34 influenza virus vaccine leads to an increase in CD4+ Foxp3+ Treg cells and increases virus-specific in CD4+ Foxp3+ cells' evolution. Although increasing the number of Foxp3+ Treg cells did not alter PR8-specific primary B cells' response, it suppressed the responses of primary helper T cells and PR8-specific memories induced by vaccination. Conversely, T cell responses increased in the absence of CD4+ Foxp3+ Treg cells [84]. Another study showed CD4+ CD25+ FoxP3+ cells induce immunosuppression after influenza vaccine injection so that adoptive transfer of these cells reduced the protective immune function induced by formalin-inactivated H5N1 virus vaccination [85]. Human studies data also show that Tregs increase after influenza vaccine injection. Also, the level of TGF-β, which is one of the cytokines of the Treg index, has a negative correlation with the anti-influenza antibodies titer [86]. Because CD4+ helper T cells play a role in inducing protection against the flu, suppressing Treg cells' function before vaccination can increase the effectiveness of the flu vaccine. According to this theory, the Foxp3 vaccination strategy is used in this study to suppress Treg cells' function. The effectiveness of this method was proven in a previous study [41]. The present study results show that the expression levels of IFN-γ and IL-12 cytokines and IgG2a antibody titer were higher in the suppressed Treg vaccinated groups than the same Treg competent vaccinated groups. On the other hand, the results of virus titer measurements show that the homologous and heterologous virus titers in Treg suppressed vaccinated groups' lungs are lower than the same Treg competent vaccinated groups. These results could be a document to the effectiveness of the Foxp3 vaccination strategy in suppressing the Treg system and enhancing Th1 cellular immune responses. On the other hand, it was observed that IL-17A and IL-22 cytokines' expression level is also higher in some Treg suppressed vaccinated groups than in Treg competent vaccinated groups. This phenomenon may be due to inflammatory responses.

CRediT authorship contribution statement

Mojtaba Taheri: Writing – original draft, Investigation. **Mehran Nemattalab:** Writing – original draft, Investigation. **Mohammad Mahjoob:** Investigation. **Elham Hasan-alizadeh:** Investigation, Writing – review & editing. **Nina Zamani:** Writing – review & editing. **Iraj Nikookar:** Project administration. **Mehdi Evazalipour:** Project administration. **Bahram Soltani Tehrani:** Project administration. **Mohammad Shenagari:** Investigation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] R. Nachbagauer, P. Palese, Is a universal influenza virus vaccine possible? Ann. Rev. Med. 71 (1) (2020) 315–327.
[2] J. Yavarian, N.Z. Shafiei Jandaghi, F. Rezaei, T. Mokhtari-Azad, Adamantane and Neuraminidase resistant influenza A/H3N2 isolated in Iran from 2005 to 2013, Tehran Univ. Med. J. 72 (2014) 40–45.
[3] T. Lampejo, Influenza and antiviral resistance: an overview, Eur. J. Clin. Microbiol. Infect. Dis. 39 (7) (2020) 1201–1208.
[4] F. Krammer, The human antibody response to influenza A virus infection and vaccination, Nat. Rev. Immunol. 19 (6) (2019) 383–397.
[5] K.E.C. Ainslie, M. Haber, W.A. Orenstein, Challenges in estimating influenza vaccine effectiveness, Exp. Rev. Vaccines. 18 (6) (2019) 615–628.
[6] J.C. de Jong, W.E.P. Beyer, A.M. Palache, G.F. Rimmelzwaan, A.D.M.E. Osterhaus, Mismatch between the 1997/1998 influenza vaccine and the major epidemic A (H3N2) virus strain as the cause of an inadequate vaccine-induced antibody response to this strain in the elderly, J. Med. Virol. 61 (1) (2000) 94–99.
[7] O.A. Vogel, B. Manicassamy, Broadly protective strategies against influenza viruses: universal vaccines and therapeutics, Front. Microbiol. 11 (135) (2020).
[8] H. Kim, R.G. Webster, R.J. Webby, Influenza virus: dealing with a drifting and shifting pathogen, Viral Immunol. 31 (2) (2018) 174–183.
[9] E.A. Belongia, B.A. Kieke, J.G. Donahue, L.A. Coleman, S.A. Irving, J.K. Meece, M. Vandermause, S. Lindstrom, P. Gargiullo, D.K. Shay, Influenza vaccine effectiveness in Wisconsin during the 2007–08 season: comparison of interim and final results, Vaccine. 29 (38) (2011) 6558–6563.
[10] M.A. Rolfes, B. Flannery, J.R. Chung, A. O'Halloran, S. Garg, E.A. Belongia, et al., Effects of influenza vaccination in the United States during the 2017–2018 influenza season, Clin. Infect. Dis. 69 (11) (2019) 1845–1853.
[11] A.H. Ellebedy, R.J. Webby, Influenza vaccines, Vaccine. 27 (2009) D65–D68.
[12] S.R. McMaster, J.J. Wilson, H. Wang, J.E. Kohlmeier, Airway-resident memory CD8 T cells provide antigen-specific protection against respiratory virus challenge through rapid IFN-γ production, J. Immunol. 195 (1) (2015) 203–209.
[13] B. Piet, G.J. de Bree, B.S. Smids-Dierdorp, C.M. van der Loos, E.B.M. Remmerswaal, J.H. von der Thüsen, J.M.W. van Haarst, J.P. Eerenberg, A. ten Brinke, W. van der Bij, W. Timens, R.A.W. van Lier, R.E. Jonkers, $CD8^+$ T cells with an intraepithelial phenotype upregulate cytotoxic function upon influenza infection in human lung, J. Clin. Invest. 121 (6) (2011) 2254–2263.
[14] A. Pizzolla, L.M. Wakim, Memory T cell dynamics in the lung during influenza virus infection, J. Immunol. 202 (2) (2019) 374–381.
[15] E.G.M. Berkhoff, A.C.M. Boon, N.J. Nieuwkoop, R.A.M. Fouchier, K. Sintnicolaas, A.D.M.E. Osterhaus, et al., A Mutation in the HLA-B[*]2705-restricted NP₃₈₃₋₃₉₁ epitope affects the human influenza A virus-specific cytotoxic T-lymphocyte response in vitro, J. Virol. 78 (10) (2004) 5216.

[16] T.-S. Kim, E.-C. Shin, The activation of bystander CD8+ T cells and their roles in viral infection, Exp. Mol. Med. 51 (12) (2019) 1–9.
[17] A. Portela, P. Digard, The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication, J. Gen. Virol. 83 (Pt 4) (2002) 723–734.
[18] J. Kaiser, A one-size-fits-all flu vaccine? Science (New York, NY). 312 (5772) (2006) 380–382.
[19] R. Bullido, P. Gómez-Puertas, C. Albo, A. Portela, Several protein regions contribute to determine the nuclear and cytoplasmic localization of the influenza A virus nucleoprotein, J. Gen. Virol. 81 (Pt 1) (2000) 135–142.
[20] P. Digard, D. Elton, K. Bishop, E. Medcalf, A. Weeds, B. Pope, Modulation of nuclear localization of the influenza virus nucleoprotein through interaction with actin filaments, J. Virol. 73 (3) (1999) 2222–2231.
[21] G. Neumann, M.R. Castrucci, Y. Kawaoka, Nuclear import and export of influenza virus nucleoprotein, J. Virol. 71 (12) (1997) 9690–9700.
[22] K. Ohba, S. Yoshida, M.d. Zahidunnabi Dewan, H. Shimura, N. Sakamaki, F. Takeshita, N. Yamamoto, K. Okuda, Mutant influenza A virus nucleoprotein is preferentially localized in the cytoplasm and its immunization in mice shows higher immunogenicity and cross-reactivity, Vaccine. 25 (21) (2007) 4291–4300.
[23] P. Öhlschläger, M. Quetting, G. Alvarez, M. Dürst, L. Gissmann, A.M. Kaufmann, Enhancement of immunogenicity of a therapeutic cervical cancer DNA-based vaccine by co-application of sequence-optimized genetic adjuvants, Int. J. Cancer. 125 (1) (2009) 189–198.
[24] O. Haigh, J. Kattenbelt, M. Cochrane, S. Thomson, A. Gould, R. Tindle, Hepatitis B surface antigen fusions delivered by DNA vaccination elicit CTL responses to human papillomavirus oncoproteins associated with tumor protection, Cancer Gene Ther. 17 (10) (2010) 708–720.
[25] L.Y.Y. Lee, L. Izzard, A.C. Hurt, A review of DNA vaccines against influenza, Front. Immunol. 9 (2018) 1568.
[26] J.A. Dudakov, A.M. Hanash, M.R.M. van den Brink, Interleukin-22: immunobiology and pathology, Annu. Rev. Immunol. 33 (1) (2015) 747–785.
[27] K. Wolk, S. Kunz, E. Witte, M. Friedrich, K. Asadullah, R. Sabat, IL-22 increases the innate immunity of tissues, Immunity 21 (2) (2004) 241–254.
[28] C.A. Lindemans, M. Calafiore, A.M. Mertelsmann, M.H. O'Connor, J.A. Dudakov, R.R. Jenq, E. Velardi, L.F. Young, O.M. Smith, G. Lawrence, J.A. Ivanov, Y.-Y. Fu, S. Takashima, G. Hua, M.L. Martin, K.P. O'Rourke, Y.-H. Lo, M. Mokry, M. Romera-Hernandez, T. Cupedo, L.E. Dow, E.E. Nieuwenhuis, N.F. Shroyer, C. Liu, R. Kolesnick, M.R.M. van den Brink, A.M. Hanash, Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration, Nature 528 (7583) (2015) 560–564.
[29] S. Ivanov, J. Renneson, J. Fontaine, A. Barthelemy, C. Paget, E.M. Fernandez, F. Blanc, C. De Trez, L. Van Maele, L. Dumoutier, M.-R. Huerre, G. Eberl, M. Si-Tahar, P. Gosset, J.C. Renauld, J.C. Sirard, C. Faveeuw, F. Trottein, Interleukin-22 reduces lung inflammation during influenza A virus infection and protects against secondary bacterial infection, J. Virol. 87 (12) (2013) 6911–6924.
[30] P.P. Hernández, T. Mahlakõiv, I. Yang, V. Schwierzeck, N. Nguyen, F. Guendel, K. Gronke, B. Ryffel, C. Hölscher, L. Dumoutier, J.-C. Renauld, S. Suerbaum, P. Staeheli, A. Diefenbach, Interferon-λ and interleukin 22 act synergistically for the induction of interferon-stimulated genes and control of rotavirus infection, Nat. Immunol. 16 (7) (2015) 698–707.
[31] P. Kumar, M.S. Thakar, W. Ouyang, S. Malarkannan, IL-22 from conventional NK cells is epithelial regenerative and inflammation protective during influenza infection, Mucosal Immunol. 6 (1) (2013) 69–82.
[32] S. Xu, X. Cao, Interleukin-17 and its expanding biological functions, Cell. Mol. Immunol. 7 (3) (2010) 164–174.
[33] D. Gong, K. Farley, M. White, K.L. Hartshorn, C. Benarafa, E. Remold-O'Donnell, Critical role of serpinB1 in regulating inflammatory responses in pulmonary influenza infection, J. Infect. Dis. 204 (4) (2011) 592–600.
[34] C. Xue, M. Wen, L. Bao, H. Li, F. Li, M. Liu, Q.i. Lv, Y. An, X. Zhang, B. Cao, Vγ4(+)γδT cells aggravate severe H1N1 influenza virus infection-induced acute pulmonary immunopathological injury via secreting interleukin-17A, Front Immunol. 8 (2017) 1054, https://doi.org/10.3389/fimmu.2017.01054.
[35] S. Akira, The role of IL18 in innate immunity, Curr. Opin. Immunol. 12 (1) (2000) 59–63.
[36] D.H. Barouch, S. Santra, K. Tenner-Racz, P. Racz, M.J. Kuroda, J.E. Schmitz, S. S. Jackson, M.A. Lifton, D.C. Freed, H.C. Perry, M.-E. Davies, J.W. Shiver, N. L. Letvin, Potent CD4+ T cell responses elicited by a bicistronic HIV-1 DNA vaccine expressing gp120 and GM-CSF, J. Immunol. 168 (2) (2002) 562–568.
[37] M. Zavvar, A. Moshiri, F. Motevali, M.H. Pouriayevali, G. Bahramali, Construction of pIRES2 vector encoding truncated NS3 of HCV and IL-18 for DNA vaccine studies, Vacres. 2 (3) (2015) 24–28.
[38] N.M. Niri, A. Memarnejadian, J. Hadjati, M.R. Aghasadeghi, M. Shokri, Y. Pilehvar-Soltanahmadi, et al., Construction and production of Foxp3-Fc (IgG) DNA vaccine/fusion protein, Avicenna J. Med. Biotechnol. 8 (2) (2016) 57.
[39] M. Nemattalab, M. Shenagari, M. Taheri, M. Mahjoob, F. Nazari Chamaki, A. Mojtahedi, E. Hasan-alizadeh, B. Ashrafkhani, N. Mousavi Niri, Co-expression of Interleukin-17A molecular adjuvant and prophylactic Helicobacter pylori genetic vaccine could cause sterile immunity in Treg suppressed mice, Cytokine 126 (2020) 154866, https://doi.org/10.1016/j.cyto.2019.154866.
[40] M.H. Pouriayevali, A.R. Memarnejadian, S.M. Sadat, M. Zavva, S.D. Siadat, C. Hartoonian, et al., Designing and construction of bicistronic plasmid pIRES-Igk/mIL18/Fc: potential implications for vaccine investigations, Pathobiol. Res. 14 (2) (2011) 13–23.
[41] N. Mousavi Niri, A. Memarnejadian, Y. Pilehvar-Soltanahmadi, M. Agha Sadeghi, M. Mahdavi, N. Kheshtchin, et al., Improved anti-Treg vaccination targeting Foxp3 efficiently decreases regulatory T cells in mice, J. Immunother. 39 (7) (2016) 269–275.
[42] N.S. Heaton, D. Sachs, C.-J. Chen, R. Hai, P. Palese, Genome-wide mutagenesis of influenza virus reveals unique plasticity of the hemagglutinin and NS1 proteins, Proc. Natl. Acad. Sci. 110 (50) (2013) 20248–20253.
[43] R. Nachbagauer, F. Krammer, Universal influenza virus vaccines and therapeutic antibodies, Clin. Microbiol. Infect. 23 (4) (2017) 222–228.
[44] S. Sebastian, T. Lambe, Clinical advances in viral-vectored influenza vaccines, Vaccines. 6 (2) (2018) 29.
[45] J. Ostrowsky, M. Arpey, K. Moore, M. Osterholm, M. Friede, J. Gordon, D. Higgins, J. Molto-Lopez, J. Seals, J. Bresee, Tracking progress in universal influenza vaccine development, Curr. Opin. Virol. 40 (2020) 28–36.
[46] S. Khodamoradi, M. Shenagari, M.T. Kheiri, F. Sabahi, A. Jamali, A. Heidari, B. Ashrafkhani, IRES-based co-expression of influenza virus conserved genes can promote synergistic antiviral effects both in vitro and in vivo, Arch. Virol. 163 (4) (2018) 877–886.
[47] M. Zheng, J. Luo, Z. Chen, Development of universal influenza vaccines based on influenza virus M and NP genes, Infection 42 (2) (2014) 251–262.
[48] J.B. Ulmer, T.-M. Fu, R.R. Deck, A. Friedman, L. Guan, C. DeWitt, X.u. Liu, S. u. Wang, M.A. Liu, J.J. Donnelly, M.J. Caulfield, Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA, J. Virol. 72 (7) (1998) 5648–5653.
[49] J.L. Schulman, E.D. Kilbourne, Induction of partial specific heterotypic immunity in mice by a single infection with influenza A virus, J. Bacteriol. 89 (1) (1965) 170–174.
[50] J.H.C.M. Kreijtz, R. Bodewes, G. van Amerongen, T. Kuiken, R.A.M. Fouchier, A.D. M.E. Osterhaus, G.F. Rimmelzwaan, Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice, Vaccine. 25 (4) (2007) 612–620.
[51] Z.-R. Li, V.I. Zarnitsyna, A.C. Lowen, D. Weissman, K. Koelle, J.E. Kohlmeier, R. Antia, A. García-Sastre, Why are CD8 T cell epitopes of human influenza A virus conserved? J. Virol. 93 (6) (2019) https://doi.org/10.1128/JVI.01534-18.
[52] D.J. Laddy, J. Yan, A.S. Khan, H. Andersen, A. Cohn, J. Greenhouse, M. Lewis, J. Manischewitz, L.R. King, H. Golding, R. Draghia-Akli, D.B. Weiner, Electroporation of synthetic DNA antigens offers protection in nonhuman primates challenged with highly pathogenic avian influenza virus, J. Virol. 83 (9) (2009) 4624–4630.
[53] L. Li, N. Petrovsky, Molecular mechanisms for enhanced DNA vaccine immunogenicity, Exp. Rev. Vaccines. 15 (3) (2016) 313–329.
[54] J.F. Cros, A. García-Sastre, P. Palese, An unconventional NLS is critical for the nuclear import of the influenza A virus nucleoprotein and ribonucleoprotein, Traffic. 6 (3) (2005) 205–213.
[55] A. Sakaguchi, E. Hirayama, A. Hiraki, Y.-o.-I. Ishida, J. Kim, Nuclear export of influenza viral ribonucleoprotein is temperature-dependently inhibited by dissociation of viral matrix protein, Virology 306 (2) (2003) 244–253.
[56] A. Stachyra, A. Góra-Sochacka, A. Sirko, DNA vaccines against influenza, Acta Biochim. Pol. 61 (3) (2014), https://doi.org/10.18388/abp.2014_1873.
[57] J.-P. Scheerlinck, Genetic adjuvants for DNA vaccines, Vaccine. 19 (17-19) (2001) 2647–2656.
[58] F. Saade, N. Petrovsky, Technologies for enhanced efficacy of DNA vaccines, Exp. Rev. Vaccines. 11 (2) (2012) 189–209.
[59] A. Henke, N. Rohland, R. Zell, P. Wutzler, Co-expression of interleukin-2 by a bicistronic plasmid increases the efficacy of DNA immunization to prevent influenza virus infections, Intervirology 49 (4) (2006) 249–252.
[60] A. Henke, C. Chiang, R. Zell, A. Stelzner, Co-expression of interleukin-2 to increase the efficacy of DNA vaccine-mediated protection in coxsackievirus B3-infected mice, Antiviral Res. 64 (2) (2004) 131–136.
[61] C.R. Ferrone, M.-A. Perales, S.M. Goldberg, C.J. Somberg, D. Hirschhorn-Cymerman, P.D. Gregor, M.J. Turk, T. Ramirez-Montagut, J.S. Gold, A. N. Houghton, J.D. Wolchok, Adjuvanticity of plasmid DNA encoding cytokines fused to immunoglobulin Fc domains, Clin. Cancer Res. 12 (18) (2006) 5511–5519.
[62] E. Seki, H. Tsutsui, H. Nakano, N.M. Tsuji, K. Hoshino, O. Adachi, K. Adachi, S. Futatsugi, K. Kuida, O. Takeuchi, H. Okamura, J. Fujimoto, S. Akira, K. Nakanishi, Lipopolysaccharide-induced IL-18 secretion from murine Kupffer cells independently of myeloid differentiation factor 88 that is critically involved in induction of production of IL-12 and IL-1β, J. Immunol. 166 (4) (2001) 2651–2657.
[63] L.H. O'Donovan, E.L. McMonagle, S. Taylor, D.J. Argyle, L. Nicolson, Bioactivity and secretion of interleukin-18 (IL-18) generated by equine and feline IL-18 expression constructs, Vet. Immunol. Immunopathol. 102 (4) (2004) 421–428.
[64] T. Osaki, W. Hashimoto, A. Gambotto, H. Okamura, P.D. Robbins, M. Kurimoto, M. T. Lotze, H. Tahara, Potent antitumor effects mediated by local expression of the mature form of the interferon-γ inducing factor, interleukin-18 (IL-18), Gene Ther. 6 (5) (1999) 808–815.
[65] S. Kim, D. Cho, T.S. Kim, Induction of in vivo resistance to Mycobacterium avium infection by intramuscular injection with DNA encoding interleukin-18, Immunology 102 (2) (2001) 234–241.
[66] L. Loh, Z. Wang, S. Sant, M. Koutsakos, S. Jegaskanda, A.J. Corbett, L. Liu, D. P. Fairlie, J. Crowe, J. Rossjohn, J. Xu, P.C. Doherty, J. McCluskey, K. Kedzierska, Human mucosal-associated invariant T cells contribute to antiviral influenza immunity via IL-18–dependent activation, Proc. Natl. Acad. Sci. 113 (36) (2016) 10133–10138.
[67] B. van Wilgenburg, L. Loh, Z. Chen, T.J. Pediongco, H. Wang, M. Shi, Z. Zhao, M. Koutsakos, S. Nüssing, S. Sant, Z. Wang, C. D'Souza, X. Jia, C.F. Almeida, L. Kostenko, S.B.G. Eckle, B.S. Meehan, A. Kallies, D.I. Godfrey, P.C. Reading, A. J. Corbett, J. McCluskey, P. Klenerman, K. Kedzierska, T.S.C. Hinks, MAIT cells

contribute to protection against lethal influenza infection in vivo, Nat. Commun. 9 (1) (2018), https://doi.org/10.1038/s41467-018-07207-9.
[68] K.H. Ely, L.S. Cauley, A.D. Roberts, J.W. Brennan, T. Cookenham, D.L. Woodland, Nonspecific recruitment of memory CD8+ T cells to the lung airways during respiratory virus infections, J. Immunol. 170 (3) (2003) 1423–1429.
[69] J.E. Kohlmeier, T. Cookenham, A.D. Roberts, S.C. Miller, D.L. Woodland, Type I interferons regulate cytolytic activity of memory CD8+ T cells in the lung airways during respiratory virus challenge, Immunity 33 (1) (2010) 96–105.
[70] G.D. Sckisel, J.K. Tietze, A.E. Zamora, H.-H. Hsiao, S.O. Priest, D.E.C. Wilkins, L. L. Lanier, B.R. Blazar, N. Baumgarth, W.J. Murphy, Influenza infection results in local expansion of memory CD 8+ T cells with antigen non-specific phenotype and function, Clin. Exp. Immunol. 175 (1) (2014) 79–91.
[71] H. Guo, D.J. Topham, Interleukin-22 (IL-22) production by pulmonary Natural Killer cells and the potential role of IL-22 during primary influenza virus infection, J. Virol. 84 (15) (2010) 7750–7759.
[72] C. Paget, S. Ivanov, J. Fontaine, J. Renneson, F. Blanc, M. Pichavant, L. Dumoutier, B. Ryffel, J.C. Renauld, P. Gosset, P. Gosset, M. Si-Tahar, C. Faveeuw, F. Trottein, Interleukin-22 is produced by invariant natural killer T lymphocytes during influenza a virus infection potential role in protection against lung epithelial damages, J. Biol. Chem. 287 (12) (2012) 8816–8829.
[73] D.A. Pociask, E.V. Scheller, S. Mandalapu, K.J. McHugh, R.I. Enelow, C.L. Fattman, J.K. Kolls, J.F. Alcorn, IL-22 is essential for lung epithelial repair following influenza infection, Am. J. Pathol. 182 (4) (2013) 1286–1296.
[74] M.A. Gessner, J.L. Werner, L.M. Lilly, M.P. Nelson, A.E. Metz, C.W. Dunaway, Y. R. Chan, W. Ouyang, G.D. Brown, C.T. Weaver, C. Steele, G.S. Deepe, Dectin-1-dependent interleukin-22 contributes to early innate lung defense against Aspergillus fumigatus, Infect. Immun. 80 (1) (2012) 410–417.
[75] K.L. Wozniak, C.R. Hole, J. Yano, P.L. Fidel Jr, F.L. Wormley Jr, Characterization of IL-22 and antimicrobial peptide production in mice protected against pulmonary Cryptococcus neoformans infection, Microbiology 160 (Pt 7) (2014) 1440.
[76] B. Zhang, B. Chassaing, Z. Shi, R. Uchiyama, Z. Zhang, T.L. Denning, S.E. Crawford, A.J. Pruijssers, J.A. Iskarpatyoti, M.K. Estes, T.S. Dermody, W. Ouyang, I. R. Williams, M. Vijay-Kumar, A.T. Gewirtz, Prevention and cure of rotavirus infection via TLR5/NLRC4–mediated production of IL-22 and IL-18, Science (New York, NY). 346 (6211) (2014) 861–865.
[77] X. Wang, C.CS. Chan, M. Yang, J. Deng, V.K.M. Poon, V.HC. Leung, K.-H. Ko, J. Zhou, K. Yung Yuen, B.-J. Zheng, L. Lu, A critical role of IL-17 in modulating the B-cell response during H5N1 influenza virus infection, Cell. Mol. Immunol. 8 (6) (2011) 462–468.
[78] K.K. McKinstry, T.M. Strutt, A. Buck, J.D. Curtis, J.P. Dibble, G. Huston, M. Tighe, H. Hamada, S. Sell, R.W. Dutton, S.L. Swain, IL-10 deficiency unleashes an influenza-specific Th17 response and enhances survival against high-dose challenge, J. Immunol. 182 (12) (2009) 7353–7363.
[79] C.R. Crowe, K. Chen, D.A. Pociask, J.F. Alcorn, C. Krivich, R.I. Enelow, T.M. Ross, J.L. Witztum, J.K. Kolls, Critical role of IL-17RA in immunopathology of influenza infection, J. Immunol. 183 (8) (2009) 5301–5310.
[80] J. Bayry, Harnessing the regulators to enhance viral vaccine efficacy. Future, Science 9 (12) (2017) 1319–1321.
[81] E. Stephen-Victor, I. Bosschem, F. Haesebrouck, J. Bayry, The Yin and Yang of regulatory T cells in infectious diseases and avenues to target them, Cell. Microbiol. 19 (6) (2017) e12746, https://doi.org/10.1111/cmi.v19.610.1111/cmi.12746.
[82] M.C. Boer, S.A. Joosten, T.H. Ottenhoff, Regulatory T-cells at the interface between human host and pathogens in infectious diseases and vaccination, Front Immunol. 6 (2015) 217.
[83] J. Bayry, Regulatory T cells as adjuvant target for enhancing the viral disease vaccine efficacy, Virusdisease. 25 (1) (2014) 18–25.
[84] J. Surls, C. Nazarov-Stoica, M. Kehl, S. Casares, T.-D. Brumeanu, Differential effect of CD4+ Foxp3+ T-regulatory cells on the B and T helper cell responses to influenza virus vaccination, Vaccine. 28 (45) (2010) 7319–7330.
[85] D. Meng, Y. Hu, C. Xiao, T. Wei, Q. Zou, M. Wang, Chronic heat stress inhibits immune responses to H5N1 vaccination through regulating CD4+ CD25+ Foxp3+ tregs, Biomed Res. Int. 2013 (2013).
[86] S.-M. Wang, M.-H. Tsai, H.-Y. Lei, J.-R. Wang, C.-C. Liu, The regulatory T cells in anti-influenza antibody response post influenza vaccination, Human Vaccines Immunotherap. 8 (9) (2012) 1243–1249.