RESEARCH PAPER

Enhanced CD8+ T-cell response in mice immunized with NS1-truncated influenza virus

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ABSTRACT

Influenza viruses with truncated NS1 protein stimulate a more intensive innate immune response compared to their wild type counterparts. Here, we investigate how the shortening of the NS1 protein influence the immunogenicity of the conserved T-cellular epitopes of influenza virus. Using flow cytometry, we showed that the intraperitoneal immunization of mice with influenza virus encoding 124 N-terminal amino acid residues of the NS1 protein (A/PR8/NS124) induced higher levels of CD8+ T-cells recognizing immunodominant ($NP_{366-374}$) and sub-immunodominant ($NP_{161-175}$, $NP_{196-210}$, $HA_{323-337}$, $HA_{474-483}$, $NA_{427-433}$) epitopes compared to immunization with the virus expressing full-length NS1 (A/PR8/full NS). It is noteworthy that the response to the immunodominant influenza epitope $NP_{366-374}$ was achieved with the lower immunization dose of A/PR8/NS124 virus compared to the reference wild type strain. Despite the fact that polyfunctional CD8+ effector memory T-lymphocytes simultaneously producing two (IFNγ and TNFα) or three (IFNγ, IL2 and TNFα) cytokines prevailed in the immune response to both viruses, the relative number of such T-cells was higher in A/PR8/NS124-immunized mice. Furthermore, we have found that polyfunctional populations of lymphocytes generated upon the immunization of mice with the mutant virus demonstrated an increased capacity to produce IFNγ compared to the corresponding populations derived from the A/PR8/full NS-immunized mice. Therefore, immunization with the attenuated influenza virus encoding truncated NS1 protein ensures a more potent CD8+ T-cell immune response.

INTRODUCTION

According to meta-analysis [1], the effectiveness of modern influenza vaccines in people aged 18-65 years is about 60%. Inactivated vaccines mostly induce a humoral immune response and effective protection only when the antigenic structure of surface influenza proteins in vaccine strains coincides with the antigenic structure of surface proteins of circulating viruses [2]. Live attenuated influenza vaccines (LAIVs) induce not only humoral but also mucosal and T-cellular immunity. The ability of LAIV strains to replicate in the upper respiratory tract provides a way to trigger the MHC-I/II-dependent presentation of conserved epitopes to CD8+ and CD4+ T-cells. It has been shown in recent studies that circulating influenza viruses of types A, B, and C share common CD8+ T-cellular epitopes that could induce the cross-protective immune response [3]. Memory CD8+ T-cells directed against these universal epitopes were found in the blood and lungs of healthy humans [3]. Despite the existence of such highly conserved cross-protective influenza epitopes, the T-cellular immune response induced by LAIVs is insufficient to provide a broad cross-protection [4]. It is noteworthy that all of the licensed LAIVs contain viruses comprising active NS1 protein which inhibits the host immune response by interacting with the components of the interferon (IFN) signaling system [5, 6]. Therefore, LAIVs can prevent the formation of an immune response to the weakly immunogenic conserved epitopes of influenza proteins and, consequently, suppress the ability of the vaccine strain to provide cross-protection against a broad range of influenza viruses. The development of approaches to increase the immunogenicity of conserved influenza epitopes can be a promising strategy for creating a new generation of live influenza vaccines with enhanced cross-protection activity. It is known that the abrogation of the immunosuppressive activity of NS1 protein by the truncation of its effector domain increases the innate cytokine immune response to influenza virus and reduces its reproductive activity in the respiratory tract of the host organism [7]. It is also known that immunization with live influenza virus with a modified *NS* gene protects mice when challenged with heterologous strains of the same virus subtype [8, 9].

Previously, we showed that the influenza virus encoding NS1 protein, when shortened to 124 amino acids (aa), induced higher proinflammatory cytokine production and enhanced innate immune cell stimulation compared

to influenza A/PR8/34 wild type virus after the intra-peritoneal (i.p.) immunization of mice [10]. Here, we investigate whether the enhanced ability of the influenza virus with truncated NS1 protein to activate the innate immune system could be translated into a more efficient adaptive T-cellular immune response against immunodominant and sub-immunodominant influenza epitopes. Since the importance of polyfunctional T-lymphocytes in mediating the protective immune response against different bacteria and viruses is well recognized [11–15], we analyzed the level and functional activity of the variety of T-cells producing IFNγ, IL2, and TNFα. To equalize the antigenic load of two viruses with different reproduction activity in the respiratory tract of immunized mice, we used the i.p. route of immunization, as it was previously shown that influenza virus does not replicate in the peritoneal cavity but retains the ability to induce a humoral and T-cellular immune response [16].

MATERIALS AND METHODS

Viruses

Two strains based on the influenza A/Puerto Rico/8/1934 (H1N1) virus were used for the immunization of mice: (1) the virus, encoding full-length NS1 protein (A/PR8/full NS) and (2) A/PR8/NS124 strain, encoding shortened to 124 aa NS1 protein. The strains were obtained by the method of reverse genetics [17] assembled in developing chicken embryos and purified by fractionation in a sucrose density gradient according to the standard procedure.

Laboratory animals

This study was performed in C57BL/6 female mice obtained from the Biomedical Science Center (Stolbovaya, Russia). All of the experiments with mice in this research study were conducted according to the guidelines for care and work with laboratory animals [18].

Immunization

The A/PR8/full NS and A/PR8/NS124 viruses were titrated on Vero cells to equalize the immunization dose. A limiting dilution assay was performed in 96-well culture plates (Nunc, Denmark) by the addition of 100 μl of prepared dilutions of the virus-containing material in OptiPRO SFM (Invitrogen) with 2% L-glutamine (Invitrogen) and 5 μg/ml of trypsin (Sigma-Aldrich) into the wells and subsequent incubation for 5 days at 34℃ and 5% CO_2. The results were evaluated visually by the estimation of the cytopathic effect. Hemagglutination assay (using a 0.5% suspension of chicken red blood cells) was used as the control. The calculation of virus 50% tissue culture infectious dose ($TCID_{50}$) was performed using the Reed and Munch method [19]; the viral titer was expressed as $\log_{10} TCID_{50}/ml$. In order to assess the immunogenicity of strains mice were immunized i.p. with 4.0 or 7.0 $\log_{10} TCID_{50}/ml$ of each virus (500 μl of a virus suspension in sodium phosphate buffer (PBS, Biolot)).

The control group received PBS in an equivalent volume. Each group included 5 mice. In total, 45 experimental animals were used in the present study.

Flow cytometry

T-cellular immune response was evaluated in splenocytes obtained on the 8th and 21st days post-immunization (d.p.i.). The spleens were manually homogenized using pestle homogenizers (Eppendorf, Germany). Red blood cells were lysed using the RBC Lysis Buffer reagent (Biolegend, USA). Cells were cultivated in RPMI1640 media (Gibco), containing 10% of fetal bovine serum (FBS, Gibco) and 1% of penicillin/streptomycin solution (Gibco). To stimulate cytokine production splenocytes were incubated with $NP_{366-374}$, $NP_{161-175}$, $NP_{196-210}$, $HA_{474-483}$, $HA_{523-337}$ or $NA_{427-433}$ peptides and brefeldin A (Biolegend) for 6 h at 37℃ and 5% CO_2. Epitopes were selected using the IEDB database (www.iedb.org). Peptides were synthesized by Verta Ltd. (St. Petersburg, Russia). The sample of each peptide (10 mg) was dissolved in DPBS (Biolot, Russia) at 10 mg/ml and stored in small aliquots at −20℃. The purity of the peptides was >90% as determined by high-performance liquid chromatography. After the stimulation, the cells were stained with CD8-PE/Cy7, CD4-PerCP-Cy5.5, CD44-BV510, CD62L-APC/Cy7, IFNγ-FITC, TNFα-BV421, and IL2-PE antibodies using the Fixation and Permeabilization Solution reagent kit (BD Biosciences, USA) according to the manufacturer's instructions. Zombie Red viability marker (BioLegend, USA) was used to identify the dead cells. True Stain reagent, containing antibodies to CD16/CD32, was used to block non-specific antibody binding (BioLegend, USA). The data were collected on a BD FACSCanto II flow cytometer (BD Biosciences, USA). The results were analyzed using the Kaluza Analysis 1.5a program (Beckman Coulter, USA). In order to estimate the increase in the cytokine production levels upon the peptide stimulation, the background values obtained from the non-stimulated cells were subtracted from the corresponding values of stimulated samples before the statistical analysis.

Statistical analysis

RStudio Desktop 1.0.153 (RStudio Inc, USA) was used for statistical data analysis. The Dunnet test was used to compare several experimental groups with one control group. A comparison of two experimental groups was carried out using a Student's t-test. Multiple comparisons of several groups were performed using univariate analysis of variance (ANOVA) followed by a pairwise comparison of groups using the Tukey criterion. Cellular polyfunctionality index (PI) was calculated using the formula: $PI = \sum_{i=1}^{n} F_i * (\frac{i}{n})$ (where n is the number of analyzed functions (n=3 for IFNγ, IL-2, and TNFα) and F is the percentage of cells performing i functions) as described previously [14, 20]. Integrated mean fluorescence intensity (iMFI) was calculated by multiplying the frequency of the particular population by the MFI of IFNγ, IL2, or TNFα of this population.

RESULTS

Selection of epitopes for T-cell immune response analysis

The description of the sequence and the conservancy of the selected for this study epitopes are shown in Table 1. These peptides allowed us to evaluate the effect of NS1 protein modification on the immunogenicity of the immunodominant and sub-immunodominant epitopes from the internal (NP) and surface (HA, NA) proteins of influenza virus.

Table 1. T-cell epitopes of influenza virus

Epitope	Sequence	Conservancy
$NP_{366-374}$	ASNENMETM	1.8% (63 / 3448)
$NP_{161-175}$	PRMCSLMQGSTLPRR	98.9% (3411 / 3448)
$NP_{196-210}$	MIKRGINDRNFWRGE	67.28% (2320 / 3448)
$HA_{323-337}$	YVKSTKLRLATGLRN	41.9% (4174 / 9949)
$HA_{474-483}$	KEIGNGCFEF	47.7% (4750 / 9949)
$NA_{427-433}$	SISFCGV	42.2% (3288 / 7776)

The level of conservancy for each epitope was determined by calculating the relative number of unique sequences of the corresponding protein of influenza A virus containing a given epitope. The sequences presented in the Influenza Research Database were used for the conservancy analysis. Data on the immunogenicity of epitopes were obtained from publications that compare the immune response to different epitopes of the influenza virus [21-26].

A/PR8/NS124 mutant virus induces CD8+ response at a lower dose of immunization

In order to determine the optimal immunizing dose, C57BL/6 mice were injected i.p. with A/PR8/full NS and A/PR8/NS124 influenza viruses at a dose of 4.0 and 7.0 log $TCID_{50}$/mouse. The antigen-specific T-cellular response to immunodominant $NP_{366-374}$ $CD8^+$ epitope was evaluated in spleens 8 d.p.i. Cells were stimulated *in vitro* with this peptide for 6 h after which the levels of IFNγ, IL2, and TNFα-producing $CD8^+CD44^+CD62L^-$ effector memory ($CD8^+$ EM) T-cells were estimated by flow cytometry.

The immune response to both strains was dose-dependent. After the immunization of mice with A/PR8/NS124 at 4.0 $\log_{10}$ $TCID_{50}$/mouse, 2.88±2.01% of $CD8^+$ effector T-lymphocytes produced cytokines in response to the *in vitro* stimulation. On the other hand, after the immunization with A/PR8/full NS at the same dose, the number of antigen specific cells was 0.34±0.10% while in the control group it was 0.26±0.16%. After immunization with 7.0 $\log_{10}$ $TCID_{50}$/mouse of each virus, the level of antigen-specific $CD8^+$ EM T-lymphocytes was 15.07±2.77% of total $CD8^+$ EM T-cells in the full NS group and 26.86±1.72% in the NS124 group (p=0.013, Fig. 1). Therefore, A/PR8/NS124 virus was capable of inducing $CD8^+$ T-cell response to $NP_{366-374}$ epitope at a lower dose than the influenza strain with the full-length NS1 protein and was more immunogenic at a dose that induces a substantial immune response to both tested viruses.

The shortening of NS1 protein enhances the immunogenicity of sub-immunodominant CD8+ T-cell epitopes

Next, we assessed the immunogenicity of sub-immunodominant T-cell epitopes using the highest immunizing dose of 7.0 $\log_{10}$ $TCID_{50}$/mouse. According to Cox *et al.* [15], the peak of $CD8^+$ T-cell response occurs 8-10 days after the immunization, followed by a decrease in the
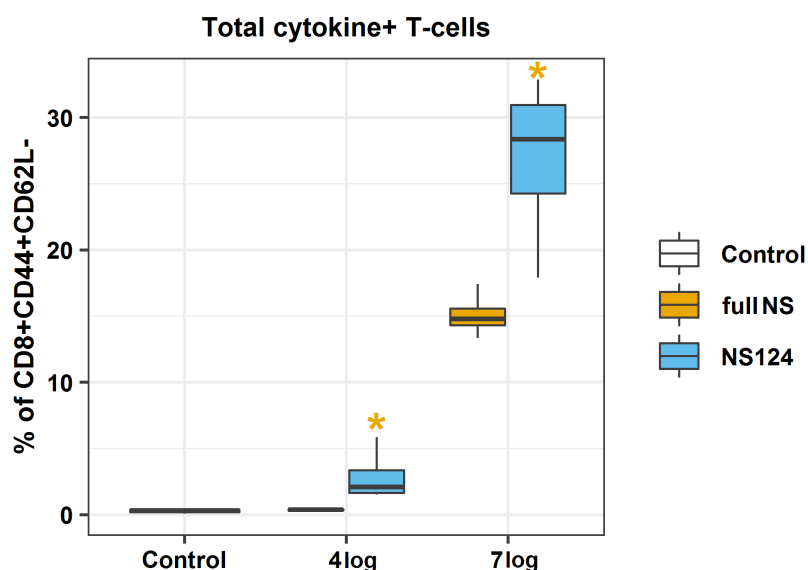
Fig. 1. $CD8^+$ T-cellular immune response to different doses of A/PR8/full NS or A/PR8/NS124 influenza strains. The total levels of cytokine-producing effector memory $CD8^+CD44^+CD62L^-$ T-lymphocytes after 6 h of *in vitro* stimulation of splenocytes of C57BL/6 mice with the $NP_{366-374}$ peptide. Groups were compared using the Student's t-test (*: p<0.05, n=5).

number of antigen-specific T-cells. At the same time, the spectrum of epitopes inducing adaptive immune response shrinks at later stages after immunization [27]. Considering these data, we estimated the immune response to influenza epitopes on the 8th and 21st days after the i.p. immunization.

Fig. 2 shows the levels of cytokine-producing EM (CD44$^+$CD62L$^-$) CD8$^+$ T-cells. Eight days after immunization, A/PR8/NS124 virus induced a higher CD8$^+$ T-cell immune response to NP and HA than A/PR8/full NS while the immune response to NA protein epitopes was comparable (Fig. 2A). The most pronounced differences in the total amount of cytokine-producing cells between full NS and NS124 groups were obtained upon stimulation with immunodominant NP$_{366-374}$ epitope. However, stimulation with HA$_{474-483}$, NA$_{427-433}$, and NP$_{196-210}$ peptides, representing sub-immunodominant epitopes, also significantly increased the immunogenicity of A/PR8/NS124 virus: for NA$_{427-433}$ 10.7±0.8% of CD8$^+$ EM T-cells in NS124 group versus 8.4±1.9% in full NS group (p=0.05); for HA$_{474-483}$ 9.9±0.9% of CD8$^+$ EM T-cells in NS124 group versus 7.2±1.8% in full NS group (p=0.03); and for NP$_{196-210}$ 11.6±1.7% in NS124 group versus 8.3±2.0% in full NS group (p=0.05) (Fig. 2A). *In vitro* stimulation with either of the studied epitopes did not result in the induction of the significant CD4$^+$ T-cellular response (data not shown).

On the 21st d.p.i., only two epitopes (NP$_{366-374}$ and NA$_{427-433}$) retained the ability to induce cytokine production in CD8$^+$ EM T-cells upon *in vitro* stimulation in both A/PR8/full NS and A/PR8/NS124-immunized groups (Fig.2B). The proportion of cytokine-producing T-cells was 9.4±1.5% and 14.0±1.3% for NP$_{366-374}$ and 7.0±0.8% and 11.1±2.4% for NA$_{427-433}$ in full NS and NS124 groups, respectively (p=0.003, p=0.01). Therefore, the total CD8$^+$

EM T-cellular immune response was higher in the NS124 group compared to A/PR8/full NS group on the 21st d.p.i.

The percentage of distinct cytokine-producing populations of the total amount of CD8$^+$ EM T-cells or of the total number of cytokine-producing CD8$^+$ EM T-cells on the 8th and 21st d.p.i. in full NS and NS124 groups is shown in Fig. 3. Upon stimulation with NP$_{366-374}$ peptide, the majority of antigen-specific T-cells in both full NS and NS124 groups were polyfunctional CD8$^+$ T-cells (IFNγ$^+$IL2$^+$TNFα$^+$). The second dominant population of antigen-specific T-lymphocytes comprised double cytokine-producers (IFNγ$^+$IL2$^-$TNFα$^+$). The rest (approx. 25% of the total cytokine-producing T-cells in both groups) was represented by the minor populations of single IFNγ-, IL2-, or TNFα-producers or double-positive T-cells (IFNγ$^-$IL2$^+$TNFα$^+$ and IFNγ$^+$IL2$^+$TNFα$^-$). The peptides corresponding to the sub-immunodominant influenza epitopes as well as NP$_{366-374}$ epitope, induced a higher level of polyfunctional IFNγ$^+$IL2$^-$TNFα$^+$ and IFNγ$^+$IL2$^+$TNFα$^+$ CD8$^+$ T-lymphocytes in the NS124 group compared to the full NS group on the 8th d.p.i. In contrast to NP$_{366-374}$ specific response, all of the evaluated sub-immunodominant epitopes induced an increased level of IFNγ$^+$IL2$^-$TNFα$^-$ single-producers in the NS124 group. At the same time, A/PR8/full NS strain induced a higher proportion of IFNγ$^-$IL2$^-$TNFα$^+$ T-cells. The stimulation of CD8$^+$ EM T-cells with HA$_{474-483}$ versus NP$_{161-175}$ revealed significant differences between the experimental groups in the amount of TNFα single-producers (full NS: 0.8±0.2%, NS124: 0.4±0.3% (p=0.04) after HA$_{474-483}$ stimulation; 1.1±0.4%, 0.7±0.3% (p= 0.05) correspondingly after NP$_{161-175}$ stimulation).

The contribution of polyfunctional T-lymphocytes (IFNγ$^+$IL2$^-$TNFα$^+$ and IFNγ$^+$IL2$^+$TNFα$^+$) to the cumulative cytokine response increased on the 21st d.p.i. Only
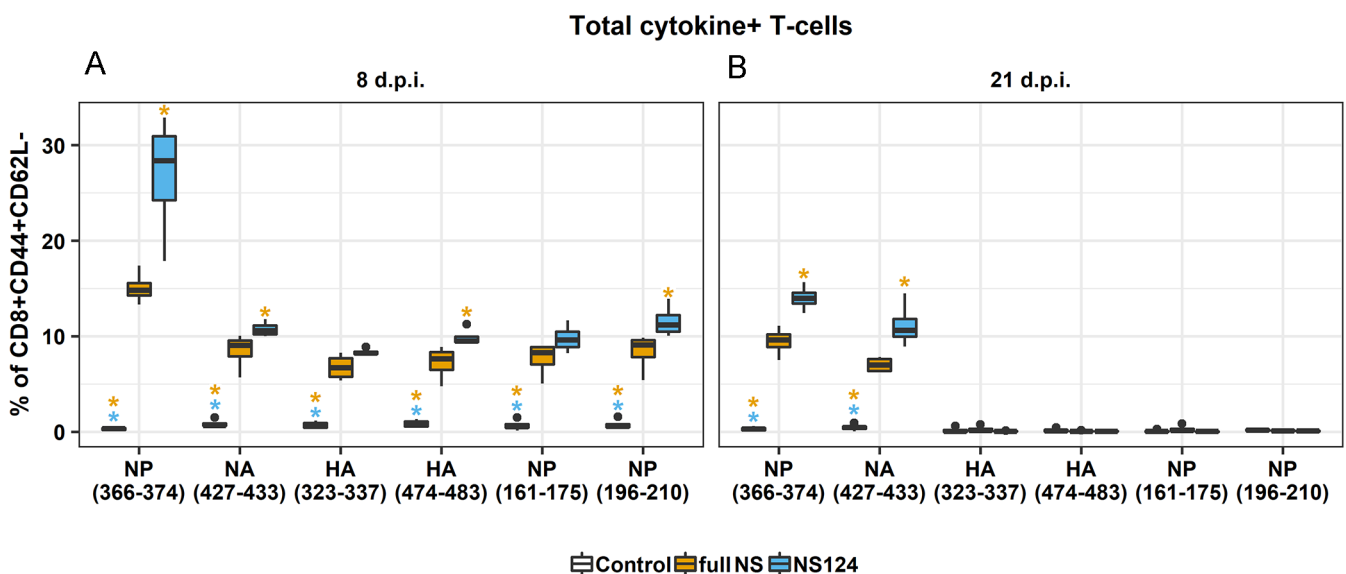
Fig. 2. T-cellular immune response to different epitopes of influenza virus in the spleens of C57BL/6 mice on 8th (A) and 21th (B) days after i.p. immunization with A/PR8/full NS and A/PR8/NS124 influenza strains. Box-plots represent the total levels of cytokine-producing CD8$^+$ EM T-lymphocytes after 6 h of *in vitro* stimulation of splenocytes with peptides corresponding to sub-immunodominant conservative influenza epitopes. Groups were compared using ANOVA followed by a Tukey's post-hoc comparison. Significant differences between groups (p<0.05, n=5) are marked by *.

a minor part of the total influenza-specific cells was represented by IFNγ single producers in both full NS and NS124 groups. The IFNγ+IL2+TNFα- CD8+ EM T-cells were observed only in A/PR8/NS124-immunized animals. A statistically significant difference in the proportion of IFNγ+IL2+TNFα+ T-cells was found between the full NS and NS124 groups after stimulation with NP$_{366-374}$ peptide (p=0.0003). The immune response to NA$_{427-433}$ epitope was characterized by the formation of the higher amount of IFNγ+IL2-TNFα+ and IFNγ+IL2+TNFα+ T-lymphocytes in the NS124 group (p=0.02 and p=0.01, respectively).

Therefore, the immune response to the sub-immunodominant epitopes of A/PR8/NS124 influenza strain was characterized by a higher level of total cytokine-producing T-cells and an increase of the proportion of minor IFNγ+IL2+TNFα- population of CD8+ EM T-lymphocytes compared to A/PR8/full NS virus on the 8th and 21st d.p.i. However, the number of epitopes inducing the CD8+ T-cell response simultaneously decreased in both groups on the 21st d.p.i.

The A/PR8/NS124 mutant virus promotes the appearance of polyfunctional CD8+ lymphocytes with enhanced IFNγ expression

In order to estimate the difference in the functional activity of cytokine-producing T-cell populations in mice upon immunization with A/PR8/full NS or A/PR8/NS124 viruses, we analyzed the expression levels (mean fluorescence intensity, MFI) of IFNγ, IL2, and TNFα in different populations of cytokine-producing T-cells on the 8th and 21st d.p.i. The distribution of CD8+ T-cells from A/PR8/NS124-immunized mice (8th d.p.i.) by their fluorescence intensity of IFNγ, IL2, and TNFα corresponds to their expression levels as shown in Fig. 4A. The polyfunctional
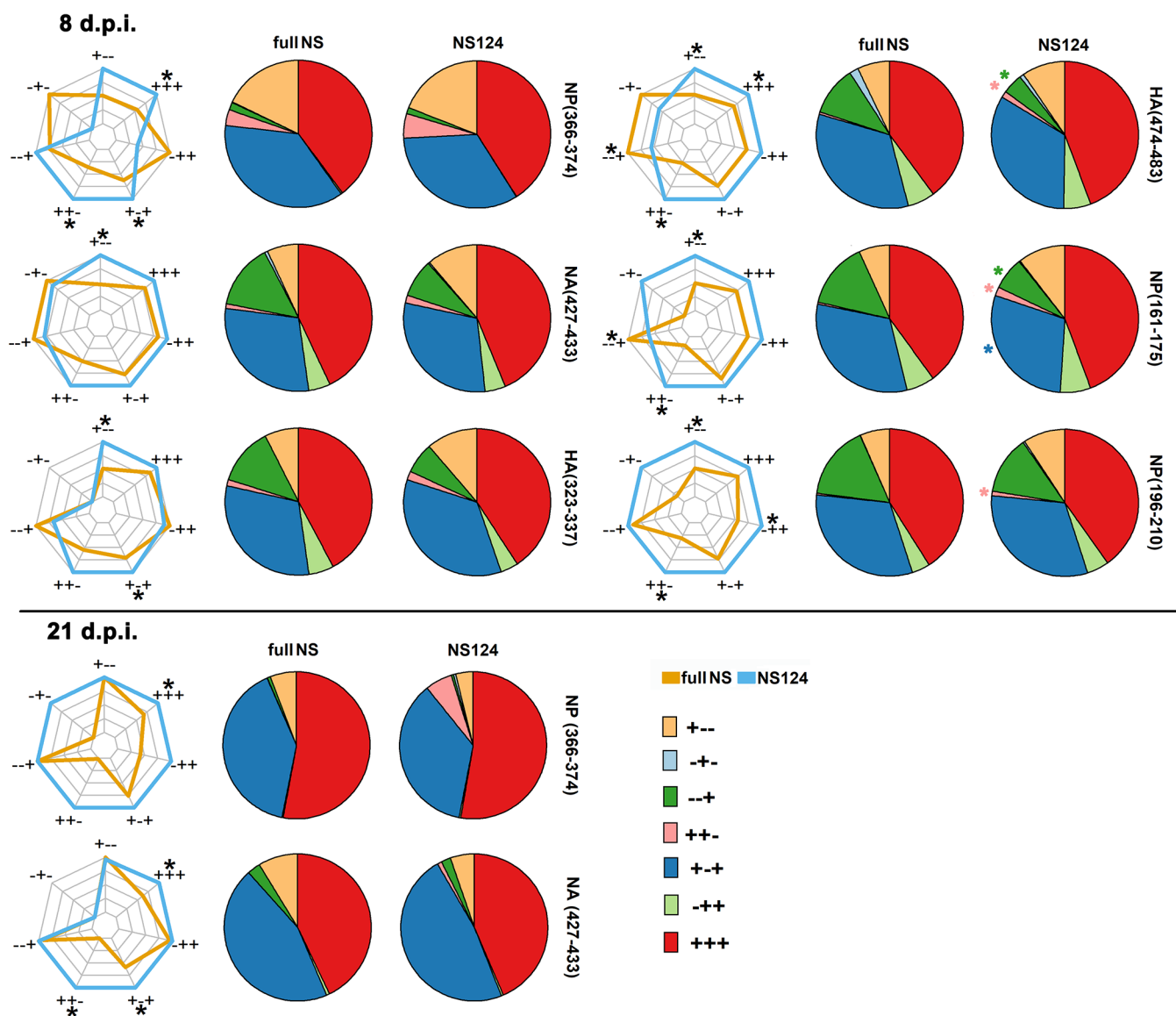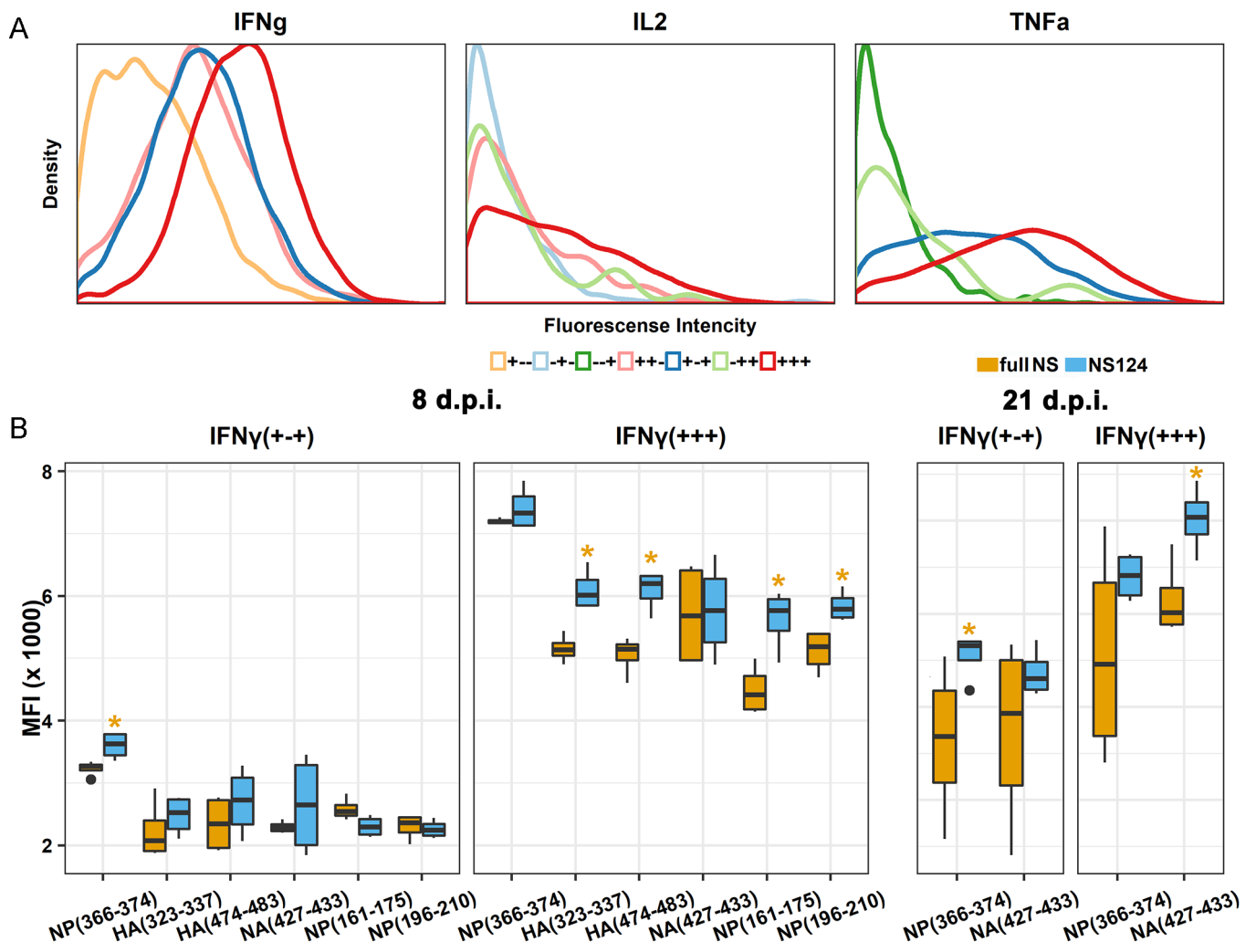

Fig. 3. Relative content of different cytokine-producing populations of CD8+ EM T-lymphocytes on the 8th and 21st days after the i.p. immunization with A/PR8/full NS and A/PR8/NS124 influenza strains. Radar charts represent the differences in the mean values of epitope-specific immune response of each population of cytokine-producing CD8+ EM T-cells of immunized mice. Each point is located in the range from 0 to the maximal mean value of the corresponding population in full NS or NS124 group. The pie charts represent the percentage of cells, producing any combination of IFNγ, IL2, or TNFα cytokines in the total cytokine-producing CD8+ EM T-cell subset. (*: p<0.05, Student's t-test, n=5).

IFNγ+IL2-TNFα+ and IFNγ+IL2+TNFα+ subpopulations were characterized by the highest cytokine-producing activity among all antigen-specific T-lymphocytes. In particular, IFNγ+IL2+TNFα+ triple-producers secreted more IFNγ, IL2, and TNFα than their bifunctional or monofunctional analogs. IFNγ+IL2-TNFα+ T-cells produced higher levels of IFNγ and TNFα than the single-producers expressing these cytokines (Fig. 4A). The similar fluorescence intensity (FI) distributions were obtained for A/PR8/full NS-immunized animals (data not shown). These results underline the dominance of polyfunctional T-cells in the mediation of CD8+ T-cell cytokine response after i.p. immunization regardless of NS1 functionality.

Nevertheless, the comparison of the full NS and NS124 groups showed that A/PR8/NS124 virus induced higher levels of the IFNγ MFI in IFNγ+IL2-TNFα+ T-cells upon the stimulation with $NP_{366-374}$ peptide (Fig. 4B). Similarly, $NP_{161-175}$, $NP_{196-210}$, $HA_{323-337}$, and $HA_{474-483}$ peptides induced higher values of IFNγ MFI in IFNγ+IL2+TNFα+ CD8+ EM

T-cells in the NS124 group compared to the full NS group ($p<0.05$). No statistically significant differences between the experimental groups were found in the MFI expression levels of IL2 and TNFα (data not shown). The observed differences in the expression level of IFNγ in the polyfunctional T-lymphocytes remained during 21 days after the immunization. The expression level of IFNγ MFI of IFNγ+IL2-TNFα+ was higher in the NS124 group compared to the full NS group after the stimulation with $NP_{366-374}$ ($p<0.001$). The $NA_{427-433}$ peptide induced the enhanced level of the IFNγ MFI in the IFNγ+IL2+TNFα+ T-cells in the A/PR8/NS124-immunized animals ($p=0.02$).

Based on the foregoing data, we can conclude that i.p. administration of influenza A/PR8/NS124 virus not only stimulates a greater amount of antigen-specific CD8+ T-lymphocytes than the A/PR8/full NS strain, but it also ensures the formation of polyfunctional T-cells with the enhanced IFNγ-producing activity.

Fig. 4. IFNγ expression level in IFNγ+IL2-TNFα+ and IFNγ+IL2+TNFα+ cytokine-producing populations of CD8+ EM T-lymphocytes on 8th and 21st days after the i.p. immunization with A/PR8/full NS and A/PR8/NS124 influenza strains. (A) The distribution of fluorescence intensity of cytokine-producing cells from mice immunized with 7.0 log $TCID_{50}$/mouse of the A/PR8/NS124 influenza strain. The density plots represent the differences in the cytokine expression level between the subsets of cytokine-producing cells. (B) IFNγ mean fluorescence intensity (MFI) values of the polyfunctional subpopulations after peptide stimulation were compared using the Student's t-test (*: $p<0.05$, n=5).

DISCUSSION

CD8 T-cell immune response to conserved antigens is essential for cross-protection against a broad variety of influenza viruses [3, 28]. T-lymphocytes recognize the linear (8-24 aa) epitopes of the internal influenza antigens [29, 30]. Unfortunately, the majority of the conserved influenza epitopes maintain low immunogenicity during the course of natural influenza infection and vaccination. Therefore, the development of new approaches for vaccination that will ensure cell-mediating immune responses is of great importance.

Adaptive T-cell immune response is strongly dependent on the cytokine milieu and activated antigen-presenting cells generated during the innate immune response. Previously, we showed that A/PR8/NS124 influenza virus injected i.p. induces the production of IFNβ at a 300 times higher level than the virus expressing full-length NS1 protein in the peritoneal washes as well as promotes the increased expression of co-stimulatory CD86-molecules participating in the T-cell differentiation process [10]. In this study, we estimated the effect of the modification of the NS1 protein on the immunogenicity of the immunodominant and sub-immunodominant epitopes of the internal and surface proteins of the influenza virus. It was shown that the lower immunization dose of A/PR8/NS124 is required for triggering the CD8$^+$ response compared to NS1 competent virus. In addition, the NS1 mutant virus induced the formation of an increased number of antigen-specific effector CD8$^+$ T-lymphocytes compared to the A/PR8/full NS strain at the highest immunization dose. The shortening of the NS1 protein resulted in the simultaneous enhancement of the immunogenicity of all the studied epitopes. This was reflected in the increased formation of polyfunctional CD8$^+$ IFNγ$^+$IL2$^-$TNFα$^+$ and IFNγ$^+$IL2$^+$TNFα$^+$ T-lymphocytes and monofunctional IFNγ$^+$IL2$^-$TNFα$^-$ T-cells in mice immunized with A/PR8/NS124. Antigen-specific polyfunctional T-cells play a key role in providing protection against reinfection with different microbial and viral pathogens [11, 12, 31-34]. It is known that polyfunctional T-lymphocytes have a prolonged life cycle compared to monofunctional subpopulations and serve as a source for the formation of long-lived memory cells [33]. Polyfunctional T-lymphocytes also provide a significant contribution to the formation of the immune response to conserved influenza antigens and could be considered as effectors for cross-protection against antigenically divergent influenza viruses [34]. In the course of this research project, we only analyzed the cytokine-producing function of T-lymphocytes. However, from previous studies, it is known that the polyfunctional T-cells demonstrate more efficient killing capacities compared to their monofunctional analogs [35-38]. Therefore, we can speculate that influenza virus-specific polyfunctional T-cells generated in response to the A/PR8/NS124 immunization may be characterized by higher cytotoxic activity compared to the T-lymphocytes formed after the immunization with A/PR8/full NS strain. This hypothesis deserves further evaluation.

The published data on the role of TLR7/8 and RIG-I signaling pathways in the activation of dendritic cells as well as the induction of T-lymphocytes maturation could be used for the explanation of the increased potency of A/PR8/NS124 virus in the induction of polyfunctional T-cells. Mice with impaired RIG-I expression are characterized by the formation of the reduced number of polyfunctional T-lymphocytes in response to the immunization and weakened protection against heterologous strains of the influenza virus [39]. Considering that NS1 protein mediates its immunosuppressive function through direct interaction with RIG-I, it should be expected that the immunization of mice with influenza virus lacking the effector domain of NS1 protein would lead to the enhanced production of the polyfunctional T-lymphocytes. We found that IFNγ$^+$IL2$^-$TNFα$^+$ and IFNγ$^+$IL2$^+$TNFα$^+$ T-cells generated after immunization with the A/PR8/NS124 strain produced a higher amount of IFNγ compared to A/PR8/full NS-immunized mice. To compare the cumulative functional properties of influenza-specific T-cell immune response to A/PR8/full NS and A/PR8/NS124 strains after the i.p. immunization, we calculated an integrated MFI (iMFI) of IFNγ in predominant polyfunctional populations and cellular polyfunctionality index (PI) for each observation as was described previously [14, 20]. The results are presented in Supplementary Tables 2S and 3S. PI and iMFI reflect both the magnitude and quality of immune response. The higher values of these two parameters in the NS124 group compared to full NS group after the stimulation with HA$_{474-483}$, HA$_{323-337}$, NP$_{366-374}$, and NP$_{196-210}$ on the 8th d.p.i. and with NP$_{366-374}$ and NA$_{427-433}$ on the 21st d.p.i. showed that the shortening of NS1 protein leads to the increase in both the magnitude of T-cell immune response to influenza virus and the functional activity of antigen-specific T-lymphocytes. Since the goal of the present study was to compare the immunogenicity of the two viruses with different reproduction activity in the respiratory system, we analyzed only the systemic immune response to A/PR8/full NS and A/PR8/NS124 viruses after the i.p. immunization. However, growing literature evidence proves the importance of the local immune response to the influenza virus in the lung tissue for the mediation of heterologous protection. It was shown that the populations of CD69$^+$CD103$^+$ tissue resident memory CD4$^+$ and CD8$^+$ T-cells (Trm) play a critical role in local immune protection by means of the direct killing of infected cells [40, 41] and cytokine release [42], promoting the recruitment of the immune cells from the circulation and switching the state of the surrounding cells to non-permissive [43]. Trms are indispensable for providing optimal heterosubtypic immunity [44, 45]. Therefore, the vaccines that induce the formation of influenza virus-specific Trm cells in the lungs provide superior protection against heterologous influenza strains [46, 47]. Moreover, as it was shown by Zhao *et al.*, Trm generated in lungs after intranasal vaccine administration were more protective against challenge with pathogenic human coronaviruses than those generated after

systemic vaccination [48]. Given the obtained data, we could expect that live attenuated vaccines based on viruses with shortened NS1 protein will have higher potential in the induction of cross-reactive Trm compared to the existing LAIVs.

It should be noted that the inhibition of the immunosuppressive function of the NS1 protein did not prevent the loss of immunogenicity of several sub-immunodominant epitopes of influenza virus on the 21st d.p.i. The importance of the conserved low immunogenic epitopes for the formation of a cross-protective immune response is well known [3]. Therefore, the influenza viruses with the shortened NS1 protein could be considered as a tool for the creation of influenza virus vectors overexpressing important cross-protective epitope sequences from various genomic fragments. Such a strategy for vaccine development might solve the problem of losing the immunogenicity of conserved subdominant epitopes at the later stages after immunization.

CONFLICT OF INTERESTS

The authors declare no commercial or financial conflict of interest.

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