

# CHICKEN ANAEMIA VIRUS IMPAIRS NITRIC OXIDE PRODUCTION IN HD11 CHICKEN MACROPHAGES

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**Abstract:** Immunosuppressive viruses cause substantial economic losses to the poultry industry. Chicken anaemia virus (CAV) causes severe disease in young chickens, whereas subclinical infection in older birds causes immunosuppression. In this study, we addressed the ability of CAV to interfere with production of antimicrobial molecule nitric oxide (NO) by macrophages. NO production in chicken macrophage cell line HD11 was induced using both Toll-like receptor 4 agonist, bacterial lipopolysaccharide, and an immune modulator, interferon- $\gamma$ . In addition, we treated macrophages with CAV propagated in chicken lymphoblastoid cells. The levels of NO were measured by the Griess reaction. Addition of CAV decreased both the interferon- $\gamma$  and the lipopolysaccharide associated induction of NO. Observed effect was not caused by CAV-related cytotoxicity, as no decrease in number of viable cells was observed. Although CAV could not completely abrogate NO production, attenuation of NO induction was clearly present. We have previously shown that CAV interferes with the expression of interferons in chickens during subclinical infection. Since the signalling pathways of expression of interferons and type 2 nitric oxide synthase, enzyme involved in NO formation, overlap, we conclude that measured decrease in NO levels is a consequence of CAV interference with interferon and NO synthase signalling. Regardless of the fact whether the attenuation of NO serves as a viral primary defence, or is only a secondary effect, it could impair the immune response to other pathogens and contribute to the global immunosuppression in chicken houses.

**Key words:** chicken; immunosuppression; chicken anaemia virus (CAV); macrophage; nitric oxide (NO)

## Introduction

The consumption of poultry, according to the USDA Foreign Agricultural Service, will increase worldwide, especially in China, The European Union (EU), Brazil and India (1). EU produced 15.2 million tonnes of poultry meat in 2018, which represents a new high and a cumulative rise since 2010 (2). The extraordinary performance of the world production and consumption of poultry meat reflects the modest and decelerating growth in world per capita consumption of red meat, which has been taking place for a wide variety of reasons.

For the high-income countries, the reasons include the near saturation of consumption (e.g. in the EU and Australia), policies of high domestic meat prices and/or preference for fish (Japan and Norway), and health and food safety reasons everywhere. The production of poultry is increasing, and the overall profit in USA only, based on the domestic consumption and on a large export to the other markets, was \$46.3 billion in 2018 (3). Extensive vaccination and disease monitoring are the most important strategies that make this global increase possible.

Immunosuppressive viruses of poultry, widely present in chicken houses all around the world, have devastating effects on the poultry industry. Immunosuppression and increased mortality are

caused by these viruses, as they interfere with vaccination, and moreover, as in the case of Chicken anaemia virus (CAV), they often cause subclinical infections with no obvious clinical signs.

CAV is a non-enveloped virus that contains a circular, single-stranded 2.3-kb DNA genome contained within an icosahedral capsid, 25 nm in diameter (4). It belongs to the Gyrovirus genus, Anelloviridae family (5). The CAV genome encodes three open reading frames: VP1 - a major structural protein, VP2 - a scaffolding protein, and VP3 - a non-structural protein, named apoptin, which is able to induce apoptosis selectively in tumour cells (6).

CAV causes severe disease in young chickens, characterized by a generalized lymphoid atrophy, severe anaemia, development of subcutaneous and intramuscular haemorrhages, and increased mortality. The important targets for viral infection are haemocytoblasts in the bone marrow and precursor lymphocytes in the thymus. Subclinical infection in older birds also presents an economical problem, since infected birds are often immunosuppressed (7, 8). Infected chickens suffer an increased incidence of secondary bacterial infections and evidence of decreased responsiveness to vaccines (9, 10, 11), all of which brought investigation of CAV pathogenesis back into the focus. Moreover, CAV infection increases susceptibility to viral infections such as avian Infectious bronchitis and Influenza (12). Additional problems arise from the fact that CAV also infects specific-pathogen free (SPF) flocks (13) that are important for vaccine production.

CAV compromises immune response through lymphoid depletion, but immunosuppression persists after repopulation of lymphoid tissues. In a transcriptomic profiling study of CAV infection in an *in vivo* model, Giotis *et al.* pointed that CAV induces a global immune deregulation with emphasis on T-cells suppression in infected host (14). It is also likely that CAV developed subtle strategies to evade immune surveillance, and we previously demonstrated that CAV interferes with transcription of chicken interferons alpha and gamma during subclinical infection (15). McConnell *et al.* (16) reported that CAV displays inhibitory effects on chicken macrophage cells that play a central role in body defences against microbial infections. Macrophages are crucial cell types in both innate immunity, for the clearance of invading microorganisms, and for adaptive immunity as one of the major antigen

presenting cells (17). Inhibition of their function severely impairs the host immune response, and consequently compromise vaccination in chicken houses.

To obtain additional insight into CAV biology and its immunosuppressive properties, we have examined CAV effects on commonly used macrophage cell line HD11 (18). Chicken macrophages, when exposed to pathogens or pathogen-associated molecular patterns (PAMPs), are activated to produce pro-inflammatory cytokines, chemokines and antimicrobial reactive oxygen species (ROS) and nitric oxide (NO) (19, 20), as do their mammalian counterparts. NO is the small inorganic radical of nitric oxide, produced by inducible type 2 nitric oxide synthase (NOS2) (21). This potent antimicrobial molecule reacts with DNA, proteins and lipids, and can inhibit replication of both DNA and RNA viruses. When PAMPs bind to Toll-like receptors (TLR), the signalling cascade results in upregulation of NOS2 and interferons. In this study, we used bacterial lipopolysaccharide (LPS) and interferon- $\gamma$  (IFN- $\gamma$ ) as NO inducing agents, one representing TLR4 agonist and another representing an important mediator of the antimicrobial response, respectively, and compared measured NO levels in HD11 cells with induced NO in cells infected with CAV.

## Materials and methods

### Reagents

Chicken recombinant IFN- $\gamma$  (22) was a gift from Dr. J. W. Lowenthal, CSIRO Livestock Industries, Geelong, Australia. Foetal bovine serum and chicken serum were purchased from Eurobio (Les Ulis Cedex B, France). RPMI 1640 medium, LPS, sulfanilamide, N (1-naphthyl) ethylenediamine dihydrochloride, methylene blue, Tryptophan (TRP) and Trypan Blue were purchased from Sigma-Aldrich (Taufkirchen, Germany). All the remaining reagents were purchased from Kemika (Zagreb, Croatia).

### Cells and viruses

Chicken cell lines used in this study: avian leukaemia virus MC29-transformed chicken macrophage cell line HD11 (a gift from Dr. Bernd Kaspers, University of Munich, Germany), Marek's

disease virus transformed chicken lymphoblastoid cell line MDCC-MSB1 and spontaneously immortalized quail fibroblast cell line CEC-32 (gift from Dr. Bernd Kaspers). All cells were maintained in RPMI 1640 medium with 8 % foetal bovine serum and 2 % chicken serum, with antibiotics. Cells were kept at 41 °C (optimal temperature for avian cells) in a humidified 5 % CO<sub>2</sub> / 95 % air atmosphere, with regular passages. The DelRoss strain of CAV was propagated and titrated in MDCC-MSB1 cells as described by Yuasa *et al.* (23). The viral titre from MDCC-MSB1 supernatant used to infect cells in CAV experiments was 10<sup>6</sup> TCID<sub>50</sub> CAV in 0.1 ml.

### Effect of CAV on the induction of NO

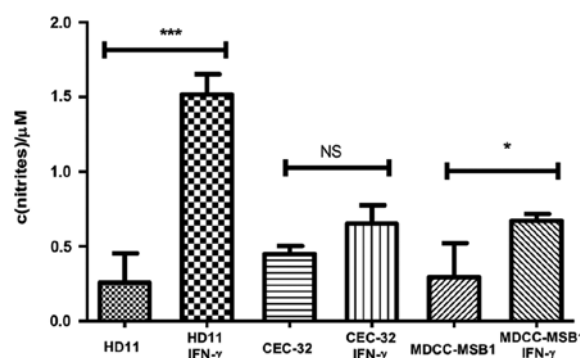
Cells were seeded at 0.5×10<sup>6</sup>/ml as quintuplicates in flat-bottomed 96 well plates. To induce NO, 100 µl of media was mixed with 100 µl of LPS or recombinant ChIFN-γ at selected concentrations (see Results and Discussion). To determine if addition of TRP would influence NO induction/inhibition, 100 µM TRP was added into cell medium. To determine if CAV influences NO induction, cells were infected with CAV (2.8 PFU/cell). Virus was added simultaneously or 1 hr prior to the stimulation of cells. The levels of NO that accumulated in cell culture media in response to various stimuli were determined by the Griess reaction after 24 hr.

### Nitric oxide analysis

The quantity of NO produced in stimulated cells was measured by the Griess reaction. Briefly, 100 µl of cell supernatant was incubated with an equal volume of Griess reagent (1 % sulfanilamide, 0.1 % N (1-naphthyl) ethylenediamine dihydrochloride in 2.5 % phosphoric acid) at room temperature for 10 min. Absorbance was measured at 570 nm, using a microtiter plate reader (Multiscan EX, Thermo LabSystems, US). Obtained absorbances were converted to micromolar values using the slope of a calibration curve established by serial dilutions of sodium nitrite from 250 µM to 1.95 µM.

### Viability assay

To test whether LPS or CAV will reduce cell number, HD11 cells at 2×10<sup>5</sup>/ml were seeded in 75 cm<sup>2</sup> flasks, cells were treated with 0.1 µg/ml and



**Figure 1:** Production of NO in avian cells upon stimulation with IFN-γ. Cells were seeded at 0.5×10<sup>6</sup>/ml in 96 well plates, and treated with IFN-γ (70 ng/ml) for 24 hr. The levels of accumulated NO were measured by the Griess reaction. Each bar represents a mean value ± s.d. from at least three individual experiments. One-way ANOVA with Tukey's post-hoc test was used for statistical analysis (NS – non-significant; \* - p < 0.05; \*\*\* - p < 0.001)

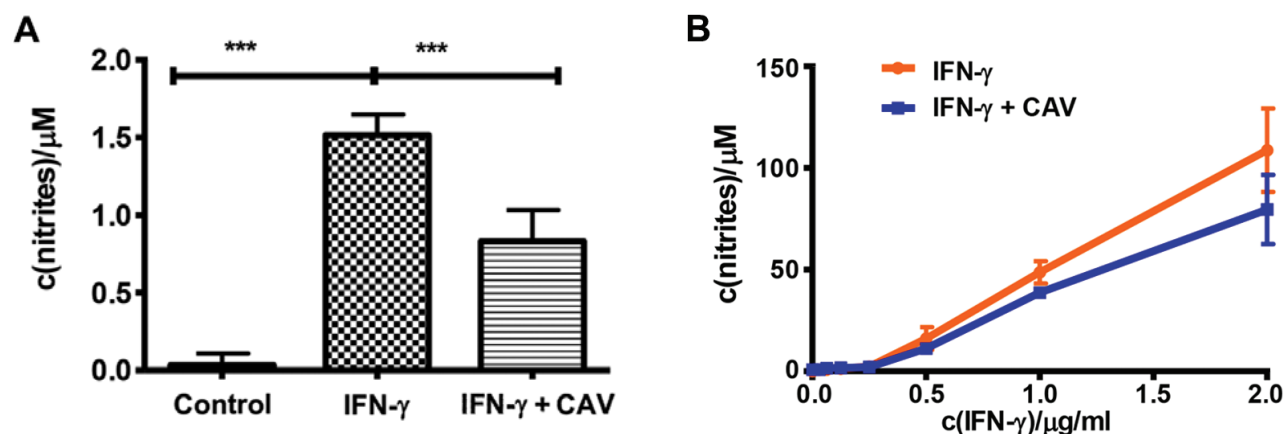
1 µg/ml LPS and incubated overnight. Cells were also exposed to CAV (2.5 PFU/cell) and incubated for 1 hr prior to the addition of LPS. Viable cells were counted in a haemocytometer the next day using Trypan Blue Exclusion staining. Briefly, cells in medium are resuspended in Trypan Blue in PBS, and only transparent cells were used to determine the percentage of viable cells.

### Statistics

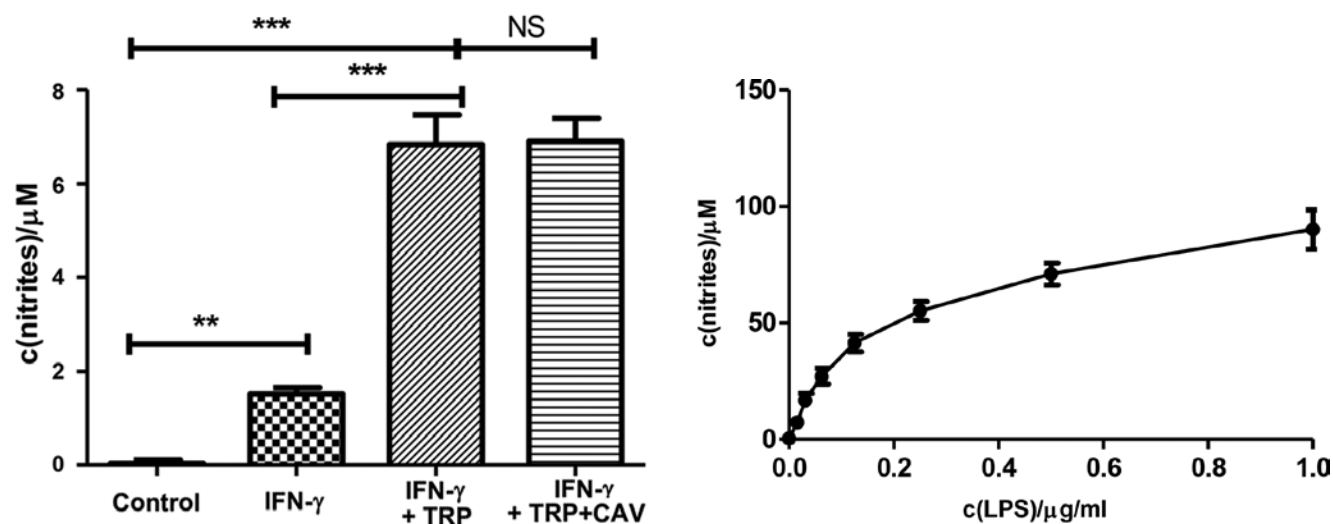
Results are represented as mean values from at least three separate experiments, except for dose-response curves and cell viability experiments where data represents mean values from two independent experiments. All graphics with error bars are presented as mean ± s.d. and were generated in GraphPad Prism 5 software. To determine statistical significance between samples, one-way ANOVA with post-hoc Tukey's multiple comparison test was performed in GraphPad Prism (NS – non-significant; \* - p < 0.05, \*\* - p < 0.01 and \*\*\* - p < 0.001).

### Results

Beside chicken macrophage cell line HD11, we analysed the induction of NO by IFN-γ in chicken lymphoblastoid cell line MDCC-MSB1 and in quail fibroblasts CEC-32. Chicken macrophages HD11 were the most inducible by IFN-γ (Figure 1). In addition, we measured a mild increase in NO production in two other cell lines. CEC-32 cell line was the least inducible in our experiments.



**Figure 2:** Effects of CAV on NO induction after stimulation with IFN- $\gamma$ . HD11 cells were seeded in 96 well plates and treated with IFN- $\gamma$  (70 ng/ml) and 2.8 PFU/cell CAV for 24 hr. The levels of accumulated NO were measured by the Griess reaction. Each bar represents a mean value  $\pm$  s.d. of at least three individual experiments. One-way ANOVA with Tukey's post-hoc test was used for statistical analysis (\*\* $p < 0.001$ ) (A). HD11 cells were seeded in 96 well plates and treated with IFN- $\gamma$  prepared as serial twofold dilutions from 2  $\mu$ g/ml to 0.03  $\mu$ g/ml and CAV for 24 hr. The levels of accumulated NO were measured by the Griess reaction. Each point represents a mean value  $\pm$  s.d. of two independent experiments (B)



**Figure 3:** TRP protects cells from CAV-induced decrease in NO induction. HD11 cells were seeded in 96 well plates and treated with IFN- $\gamma$  (70 ng/ml), TRP (100  $\mu$ M) and CAV (2.8 PFU/cell) for 24 hr. The levels of accumulated NO were measured by the Griess reaction. Each bar represents a mean value  $\pm$  s.d. of at least three individual experiments. One-way ANOVA with Tukey's post-hoc test was used for statistical analysis (NS – non-significant; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ )

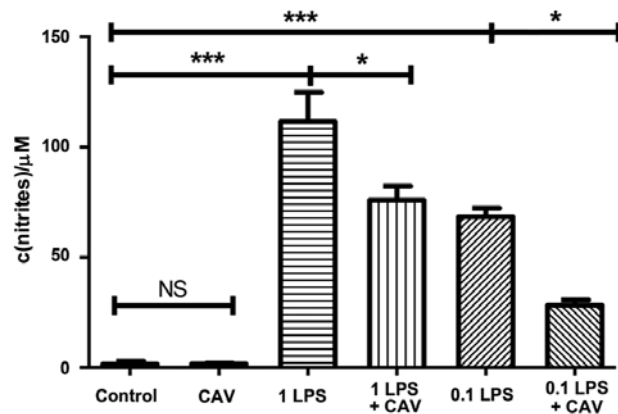
To test whether CAV influences NO production by IFN- $\gamma$  in chicken macrophages, cells were treated with IFN- $\gamma$  (70 ng/ml) and CAV for 24 hr. The addition of CAV decreased NO production (Figure 2). In addition, we titrated interferon- $\gamma$  with and without CAV to obtain dose-response curve. The inhibition of NO induction by CAV was minuscule by low dose of IFN- $\gamma$ , while in higher concentration ( $c > 1$   $\mu$ g/ml) the inhibitory

**Figure 4:** LPS dose-response curve. HD11 cells were seeded in 96 well plates and treated with LPS prepared as 1:2 serial dilutions from 1  $\mu$ g/ml to 0.0156  $\mu$ g/ml. The levels of accumulated NO were measured by the Griess reaction. Each point represents a mean value  $\pm$  s.d. of two independent experiments

effect of virus was more evident (Figure 2). Furthermore, we investigated whether addition of TRP will influence NO induction by IFN- $\gamma$ , and moreover, what effect will CAV have in this model. We added 100  $\mu$ M TRP into HD11 cell media, and treated cells as described above. Addition of TRP upregulated NO induction by IFN- $\gamma$ , whereas CAV did not have any impact on NO induction when TRP was included (Figure 3).

Induction of NO by LPS was more robust than by IFN- $\gamma$  in our experiments, with visible dose response





**Figure 5.** Effects of CAV on NO induction after stimulation with LPS. HD11 cells were seeded in 96 well plates and treated with LPS (0.1  $\mu\text{g}/\text{ml}$  and 1  $\mu\text{g}/\text{ml}$ ) and CAV (2.8 PFU/cell) for 24 hr. CAV was added 1 hr prior to the stimulation of cells. The levels of accumulated NO were measured by the Griess reaction. Each bar represents a mean value  $\pm$  s.d. of at least three individual experiments. One-way ANOVA with Tukey's post-hoc test was used for statistical analysis (NS – non-significant; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ )

(Figure 4). To test whether CAV influences NO production in chicken macrophages stimulated with LPS, HD11 cells were treated with 0.1  $\mu\text{g}/\text{ml}$  LPS and 1  $\mu\text{g}/\text{ml}$  LPS and CAV for 24 hr. CAV showed inhibitory effect on the NO induction by LPS (Figure 5), with the effect being stronger in cells treated with higher concentration of LPS. The virus itself does not induce NO production (Figure 5).

To exclude cytotoxic effects of CAV that may be responsible for decrease in measured NO levels, we performed viability assay. Incubation of cells with the virus did not reduce number of viable cells, using virus alone or in the combination with LPS (Table 1). However, we observed reduction in number of viable cells incubated with LPS alone.

## Discussion

IFN- $\gamma$  is important mediator of the immune response, and one of its effector functions is activation of macrophages and subsequent induction of antimicrobial mediator NO (24).

Differential nitric oxide production in avian cells after stimulation with interferon- $\gamma$  revealed chicken macrophages HD11 as the most inducible by IFN- $\gamma$ , what was expected according to their biological role and vast literature data. We measured a mild increase in NO production in MDCC-MSB1 cells. These cells are widely used for propagation of CAV and are well characterised in the context of host response to infection (23, 25). Giardi et al. (26) also reported that MDCC-MSB1 cells are able to produce low levels of NO after IFN- $\gamma$  stimulation. Regarding induction of NO production in CEC-32 cells, we showed that these cells produce very low levels of NO. To the best of our knowledge, no report on CEC-32 cells NO production is available, which can be attributed to the fact that CEC-32 cells are rarely used, possibly due to the fact that they were characterised as a problematic cell line, previously misidentified as a chicken cell line (27).

Although CAV did not completely abrogate NO production in macrophages in our experiments, attenuation of NO induction by both LPS and IFN- $\gamma$  was clearly present. NO is not only an effector molecule, but also an important player in the signalling cascade affecting both the adaptive and innate immunity (21, 28). It is not surprising that avian viruses, which are targeted by the macrophages/NO system, have adapted strategies to evade NO production (29, 30). Here we showed that CAV, as a part of its vast arsenal of immunosuppressive properties, inhibits IFN- $\gamma$ /macrophages/NO axis, although not to a non-induced levels.

Since it is known that production of NO interplays with several biochemical pathways that eventually lead to depletion of Tryptophan (31, 32), we included TRP in our measurements and showed that addition of TRP upregulated NO induction by IFN- $\gamma$ . Our findings are consistent with the previously published data on murine cells where addition of TRP caused a 12-fold increase in NO synthesis after IFN- $\gamma$  stimulation (32). Interestingly, CAV did not have any impact on NO induction when TRP was added, leading

**Table 1:** Cell viability assay. Chicken macrophages HD11 were seeded in 75  $\text{cm}^2$  flasks at  $2 \times 10^5/\text{ml}$  and incubated with 1  $\mu\text{g}/\text{ml}$  or 0.1  $\mu\text{g}/\text{ml}$  LPS for 24 hr. Cells were also exposed to CAV (2.5 PFU/cell) alone or in combination with LPS. Numbers represent mean values  $\pm$  s.d. from two experiments

Treatment	0.1 LPS	1 LPS	0.1LPS CAV	1 LPS CAV	CAV	HD11
Cell number after 24 hr $\times 10^5/\text{ml}$	1.9 $\pm$ 0.1	1.5 $\pm$ 0.2	2.1 $\pm$ 0.2	1.5 $\pm$ 0.3	3.9 $\pm$ 0.1	4.2 $\pm$ 0.1

to the conclusion that boost in NO production by addition of TRP protected cells from the inhibitory effect of the virus.

Induction of NO by LPS was stronger than by IFN- $\gamma$  in our experiments. Our findings correlate with previously published results by He *et al.* (33), who reported that chicken IFN- $\gamma$  itself isn't strong NO inducer in macrophages as microbial TLR4 agonist LPS.

Observed cytotoxic effect of LPS on chicken macrophages has been previously documented by others (34). There is an association between NO induction by LPS and cell death, and moreover, LPS triggers apoptosis in murine macrophages (35).

It is still not clear whether CAV is able to infect and replicate in macrophages, although it impairs their function (16). Viral proteins could bind to receptors on the macrophage membrane, but there is no data on CAV ability to bind to TLR receptors. Based on our data, we could speculate that virus doesn't bind to TLR4, since it couldn't trigger NO production by itself. Nonetheless, one plausible explanation of CAV attenuation of NO induction by other stimuli is that virus interferes with signalling pathways related to NOS2 expression in macrophages.

In general, viruses that interfere with expression of type 1 interferons often impair expression of NOS2, since these signalling pathways overlap, and as for CAV we previously showed that it interferes with both IFN- $\alpha$  and IFN- $\gamma$  expression (15). For effective NO induction by bacterial LPS, viral priming via IFN- $\gamma$  is often crucial. Hence, based on our findings, CAV interferes with both steps for effective NO induction upon bacterial infections while at the same time it interferes with interferon and NO based antiviral response. Subclinical CAV infection in chicken houses clearly involves impediment in host defence against bacteria and other viruses, and based on our previous and recent data, interference with both NO and IFN systems is involved. We can't speculate at the moment whether NO attenuation serves as a viral primary defence, or is just a secondary effect, where CAV may additionally impair NO induction on its way to evade interferon response. More importantly, virus is able to impair chicken immune response to other pathogens and to reduce effectiveness of vaccines, all of which will contribute to the global immunosuppression in chicken houses and to substantial economical losses.

We have shown for the first time that CAV interferes with NO induction in chicken macrophages. Among other avian cells, immortalised chicken macrophages HD11 were the most inducible by IFN- $\gamma$  to produce NO. Addition of CAV into cell media decreased levels of NO induced by IFN- $\gamma$ . When TRP was added into media, NO induction by IFN- $\gamma$  was upregulated and CAV didn't have any impact. Induction of NO by bacterial LPS was more robust than by IFN- $\gamma$  in HD11 cells, where CAV decreased induced NO levels upon LPS stimulation. Attenuation of NO production was not a consequence of CAV cytotoxicity, since we observed no decrease in cell viability after CAV addition. We have previously shown that CAV interferes with expression of both type 1 and type 2 interferons in chicken during subclinical infection. Since IFNs and NOS2 signalling cascades overlap, we hypothesize that CAV interferes with IFNs/NO signalling in chicken macrophages. The effect observed may contribute to the general immunosuppression in chicken houses, because NO, IFNs and their interplay are important effectors and mediators in defence against viruses and bacteria.

## Acknowledgments

The authors declare no conflict of interests.

This paper is dedicated to William L. Ragland, pioneer of molecular immunology, R&D enthusiast, mentor and friend.

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## VIRUS PIŠČANČJE ANEMIJE VPLIVA NA PROIZVODNJO DUŠIKOVIH OKSIDOV V MAKROFAGIH PIŠČANEV HD11

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**Povzetek:** Imunosupresivni virusi povzročajo velike gospodarske izgube v perutninski industriji. Virus piščančje anemije (CAV) pri mladih piščancih povzroča hudo bolezen, medtem ko subklinična okužba pri starejših pticah povzroča oslabljen imunski odziv. V tej raziskavi je bil spremljan vpliv CAV na proizvodnjo dušikovih oksidov (NO) v makrofagih. Proizvodnja NO v piščančjih makrofagih v celični liniji HD11 je bila sprožena z uporabo agonista Toll-u podobnega receptorja 4, bakterijskega lipopolisaharida in imunskega modulatorja interferona- $\gamma$ , makrofagi pa so bili okuženi s CAV, razmnoženim v piščančjih limfoblastoidnih celicah. Ravni NO so izmerili po Griessovi reakciji. Prisotnost CAV je zmanjšala proizvodnjo NO, spodbujeno tako z interferonom- $\gamma$ , kot z lipopolisaharidom. Opaženega učinka ni povzročila citotoksičnost, povezana s CAV, saj ni bilo opaziti zmanjšanja števila živih celic. Čeprav CAV ni popolnoma zavrla nastajanja NO, je bilo očitno prisotno zmanjšanje nastajanja NO. Pred tem so pokazali, da CAV moti izražanje interferonov pri piščancih med subklinično okužbo. Ker se poti znotrajceličnega prenosa urejanja izražanja interferonov in sintaze dušikovih oksidov tipa 2, encima, ki sodeluje pri tvorbi NO, prekrivajo, predvidevamo, da je izmerjeno znižanje ravni NO posledica motenj CAV pri znotrajceličnem prenosu sporočila interferona do sintaze dušikovih oksidov. Ne glede na to, ali zaviranje nastajanja NO služi kot primarna virusna obramba ali je le sekundarni učinek, lahko poslabša imunski odziv na druge patogene in prispeva k splošnemu zmanjšanju imunskega odziva v kurnikih ali na kokošjih farmah.

**Ključne besede:** piščanci; zmanjšanje imunskega odziva; virus piščančje anemije (CAV); makrofagi; dušikov oksid (NO)