



# Immunogenicity of DNA Vaccine against H5N1 Containing Extended Kappa B Site: *In Vivo* Study in Mice and Chickens

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Influenza is one of the most important illnesses in the modern world, causing great public health losses each year due to the lack of medication and broadly protective, long-lasting vaccines. The development of highly immunogenic and safe vaccines is currently one of the major problems encountered in efficient influenza prevention. DNA vaccines represent a novel and powerful alternative to the conventional vaccine approaches. To improve the efficacy of the DNA vaccine against influenza H5N1, we inserted three repeated kappa B ( $\kappa$ B) motifs, separated by a 5-bp nucleotide spacer, upstream of the cytomegalovirus promoter and downstream of the SV40 late polyadenylation signal. The  $\kappa$ B motif is a specific DNA element (10pb-long) recognized by one of the most important transcription factors NF $\kappa$ B. NF $\kappa$ B is present in almost all animal cell types and upon cell stimulation under a variety of pathogenic conditions. NF $\kappa$ B is released from I $\kappa$ B and translocates to the nucleus and binds to  $\kappa$ B sites, thereby leading to enhanced transcription and expression of downstream genes. We tested the variants of DNA vaccine with  $\kappa$ B sites flanking the antigen expression cassette and without such sites in two animal models: chickens (broilers and layers) and mice (BALB/c). In chickens, the variant with  $\kappa$ B sites stimulated stronger humoral response against the target antigen. In mice, the differences in humoral response were less apparent. Instead, it was possible to spot several gene expression differences in the spleens isolated from mice immunized with both variants. The results of our study indicate that modification of the sequence outside of the sequence encoding the antigen might enhance the immune response to the target but understanding the mechanisms responsible for this process requires further analysis.

**Keywords:** mice, DNA vaccine, kappa B sites, H5N1, influenza, chicken

## INTRODUCTION

DNA vaccines were introduced over two decades ago. This very promising technique relies on the production of an antigen by the cells of an immunized host after the introduction of a genetically engineered expression cassette for this antigen and, as a consequence, induction of humoral and cellular immune responses (1). Despite multiple advantages, the DNA vaccines have apparent

pitfalls that need to be addressed before being applied for veterinary or clinical purposes (2). One of the factors limiting the effectiveness of DNA vaccine is the transport of the plasmid DNA to the nucleus through the nuclear envelope. The links between the transfer of non-viral DNA to the nucleus and the transgene expression level has been mostly explored in relation to therapeutic purposes such as gene therapy and anticancer vaccines [for reviews, see Ref. (3–6)]. Recently, it was suggested that various proteins containing a nuclear localization signal might play a role in the intracellular trafficking of plasmid DNA to the nucleus and that the microtubule network and motors might be required for this process (7). This suggestion is in agreement with earlier observations that either covalent attachment of nuclear proteins to the plasmid or the presence of *cis*-binding sites for nuclear proteins, such as transcription factors, in the DNA of the transfected plasmid might improve the efficiency of non-viral gene transfer (8, 9).

The most promising approaches involve the use of NF- $\kappa$ B (10–12). The transcription factors of the NF- $\kappa$ B family contain an evolutionarily conserved region at their N-terminus named the Rel homology domain (RHD), which is responsible for dimerization and binding to the kappa B ( $\kappa$ B) motif in DNA (13–15). Inactive NF- $\kappa$ B dimers are sequestered in the cytoplasm because of their association with the inhibitor of NF- $\kappa$ B (I $\kappa$ B). Upon stimulation, I $\kappa$ B is phosphorylated by the action of a specific I $\kappa$ B kinase, ubiquitinated, and degraded in the proteasome. The released NF- $\kappa$ B dimers enter the nucleus, bind to  $\kappa$ B sites in the promoters of the target genes, and initiate (or enhance) their transcription (16). The observation that the  $\kappa$ B motif (5'-GGGACTTCC-3') can augment nuclear entry of modified vectors and increase the expression level of reported genes in various transfected human cells was first published in 2001 (12). Similar experiments were later performed by other researchers, who observed positive correlations between the number of  $\kappa$ B sites (i.e., 5, 10, or 20 tandem repeats) upstream the cytomegalovirus (CMV) promoter and the transgene expression level in transfected cells of the murine carcinoma colon 26 line (17). Other researchers designed DNA vectors with several  $\kappa$ B sites separated by the optimized 5-bp spacer (5'-AGCTG-3') and used luciferase as a reporter protein to monitor *in vivo* the influence of these sites on the level of transgene expression (10, 11). They found higher luciferase activity in mice transfected with the plasmid containing the expression cassette for luciferase flanked by the sequence consisting of three repeated  $\kappa$ B sites separated by spacers than in mice transfected with the plasmid containing the same cassette but without  $\kappa$ B sites. Understanding of the mechanisms responsible for the higher expression of transgenes flanked by  $\kappa$ B sites in comparison to transgenes without such motifs could help in the translation of this observation into knowledge needed for the purposes of the effective DNA vaccine development.

Influenza virus is an important pathogen, causing seasonal and sudden pandemics in humans and its avian variants can be devastating for domestic poultry. Moreover, zoonotic transmission of highly pathogenic avian influenza viruses, like H5N1, has been reported regularly. Vaccination is the most promising strategy to control the virus, but traditional vaccines are not

very useful in the case of new emerging strains, and therefore, the development of innovative, new-generation vaccines is an urgent need.

We have previously published several papers describing our work on development of DNA vaccine against H5N1 influenza (18–20). In this work, we tested two types of DNA vaccine, one containing the  $\kappa$ B sites (in triplicates; with 5-bp spacers) flanking the antigen expression cassette and the other without such sites, in two animal models, chickens and mice. Both plasmids had the same antigen expression cassette encoding full-length hemagglutinin (HA) from influenza virus strain A/swan/Poland/305-135V08/2006(H5N1). Experiments with chickens, the natural host of avian influenza virus, focused on the comparison of humoral responses in sera. In mice, not only the humoral response in serum, but also the level of cytokines secreted by splenocytes and the population of cytotoxic T cells (Tc) and helper T cells (Th) were examined. Moreover, the spleen transcriptome profiles of the selected mice groups were examined. The results indicated that in chicken the humoral response was slightly better after immunization with the plasmid containing the  $\kappa$ B sites than with the plasmid without such sites. In mice, the difference was much less apparent.

## MATERIALS AND METHODS

### Plasmid Construction

The K3/pCI plasmid, containing the full-length cDNA of HA from the highly pathogenic influenza virus strain A/swan/Poland/305-135V08/2006(H5N1, clade 2.2), has been described before as a long variant of DNA vaccine (18). The 3NF/pCI plasmid contains the same expression cassette, which is additionally flanked by 45-bp sequences. One is located upstream of the CMV promoter, and the second is located downstream of the SV40 late polyadenylation signal. Each 45-bp sequence contains three repeats of NF- $\kappa$ B-binding sites (5'-GGGACTTCC-3') separated by the optimized spacer (5'-AGCTG-3'). The NFGK/pCI plasmid consists of the identical 45-bp sequences but they flank the cassette with the modified cDNA sequence of HA, present in previously described GK/pCI plasmid (20). The plasmids were propagated in *Escherichia coli* (DH5 $\alpha$  strain) and isolated using the NucleoBond<sup>®</sup> PC 10000 EF giga-scaled purification kit (Macherey-Nagel, 740548). Before immunization, the plasmid DNA, suspended in PBS, was mixed with Lipofectin<sup>®</sup> (18292-037, Invitrogen<sup>™</sup>) in a 6:1 ratio (DNA:Lipofectin [v/v]), as described before (18).

### Immunization of Animals and Ethic Statement

#### Chickens

Broiler chickens Ross 308 and layer chickens Rosa 1 were purchased from a local commercial brooder on the day of hatching and were maintained at an experimental poultry house under standard bedding conditions. Animals were fed once a day and had free access to water. At the end of the experiment, the animals were humanely euthanized. Chickens were immunized intramuscularly with 60  $\mu$ g of plasmid in final volume of 100  $\mu$ l and blood

samples were collected from the wing vein. Two doses of vaccine were administered at 7th and 21st days of life.

## Mice

Specific pathogen-free BALB/c female mice were maintained at the experimental facility at the Mossakowski Medical Research Centre, Polish Academy of Sciences (Warsaw) under a 13-h light/11-h dark cycle with free access to water and standard mouse diet. All groups were immunized intramuscularly with 20  $\mu$ g of plasmid in final volume of 50  $\mu$ l and the blood samples were collected from a left ventricle of heart. Two doses of vaccine were administered at 35th and 49th days of life. The schedule of chickens and mice immunization experiments is summarized in **Table 1**.

All efforts were made to minimize suffering. The experiments with chickens were approved by the Second Local Ethical Committee for Animal Experiments at the Medical University of Warsaw, Permit Number 17/2009. The experiments of mice immunization were approved by the Fourth Local Ethical Committee for Animal Experiments at the National Medicines Institutes, Permit Number 03/2014.

## ELISA and Antibody Titers

### Chickens

The ELISA was performed as described earlier (18). Shortly, the MediSorp Surface (Nunc, UK) plates were coated with 300 ng of the recombinant homologous H5 HA antigen (obtained in baculovirus system; Oxford Expression Technologies, UK) and the bound IgY were detected with goat anti-chicken IgY (Fc-specific)-HRP (Pierce/Thermo Scientific, USA). For the

determination of IgY endpoint titers, twofold serial dilutions (in a range of  $10^{-3}$ – $10^{-6}$ ) of chicken sera collected on day 35 were analyzed using the ELISA protocol. The absorption curves were made using Gen5 Data Analysis Software (BioTek Instruments, Inc.), and the highest dilution giving a specific positive result was determined for each serum. The reciprocal of such dilution was defined as the endpoint serum titer.

### Mice

Sera from immunized mice were tested for antibodies directed against homologous H5 HA by a one-dilution indirect ELISA using MaxiSorp Surface (Nunc, UK) plates coated with 300 ng of recombinant H5 HA (obtained in the baculoviral system; Oxford Expression Technologies, UK). Alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma-Aldrich) was used as the secondary antibody.

## Hemagglutination Inhibition (HI)

Hemagglutination inhibition tests were performed according to the OIE standard procedures using the heterologous hemagglutinating antigen prepared from the low pathogenic H5N2 strain A/chicken/Belgium/150/1999 (DG Deventer, Netherlands). The 25- $\mu$ l aliquots of serial twofold dilutions (from 1:8 to 1:512) of sera in PBS were added to an equal volume of HA antigen containing four HA units. After incubation (25 min) in V-bottom microtiter plates at room temperature (RT), 25  $\mu$ l of a 1% suspension of chicken red blood cells was added and incubated for 25 min at RT. HI titers are defined as the reciprocal of the highest dilution of sera that completely inhibited hemagglutination.

**TABLE 1** | Details of the immunization experiments.

| Animal model | Experiment Nr (chicken type; dose)  | Group    | Size (n=) | Days of treatments <sup>a</sup> |                  |                   |
|--------------|-------------------------------------|----------|-----------|---------------------------------|------------------|-------------------|
|              |                                     |          |           | Immunization                    | Blood collection | Spleen collection |
| Chickens     | Experiment 1 (layers; 60 $\mu$ g)   | K3/pCI   | 6         | 7, 21                           | 21, 28, 35       | –                 |
|              |                                     | 3NF/pCI  | 6         |                                 |                  |                   |
|              |                                     | pCI      | 2         |                                 |                  |                   |
|              | Experiment 2 (broilers; 60 $\mu$ g) | K3/pCI   | 10        | 7, 21                           | 21, 35           | –                 |
|              |                                     | 3NF/pCI  | 10        |                                 |                  |                   |
|              |                                     | pCI      | 4         |                                 |                  |                   |
|              | Experiment 3 (layers; 60 $\mu$ g)   | K3/pCI   | 10        | 7, 21                           | 35               | –                 |
|              |                                     | 3NF/pCI  | 10        |                                 |                  |                   |
|              |                                     | NFGK/pCI | 10        |                                 |                  |                   |
| pCI          |                                     | 3        |           |                                 |                  |                   |
| Mice         | Experiment 1 (20 $\mu$ g)           | K3/pCI   | 6         | 35, 49                          | 49, 56, 63       | 63                |
|              |                                     | 3NF/pCI  | 7         |                                 |                  |                   |
|              |                                     | NFGK/pCI | 7         |                                 |                  |                   |
|              |                                     | pCI      | 2         |                                 |                  |                   |
|              | Experiment 2 (20 $\mu$ g)           | K3/pCI   | 8         | 35, 49                          | 49, 56, 63       | 63                |
|              |                                     | 3NF/pCI  | 8         |                                 |                  |                   |
|              |                                     | NFGK/pCI | 8         |                                 |                  |                   |
|              |                                     | pCI      | 7         |                                 |                  |                   |
|              | Experiment 3 (20 $\mu$ g)           | K3/pCI   | 3         | 35, 49                          | 47, 52           | 52                |
|              |                                     | 3NF/pCI  | 3         |                                 |                  |                   |
|              |                                     | pCI      | 2         |                                 |                  |                   |

<sup>a</sup>The numbers refer to the day of life of the animals.

## Cytokine Production by Mouse Splenocytes and Percentage of Tc and Th Cells in Spleens

Immunized and control mice were euthanized 2 weeks after the boost dose (day 63) and their spleens were harvested. For determining the cytokine production and percentage of Tc and Th, splenocytes were prepared from spleen isolated from three randomly selected mice immunized in Experiment 2. The spleen cells suspensions were washed in RPMI-1640 medium (Sigma-Aldrich) and treated for 5 min with lysis buffer (Becton-Dickinson, Franklin Lakes, NJ, USA) in order to remove red blood cells. To determine the levels of cytokines in culture supernatants (Experiment 1 and Experiment 2), the cells ( $2 \times 10^6$  per well) were incubated in 96-well plates (Corning, NY, USA) in complete RPMI-1640 without any supplement (negative control), with recombinant H5 HA protein (Oxford Expression Technologies, UK) (10  $\mu$ g/ml) or with concanavalin A (5  $\mu$ g/ml). Cells were incubated for 72 h (37°C, 5% CO<sub>2</sub>) and centrifuged (10 min, 1,000 rpm, 4°C). The level of cytokines was quantified in the collected supernatants using the Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine Kit (Becton-Dickinson) according to the manufacturer's instructions and a FACSVERSE™ flow cytometer (Becton-Dickinson).

For determining the levels of CD8<sup>+</sup> (Tc), CD4<sup>+</sup> (Th) cells and their corresponding activated subpopulations: CD25<sup>+</sup> (Tc), CD69<sup>+</sup> (Tc), CD25<sup>+</sup> (Th), and CD69<sup>+</sup> (Th) splenocytes ( $1 \times 10^6$  per probe) were incubated 30 min on ice with the following monoclonal antibodies (Becton-Dickinson): PerCP rat anti-mouse CD4, FITC rat anti-mouse CD8, APC rat anti-mouse CD25, and FITC rat anti-mouse CD69. After incubation, the cells were washed three times, resuspended in the Stain Buffer (Becton-Dickinson), and examined by flow cytometry using FACSVERSE™ flow cytometer (Becton-Dickinson). The levels of Tc and Th were determined in the Laboratory of Flow Cytometry, Faculty of Biology University of Warsaw.

## RNA Isolation and Microarray Analysis

Microarray expression analysis was performed using the Affymetrix Gene Atlas system according to the manufacturer's instructions. RNA was isolated from three independent individuals per treatment (K3/pCI and 3NF/pCI) or two independent control individuals (pCI), 3 days after the boosted vaccination; 100 ng of total RNA that passed the initial quality control screen was then prepared for Affymetrix whole transcriptome microarray analysis using the Ambion® WT Expression Kit (4411973). Prepared samples were hybridized to the Affymetrix® Mouse Gene 2.1 ST Array Strip (Affymetrix, Santa Clara, CA, USA). The microarrays were scanned with the Affymetrix GeneAtlas Scanner, and the intensity signals for each of the probe sets were written by Affymetrix software into CEL files. The CEL files were imported into Partek Genomic Suite v 6.6 software with the use of Robust Multiarray Averaging. During this step, a background correction was applied based on the global distribution of the PM (perfect match) probe intensities and the affinity for each of the probes (based on their sequences) was calculated. Then, the probe intensities

were quantile normalized (21), log<sub>2</sub> transformed, and the median polish summarization to each of the probe sets was applied. Qualitative analysis was performed, e.g., principal components analysis, in order to identify outliers and artifacts on the microarray. After quality check, the three-way ANOVA model using Method of Moments (22) was applied to the data, which allowed us to create lists of significantly and differentially expressed genes between biological variants (with the cutoff values:  $p$  value <0.05, 1.4 >fold change >-1.4). Functional analysis of the obtained lists of the genes was performed in Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA, <http://www.ingenuity.com>). The microarray data were deposited in Gene Expression Omnibus repository under the accession number GSE101747.

## Real-time PCR

In order to validate the microarray data, the gene expression level of the selected genes was determined using the same samples of RNA as those used for microarray analysis. The cDNA was obtained from 1.2  $\mu$ g of total RNA using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) with Oligo(dT)18 primers and was then used as the template in RT-qPCR using Thermo Scientific Luminaris Color HuGreen qPCR master mix (Thermo Scientific). PCR was performed using the PikoReal™ Real-Time PCR System (Thermo Scientific). Assays contained the cDNA template diluted 20-fold. A 10-min hot-start activation at 95°C was followed by 40 cycles of 15 s of denaturation at 95°C, 30 s of annealing at 60°C, and 30 s of extension at 72°C followed by dissociation analysis (60–95°C). Relative gene expression was calculated according to the 2<sup>- $\Delta\Delta$ Ct</sup> method using TAF8 or PGK1 as the reference gene. The list of used primers is in Table S1 in Supplementary Material.

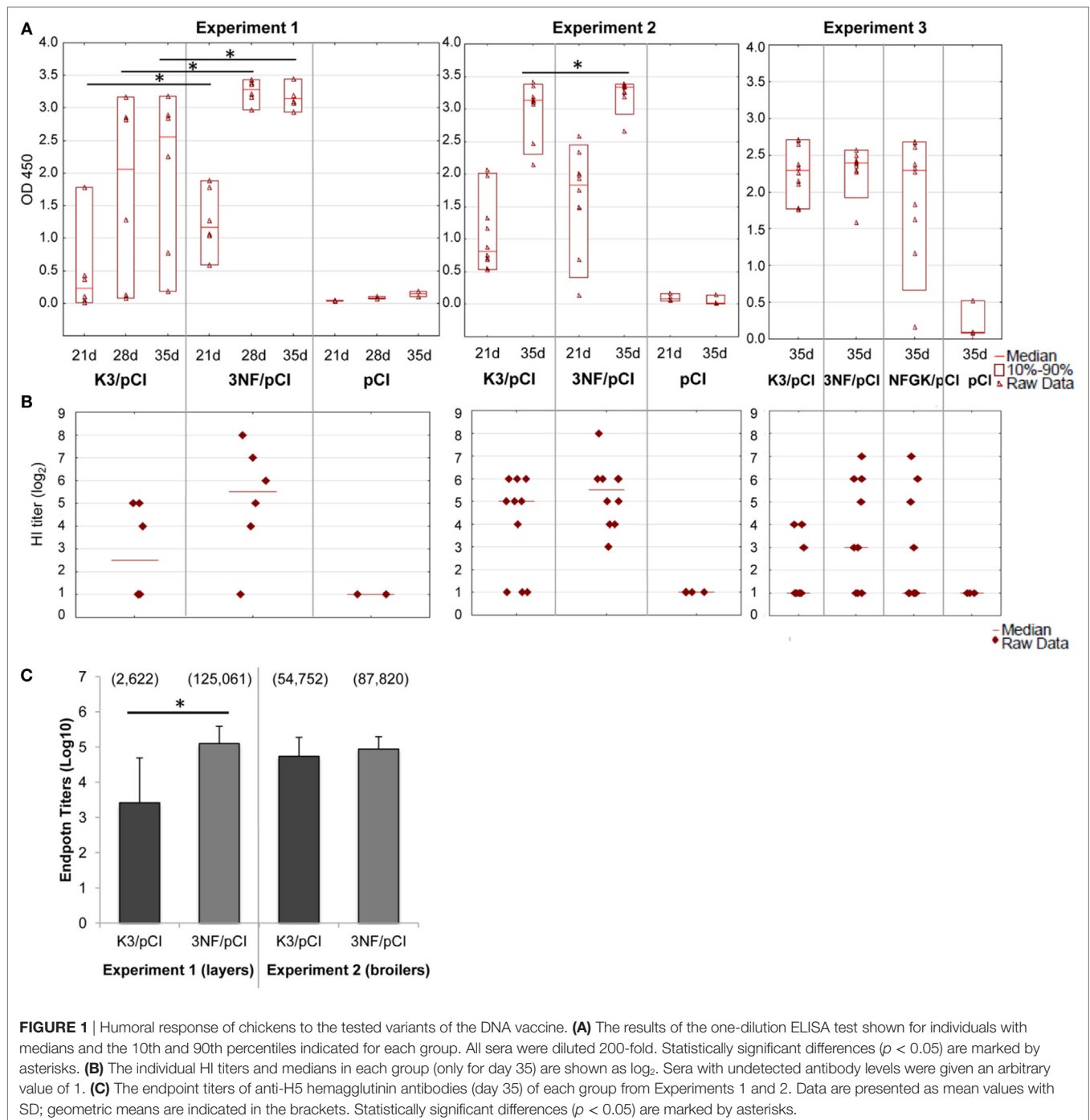
## Statistical Analysis

Non-parametric tests, such as Kruskal–Wallis (for the comparison of multiple groups) or Mann–Whitney  $U$  (for the comparison of two groups) that are components of Statistica 12 (StatSoft, Poland), were used for statistical analysis of the results of the following assays: ELISA, HI, cytokines production, and percentage of immune cells. The groups were considered significantly different if at least one of the tests was positive ( $p < 0.05$ ). The significance of the observed changes in RT-qPCR was calculated by REST-MSM (23).

## RESULTS

### Chickens Response to the Tested Variants of DNA Vaccine

The effectiveness of the three variants of DNA vaccine against H5N1 was tested first in chickens, the natural host of influenza virus. The 3NF/pCI (and NFGK/pCI used only in Experiment 3) plasmids contained  $\kappa$ B sites, while the K3/pCI plasmid did not contain such sites. The variants of the DNA vaccine were tested in chickens in three independent experiments, using either layers or broilers (Table 1). The results of the one-dilution ELISA and the HI tests are shown in Figures 1A,B, respectively. Antibody



levels in the 3NF/pCI group detected by ELISA were significantly higher than in the K3/pCI group at all three analyzed time points (days 21, 28, and 35) in Experiment 1 and at one of two analyzed time points (day 35) in Experiment 2. In Experiment 3, the group immunized with NFGK/pCI vaccine was also included. In contrast to the results obtained in Experiments 1 and 2, the differences between groups in Experiment 3 were statistically insignificant on day 35; sera from the other time points are not available (**Figure 1A**). It is worth to notice that the medians in

both groups were lower in Experiment 3 than in the previous experiments, suggesting the overall lower effectiveness of this vaccination trial.

In all experiments, the HI test was performed only with sera collected 2 weeks after the booster (day 35) using an H5N2 commercial antigen. The results shown in **Figure 1B** indicate that 3NF/pCI groups had always highest median HI than the other groups, although the applied non-parametric tests failed to confirm a statistical significance of the observed difference.

The endpoint titers of anti-H5 HA antibody were determined using sera from the final blood collection (35th day) in Experiments 1 and 2 (Figure 1C). All sera from chickens immunized with 3NF/pCI reached titers above  $10^4$  and the majority had titers above  $10^5$ . Moreover, the highest titer after immunization with 3NF/pCI was  $3.4 \times 10^5$  and  $2.2 \times 10^5$  in Experiment 1 (layers) and 2 (broilers), respectively (Figure S1 in Supplementary Material). By contrast, titers in groups K3/pCI were visibly lower and this difference was particularly appreciable in Experiment 1. In Experiment 2, titers of high responders were similar in both groups. The highest titer of K3/pCI was  $7.2 \times 10^4$  in Experiment 1 and  $2.1 \times 10^5$  in Experiment 2. In summary, addition of  $\kappa$ B sites apparently improved immunogenicity of the vaccine in chicken model.

## Mice Responses to the Tested DNA Vaccine Variants

The experiments with mice were performed independently to verify the effectiveness of the three tested variants (K3/pCI, NF/pCI, and NFGK/pCI) of the DNA vaccine. The schedule and other details of the experiments are provided in Table 1. The level of anti-H5 HA antibodies in the sera of immunized mice was monitored by one-dilution ELISA (Figure 2). The values for individuals, medians, and the 10th–90th percentiles are shown for each group at each time point. Although the medians for the NFGK/pCI group were in several cases higher than in the corresponding K3/pCI and 3NF/pCI groups, the statistically significant differences were observed only in Experiment 1 at the 56th day (between K3/pCI and NFGK/pCI) and in Experiment 2 at the 63rd day (between K3/pCI and 3NF/pCI; also between 3NF/pCI and NFGK/pCI).

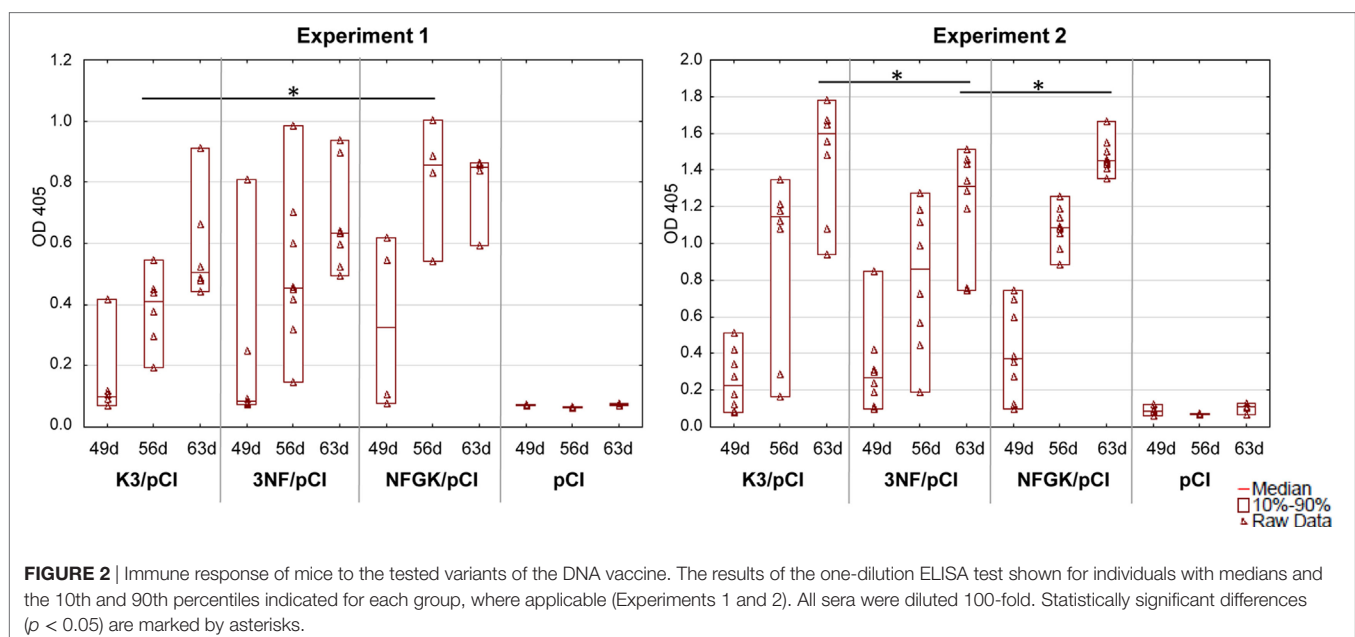
In order to further explore the differences between the mice groups, the levels of cytokines produced by the stimulated *in vitro*

splenocytes (Table 2) and the population of Tc (CD8<sup>+</sup>) and Th (CD4<sup>+</sup>) cells in the spleens (Table 3) have been assessed.

As indicated in Table 2, higher average level of IFN- $\gamma$  in K3/pCI than NFGK/pCI and 3NF/pCI was observed, while the average level of TNF was similar in K3/pCI and 3NF/pCI and slightly lower in NFGK/pCI. Unfortunately, the differences between the groups did not pass the statistical significance tests. Secretion of IL-2, IL-6, IL-4, IL-10, and IL-17a in all tested group was at the limit of detection (not shown). No secretion of TNF $\alpha$  and a very low secretion of IFN- $\gamma$  were observed in the culture of stimulated splenocytes isolated from the control group vaccinated with the empty pCI vector (not shown).

As indicated in Table 3, all vaccinated groups had higher average percentage of Tc cells as compared to the control group (pCI). The percentage of Tc CD25<sup>+</sup> cells was higher in all immunized groups, while the percentage of Tc CD69<sup>+</sup> was higher in all groups but not in 3NF/pCI. Similarly, all vaccinated groups had higher percentage of Th, Th CD25<sup>+</sup>, and Th CD69<sup>+</sup>, in the spleens than the control (pCI) group. The highest average values of CD69<sup>+</sup> (13.2%), CD25<sup>+</sup> (5.3%), and CD69<sup>+</sup> (5.6%) were in the NFGK/pCI group; however, the large SD values do not allow to qualify these values as significantly different.

More differences between the two variants (K3/pCI and 3NF/pCI) of the tested DNA vaccine were revealed by transcriptional profiling of the mice spleens isolated on day 52 (3 days after the booster) from mice immunized in Experiment 3. The analysis was limited to those 180 genes that had at least a  $\pm 1.4$ -fold difference ( $p < 0.05$ ) in at least one of the groups immunized with the DNA vaccine in comparison to the pCI group (Table S2 in Supplementary Material; Figure 3A). The 3NF/pCI group had an apparently higher percentage (41%) of downregulated genes than the K3/pCI group (23%) and lower percentage of upregulated genes: 59% in 3NF/pCI in comparison to 77% in K3/pCI. Further analysis failed to identify any significantly changed



**TABLE 2** | Cytokine levels produced by stimulated splenocytes isolated from the mice immunized with the tested variants of the DNA vaccine.

|          | <b>IFN<math>\gamma</math></b> (pg) | <b>TNF</b> (pg)    |
|----------|------------------------------------|--------------------|
| K3/pCI   | <b>1,236</b> $\pm$ 333             | <b>61</b> $\pm$ 32 |
| 3NF/pCI  | <b>960</b> $\pm$ 274               | <b>65</b> $\pm$ 30 |
| NFGK/pCI | <b>964</b> $\pm$ 520               | <b>53</b> $\pm$ 22 |

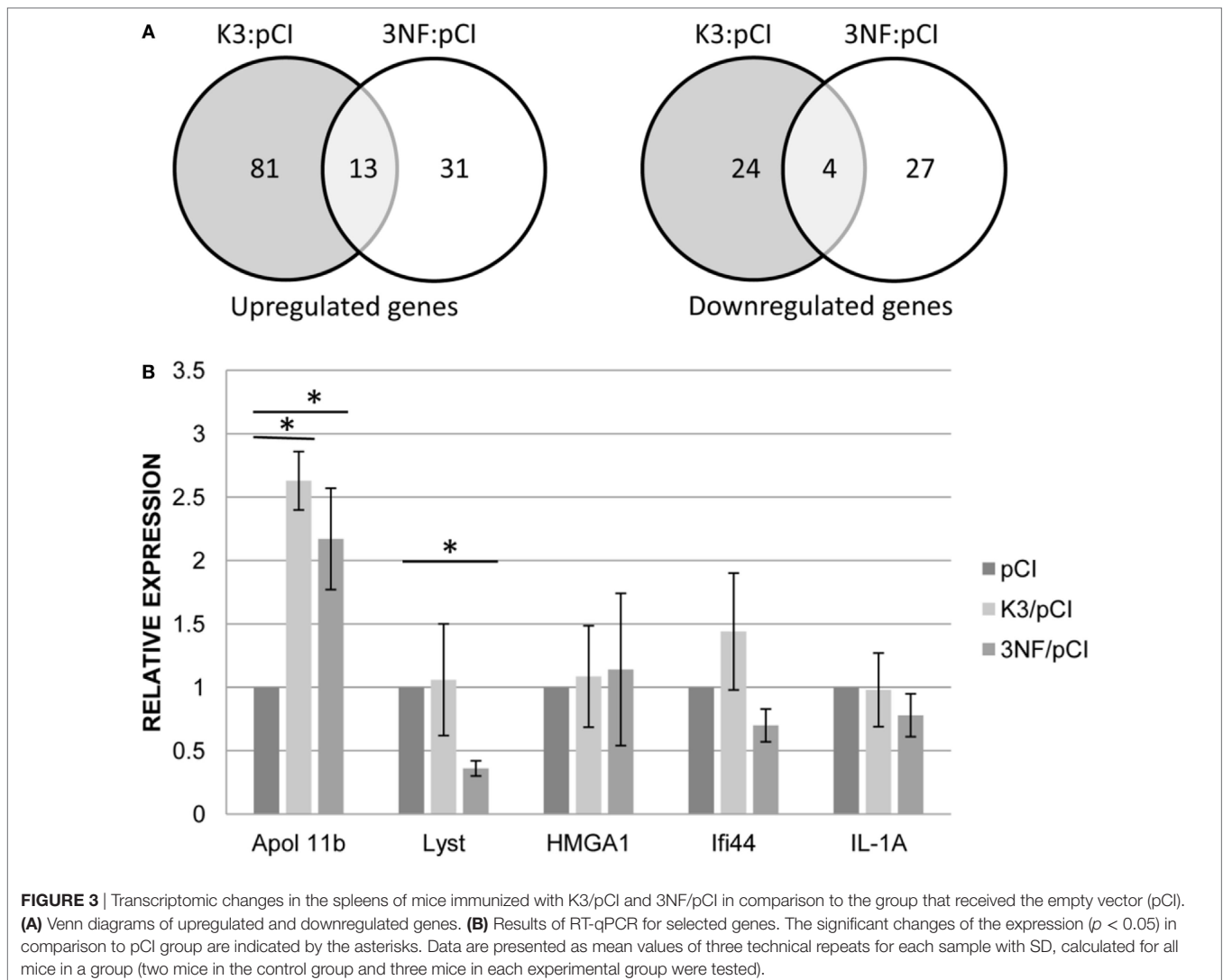
The results are means (in bold) from three individuals (n = 3) with SDs indicated.

immune-related function or pathway in the selected set of genes. Interestingly, six genes encoding miRNA were upregulated in the K3/pCI group, but only two of them (miR-669o-5p and miR-709) were also upregulated in the 3NF/pCI group. Moreover, miR-181b-1 was downregulated only in 3NF/pCI group, while miR-1186 was downregulated only in K3/pCI. The differences can be also observed in the immunoglobulins transcripts, where few of them were downregulated in K3/pCI (Igkv13-84, Igkv2-112,

**TABLE 3** | The percentage of cytotoxic T cells (Tc) and helper T cells (Th) and their subpopulations in mice spleens.

| DNA vaccine | Percentage of the indicated cells in the pull of isolated splenocytes |                                    |                                    |                        |                                    |                                    |
|-------------|---|------------------------------------|------------------------------------|------------------------|------------------------------------|------------------------------------|
|             | Tc  |                                    |                                    | Th                     |                                    |                                    |
|             | CD8 <sup>+</sup>  | CD8 <sup>+</sup> CD25 <sup>+</sup> | CD8 <sup>+</sup> CD69 <sup>+</sup> | CD4 <sup>+</sup>       | CD4 <sup>+</sup> CD25 <sup>+</sup> | CD4 <sup>+</sup> CD69 <sup>+</sup> |
| K3/pCI      | <b>26.9</b> $\pm$ 3.17  | <b>5.3</b> $\pm$ 0.65              | <b>8.2</b> $\pm$ 1.18              | <b>13.8</b> $\pm$ 0.4  | <b>3.9</b> $\pm$ 0.60              | <b>4.1</b> $\pm$ 0.43              |
| 3NF/pCI     | <b>25.0</b> $\pm$ 1.15  | <b>4.8</b> $\pm$ 0.15              | <b>7.7</b> $\pm$ 0.25              | <b>13.3</b> $\pm$ 1.37 | <b>3.0</b> $\pm$ 0.40              | <b>3.7</b> $\pm$ 0.40              |
| NFGK/pCI    | <b>26.7</b> $\pm$ 0.65  | <b>5.4</b> $\pm$ 2.26              | <b>13.2</b> $\pm$ 9.00             | <b>13.4</b> $\pm$ 1.40 | <b>5.3</b> $\pm$ 3.76              | <b>5.6</b> $\pm$ 4.00              |
| pCI         | <b>24.4</b> $\pm$ 1.60  | <b>3.9</b> $\pm$ 0.10              | <b>7.8</b> $\pm$ 0.23              | <b>12.7</b> $\pm$ 0.45 | <b>2.8</b> $\pm$ 0.25              | <b>3.3</b> $\pm$ 0.26              |

The results are means (in bold) from three individuals with SDs indicated.



Igkv3-5, Igkv4-56, Igkv4-74) and none was changed in the 3NF/pCI group (Table S2 in Supplementary Material).

Several genes (Apol11b, Lyst, HMGA1, Ifi44, IL-1A; the last two genes were not significantly changed in microarrays data) were selected for validation of expression by quantitative real-time PCR. The significant differences between the groups were observed only for Apol11b (upregulated in both K3/pCI and 3NF/pCI group) and Lyst (downregulated only in 3NF/pCI). The results of RT-qPCR analysis are shown in **Figure 3B**.

Summarizing the above results, due to the high individual variability, we do not have strong evidence to conclude that addition of  $\kappa$ B sites improves the immunogenicity of the vaccine in mouse model.

## DISCUSSION

We demonstrate for the first time in two animal models that the binding sites for NF- $\kappa$ B might improve the efficacy of a DNA vaccine against influenza. It is known that  $\kappa$ B motifs can augment nuclear entry of modified vectors and increase the expression level of reported genes in various transfected cells (10–12). The NF- $\kappa$ B proteins and  $\kappa$ B sites are evolutionarily conserved and similar signaling pathways are found in mice and chickens (16, 24–26). It has been shown that RHD, p50, is particularly highly conserved between chicken and mammalian, and *in vitro*-synthesized, truncated chicken's p105 protein (precursor of p50 protein), containing sequences that correspond to the predicted p50 protein, were able to bind the DNA fragment with the consensus  $\kappa$ B site in an electrophoretic mobility shift assay (27). Other researchers demonstrated *in vitro* that human NF- $\kappa$ B/Rel-like proteins can bind to the  $\kappa$ B-like sequence in the promoter of the gene encoding chicken lysozyme (26). The  $\kappa$ B sites used in this work are similar to that found in the promoter of the gene encoding Ig $\kappa$  light chain in human and in the LTR of HIV-1.

Also, numerous studies confirmed that codon optimization to the codon bias of the antigen improves the efficacy of DNA vaccine. Several studies with mammalian cells suggest that increasing the GC content provides better mRNA stability, processing, and nucleocytoplasmic transport. Since we have previously indicated the moderate superiority of GK/pCI vaccine over the K3/pCI vaccine (20), it was of interest to check the effects of the NFGK/pCI variant of vaccine, containing both, the  $\kappa$ B motifs and the sequence of HA with the increased percentage of GC in the third position (GC3). One could expect that NFGK/pCI DNA vaccine would be the highly immunogenic. Apparently, we have not observed an anticipated improvement of humoral response after applying of the NFGK/pCI vaccine. Presumably, the large amounts of plasmid DNA or an intracellular protein antigen (translated from the expression cassette provided by the DNA vaccine) might generate too vigorous cellular immune response which kills off the transfected cells too early to maintain immune stimulation, thereby resulting in attenuated vaccine response (28, 29).

The immunological humoral responses induced by the tested plasmids seem to work differently in the used animal models and they slightly vary depending on the experiment.

In chickens, the 3NF/pCI worked better than K3/pCI, while a single immunization trial with NFGK/pCI (only in Experiment 3; **Figure 1**) failed to show its superiority or inferiority (see the above paragraph). The highest ELISA antibody levels were induced by the 3NF/pCI plasmid similarly in three independent immunization trials, although not all experiments gave results that passed rigorous statistical tests verifying differences between the groups that received the plasmid with and without the  $\kappa$ B sites. Moreover, the lack of low responders in 3NF/pCI groups suggest that the addition of  $\kappa$ B sites could increase the chances of responding to vaccination, what is very desirable. Both, the HI titers and the IgY endpoint titers were higher in the 3NF/pCI groups than in the K3/pCI groups, confirming that the presumed more effective nuclear entry of the modified plasmid can indirectly improve immunogenicity of the vaccine. Furthermore, some differences in the strength and dynamics of the responses observed in different experiments might be attributed to the different genetic backgrounds of the two chicken lines used, as well as their metabolism, growth, and development, which are the results of intensive genetic selection (30). So far, not many studies have been performed on immunological differences between the broiler and layer chicken lines. In the experimental vaccination studies, usually the SPF White Leghorn breed is used, thus this is one of the very first reports studying the humoral response to a DNA vaccine in layer and broiler chickens.

In mice, the differences between the variants of DNA vaccine at humoral level were much less pronounced than in chickens, probably because mice are not the natural host of influenza virus. However, in Experiment 1 (**Figure 2**), the humoral response in 3NFGK/pCI group is significantly better than that in K3/pCI group on day 56. The subsequent assays, such as monitoring the level of cytokines secreted by stimulated splenocytes (isolated 2 weeks after the booster) and the percentage of Tc and Th population and their CD25<sup>+</sup> and CD69<sup>+</sup> subpopulations, might also suggest that the compared variants of the DNA vaccine differentially affect mice immune responses in the spleen. However, high individual variability does not allow us to draw strong conclusions about differences between the immunized groups.

The very low (at the detection limit, not shown) levels of IL-2, IL-6, and IL-10 and absence of IL-4 and IL-17a might be explained by the conditions of the assay and splenocytes cultivation (and induction), which were optimal for IFN- $\gamma$ , and also by the short half-life of IL-4 (31) and by the high concentration of IFN- $\gamma$ , which negatively regulates the induction of Th17 cells (32).

Comparison of the transcriptional patterns of splenocytes isolated from mice immunized with the two types of vaccine (K3/pCI and 3NF/pCI) with the transcriptional pattern of the control mice (pCI) revealed some differences between the variants. For example, differences in the transcripts level of two microRNA were detected (Table S2 in Supplementary Material): mir181-b was downregulated in the 3NF/pCI, while mir186 was downregulated in K3/pCI. Interestingly, mir181-b inhibits the expression of importin- $\alpha$ 3 that is crucial for translocation of NF- $\kappa$ B from cytoplasm to nucleus. The level of mir181-b is reduced after proinflammatory stimulation, e.g., by TNF- $\alpha$  and the transcription



of NF- $\kappa$ B-dependent genes can be activated (33). Unfortunately, little is known about function of mir1186 but it is suggested that its upregulation might stimulate cell proliferation (34).

Expression of two genes (Apol11b and Lyst) has been positively verified by RT-qPCR. Information about the function of Apol11b and Lyst, two genes with the expression verified by RT-qPCR, is rather limited. The Apol11b gene, upregulated in both tested groups (3NF/pCI and K3/pCI), encodes the Apolipoprotein L variant specific for the spleen (35). Apolipoproteins L share functional similarities with proteins of the Bcl-2 family, taking part in the regulation of apoptosis, and are encoded by genes induced under inflammatory conditions, i.e., type-1 INF. The second gene downregulated in the 3NF/pCI group (but not in the K3/pCI group) was LYST, a lysosomal trafficking regulator. A deficiency in this gene results in the enlargement of lysosomes with abnormal morphology in granulocytes and some other leukocytes (36). Recent data suggest that the LYST protein might be required for the maturation of perforin-containing granules into exocytosis-competent secretory granules (37). Moreover, silencing of the Lyst gene results in the induction of apoptosis in RPMI 8226 myeloma cells (38). Based on these findings, it can be hypothesized that vaccination with 3NF/pCI facilitates and enhances the immune response in comparison to the vaccination with K3/pCI. This is reflected in increased apoptosis, resulting in the removal of immune effector cells that have fulfilled their function and leading to immune response silencing, thereby protecting the vaccinated organism from uncontrolled inflammatory and enabling the preservation of homeostasis (39). This assumption is in agreement with the dynamics of humoral responses, since a substantial increase in induced antibodies was observed earlier in the 3NF/pCI group than in the K3/pCI group.

Despite extensive work on DNA vaccines, reports describing usage of the binding sites for nuclear factors as stimulators of antigen expression and enhancers of the immune response are quite limited. Results of our study highlight possible positive effects of such modifications on the effectiveness of DNA vaccine. However, extended analysis of the mechanisms responsible for the observed effects is needed before such modifications can be put into practice and used for development of highly immunogenic and safe DNA vaccines.

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## ETHICS STATEMENT

The experiments were approved by the Second Local Ethical Committee for Animal Experiments at the Medical University of Warsaw, Permit Number 17/2009 (chickens) or the Fourth Local Ethical Committee for Animal Experiments at the National Medicines Institutes, Permit Number 03/2014 (mice). All efforts were made to minimize suffering.

## AUTHOR CONTRIBUTIONS

PR performed mice immunization and responses analysis; AS performed chickens immunization and responses analysis; RS performed transcriptional analysis; PK involved in mice immunization; KB involved in FACS analysis; AG-S and AS conceived and designed the experiments, and prepared the final version of the manuscript. All authors were involved in writing and reviewing the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2017.01012/full#supplementary-material>.

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**Conflict of Interest Statement:** Results described in this work are subject of patent application (decision pending). No other conflict of interest declared.

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