# Gene Knock-Down in Chicken Immune Cells

## A.S. Leaflet R2328

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**Summary and Implications**

Chicken macrophages, when treated with inducible nitric oxide synthase (iNOS) short interfering RNA (siRNA) and then stimulated with recombinant chicken IFN-γ, produced significantly less nitric oxide (NO) and had lower iNOS mRNA levels compared to IFN-γ stimulated HD-11 cells not treated with siRNA.

As gene knock-outs are not readily available for most agricultural species, such as the chicken, siRNA technology to reduce gene expression could prove to be a powerful tool in advancing basic knowledge of avian immune function and immune response to infection. Our novel demonstration of siRNA-mediated knock-down of iNOS mRNA expression and NO production in HD-11 macrophages establishes the validity and feasibility of using RNAi technology in the avian immune system, thus providing a foundation for future investigations in avian immune function and the chicken immune response to bacterial pathogens of economic importance such as *Salmonella enteritidis*.

**Introduction**

RNA interference (RNAi) is a powerful tool to examine the function of specific genes and potential roles in biological pathways. When used to knock-down or silence a target gene of interest, the resulting loss of function can illuminate intricate gene interactions involved in fundamental biological processes such as growth and development, reproduction, cellular homeostasis, and immune responses. RNAi technology is an especially powerful tool for studying deleterious or lethal knock-out genes or for experiments with animal species not readily manipulated with current transgenic or knock-out procedures, such as the chicken.

Produced by macrophages that are stimulated with cytokine and/or microbial components, NO plays a powerful role in immune responses due to its antimicrobial and anti-tumor functions, and iNOS activity is primarily regulated at the transcriptional level. Macrophages, as part of the innate immune system, provide an early defensive team that patrols and protects the host from invading pathogens and potential tissue damage. Cytokines and cellular signaling molecules, including IFN-γ, IL-6, IL-1β, TGF-β1 (known as TGF-β4 in the chicken) and SOCS-3, have been implicated in the regulation and induction of iNOS-mediated NO production.

Using siRNA methodology, we investigated the hypothesis that knock-down of the iNOS gene would alter NO production in the chicken macrophage line, HD-11, without effects on mRNA expression of several upstream genes involved in iNOS-NO pathways.

**Materials and Methods**

The chicken macrophage cell line, HD-11, was routinely cultured and transfected with siRNA using siPORT *NeoFX* to deliver a 100 nM concentration of siRNA. Following four hours of incubation at 41° C and 5%$CO_2$, 200 µl of RPMI 1640 supplemented as above with recombinant chicken IFN-γ at a dilution of 1:25 was added to each individual well and cultured for 48 hours at 41° C and 5% $CO_2$. Following 48 hours of IFN-γ stimulation, assay plates were frozen at –20°C until thawed for RNA isolation and quantification of NO production using the Griess assay. A standard curve was used to calculate micro molar (uM) concentration of nitric oxide. Total RNA was isolated and gene expression levels for iNOS, IFN-γ, IL-1β, IL-6, TGF-β4, and SOCS-3 were analyzed using quantitative RT-PCR.

**Results and Discussion**

Chicken macrophages transfected with iNOS siRNA and then stimulated with IFN-γ produced significantly less NO than macrophages transfected with a non-sense iNOS siRNA and stimulated with IFN-γ, or HD-11 macrophages stimulated with IFN-γ alone (Table 1). Each iNOS siRNA decreased NO production in chicken macrophages to some degree (Table 1). On average, there was a 22% reduction in NO production by the iNOS siRNA.

To investigate iNOS siRNA-mediated changes in gene expression, we determined mRNA levels for several genes known to be involved in the IFN-γ-induced iNOS biological pathways. Transfection with iNOS siRNA did not alter mRNA expression levels for IFN-γ, IL-1β, IL-6, TGF-β4, or SOCS-3 genes in chicken macrophages (Figure 1). However, we observed a significant decrease ("knock-down") of iNOS mRNA expression in macrophages treated with each iNOS siRNA treatment, compared to cells stimulated with IFN-γ alone (Figure 1).

Chicken macrophages, when treated with iNOS siRNAs and stimulated with recombinant chicken IFN-γ, produced significantly less NO and had lower iNOS mRNA levels compared to IFN-γ stimulated cells untreated with siRNA. No alterations in mRNA levels for several other genes known to be involved in iNOS and IFN-γ pathways such as: IFN-γ, IL-1β, IL-6, TGF-β4, and

SOCS-3 were observed, suggesting that the lower NO production and decreased iNOS mRNA presented in this study are the direct result of siRNA-mediated inhibition of the iNOS gene in chicken macrophages.

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**Table 1. Nitric oxide (NO) production in HD-11 chicken macrophages treated with IFN-γ and siRNA. Optical density (OD) readings not sharing a letter are different by least squares means Student's t-test ($P < 0.05$).**

| Treatment | Optical Density 570nm | Micro Molar Nitric Oxide | Reduction Compared to IFN-γ Alone (%) |
|---|---|---|---|
| IFN-γ | 0.1032[a] | 9.05 | - |
| Neg. siRNA + IFN-γ | 0.0971[b] | 8.64 | 4.5 |
| siRNA #1 + IFN-γ | 0.0872[d,e] | 6.46 | 28.62 |
| siRNA #2 + IFN-γ | 0.0903[c,d] | 7.74 | 14.49 |
| siRNA #3 + IFN-γ | 0.0881[c] | 7.43 | 17.91 |
| siRNA #4 + IFN-γ | 0.0817[f] | 6.52 | 27.97 |
| siRNA #1-4 + IFN-γ | 0.0848[e] | 6.96 | 23.13 |

**Figure 1. mRNA expression of iNOS, IFN-γ, IL-1β, IL-6, TGF-β4, and SOCS-3 in HD-11 chicken macrophages treated with iNOS siRNA and stimulated with IFN-γ. Reduction of iNOS mRNA expression in HD-11 chicken macrophages treated with iNOS siRNA and IFN-γ. Bars not sharing a letter are different by least square means Student's t-test (*P* = 0.03).**