10-2008

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Keywords
Nitric Oxide, Macrophages, iNOS, siRNA, IFN-γ, Chicken

Disciplines
Agriculture | Animal Sciences | Genetics and Genomics | Poultry or Avian Science

Comments
This article is from *Veterinary Immunology and Immunopathology* 125 (2008): 375, doi:10.1016/j.vetimm.2008.05.015.

Available online at www.sciencedirect.com
ScienceDirect
Veterinary Immunology and Immunopathology 125 (2008) 375–380
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Short communication

Reduced nitric oxide production and iNOS mRNA expression in IFN-γ-stimulated chicken macrophages transfected with iNOS siRNAs

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Received 14 March 2008; received in revised form 14 May 2008; accepted 20 May 2008

Abstract

Utilizing RNA interference technology with siRNA in the HD11 macrophage cell line, we determined how the inhibition or knock-down of the iNOS (inducible nitric oxide synthase) gene affected IFN-γ-induced macrophage production of nitric oxide (NO) and mRNA expression of genes involved in this biological pathway in the chicken. Chicken macrophages produce NO when stimulated with recombinant chicken IFN-γ, however, when transfected with iNOS siRNAs, the production of NO is significantly decreased. We observed a 14–28% reduction in NO production by IFN-γ-stimulated HD11 cells at 48 h after initial siRNA transfection compared to non-transfected IFN-γ-stimulated macrophages. Significant knock-down of iNOS mRNA expression (15 to 50-fold lower) was observed for each of four iNOS siRNAs, when compared to non-transfected IFN-γ-stimulated macrophages and to those treated with a negative control siRNA. The IFN-γ-stimulated chicken macrophages transfected with iNOS siRNAs did not show altered levels of mRNA expression for genes involved in IFN-γ signaling and iNOS pathways (IL-1β, IL-6, IFN-γ, TGF-β4, or SOCS-3) suggesting that the observed decrease in NO production is a direct result of siRNA mediated knock-down of iNOS, rather than IFN-γ-induced changes in the other genes tested.

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1. Introduction

RNA interference or RNAi is a powerful tool to examine the function of specific genes and their potential role(s) in biological pathways (McManus and Sharp, 2002; Tuschl, 2001). 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.

RNA interference has been utilized in numerous studies investigating the role of specific gene(s) in biological pathways and disease processes including many related to immune function. Using RNA interference, TLR2 and TLR3 molecules were shown to be involved in IFN-γ-stimulated macrophage recognition of *Leishmania donovani* (Flandin et al., 2006). Silencing of IL-10 in human dendritic cells

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doi:10.1016/j.vetimm.2008.05.015

promoted Th1 responses in naïve CD4 T-cells via production of IL-12 and IFN-γ while decreasing IL-4 cytokine production (Liu et al., 2004). Inhibition of the chemokine receptor, CCR5, induced IL-6 and IL-8 protein production and blocked replication of HIV in a CD4 T-cell line (Pauls et al., 2006). RNA interference of viral replication, developmentally controlled genes, and myostatin in avian cells has also been demonstrated (Chen et al., 2007; Dai et al., 2005; Sato et al., 2006).

Inducible nitric oxide synthase (iNOS), also known as NOS-2, is an enzyme that produces nitric oxide (NO) from the amino acid L-arginine (Bogdan, 2001; Bogdan et al., 2000; Alderton et al., 2001). Produced by macrophages stimulated with cytokine and/or microbial components, NO plays a powerful role in immune responses due to its antimicrobial and anti-tumor functions (Bogdan, 2001; Blanchette et al., 2003; Bogdan et al., 2000; MacMicking et al., 1997). iNOS activity is primarily regulated at the transcriptional level, although translational and posttranslational events such as protein dimerization and stability along with phosphorylation have been shown to influence iNOS activity (Aktan, 2004; Kleinert et al., 2004).

The activation of transcription factors such as NF-κβ by LPS, TNF-α, and IL-1β induces iNOS expression and NO production in macrophages (Aktan, 2004; Kleinert et al., 2004). Additional cytokines and cellular signaling molecules have been implicated in the regulation and induction of iNOS-mediated NO production (Bogdan, 2001). Stimulation of murine, rat, and human macrophages with LPS, IFN-γ, IL-6, IL-1β, TNF-α, or a combination of these molecules induces iNOS expression (Kleinert et al., 2003, 2004). Interleukin-6 and IFN-γ activate members of the Jak/Stat pathway of intracellular signaling and transcription factors, namely Jak1 and Jak2, and Stat1 and Stat5 (Paukku and Silvennoinen, 2004; Schindler and Bogdan, 2001). Suppressor of cytokine signaling-3 (SOCS-3), intimately associated with Jak1, is induced by various cytokines such as IL-1, IL-6, IFN-γ, and TNF-α and inhibits the signaling of IL-6 and IFN-γ and other cytokine signaling pathways as well (Paukku and Silvennoinen, 2004; Tan and Rabkin, 2005). Transforming growth factor-β1 (TGF-β1) negatively regulates iNOS expression and NO production at many levels and is considered the most important negative regulator of iNOS-mediated NO production in macrophages (Vodovotz, 1997; Vodovotz et al., 1999).

Murine (Blanchette et al., 2003) and human macrophages (Bogdan, 2001) stimulated with IFN-γ produce high levels of NO. The production of NO by IFN-γ-stimulated chicken macrophages and monocytes have also been established (Okamura et al., 2005; Withanage et al., 2005; Crippen et al., 2003; Su and Austic, 1998; Chang et al., 1996; Sung et al., 1991). In a similar manner as IFN-γ, LPS has been shown to induce NO production in chicken macrophages (Hussain and Qureshi, 1997; Dil and Qureshi, 2003). Increased NO production by chicken macrophages infected with various *Salmonella* and *Eimeria* species indicates a role of NO and, therefore, likely iNOS activity, in avian immunity to disease (Babu et al., 2006; Lillehoj and Li, 2004). Using siRNA methodology, we investigated how knock-down of the iNOS gene would alter NO production in the chicken macrophage line, HD11, and the effects on mRNA expression of several genes in IFN-γ-iNOS-NO pathways.

2. Materials and methods

2.1. Macrophage culture, transfection, and IFN-γ stimulation

The chicken macrophage cell line, HD11 (Beug et al., 1979), was maintained in RPMI 1640 medium (Sigma) supplemented with 10% newborn calf serum (heat-inactivated), 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol (pH 7.3) at 41 °C and 5% CO_2. Cells were cultured in 75 cm^2 tissue flasks (Corning Inc., Corning, NY) and split approximately every 3 days. HD11 macrophages (1×10^5 cells in 200 μl media) were cultured overnight in sterile 96 well plates. Prior to transfection, the RPMI 1640 media was gently aspirated from the wells and cells were rinsed with 100 μl Opti MEM(R) I reduced-serum medium (Gibco Invitrogen, Carlsbad, CA) to remove any residual RPMI 1640 medium. siPORT *NeoFX* (Ambion, Austin, TX) transfection reagent was used according to manufacturer's instructions to deliver a 100 nM concentration of siRNA in a final volume of 100 μl. As measured by flow cytometry using Silencer FAM GAPDH siRNA (Ambion, Austin, TX), transfection efficiency averaged 30–35%. Cells not treated with siRNA were incubated in a similar manner with 100 μl Opti MEM(R) I reduced-serum medium alone. After 4 h of incubation at 41 °C and 5% CO_2, all medium was removed and cells were rinsed with 100 μl Opti MEM(R) I reduced-serum medium. 200 μl of RPMI 1640 supplemented as above and with recombinant chicken IFN-γ expressed in COS cells (Song et al., 1997) at a dilution of 1:25 was added to each

individual well and cultured for 48 h at 41 °C and 5% CO_2. After 48 h of IFN-γ stimulation, assay plates were frozen at −20 °C until thawed for RNA isolation and quantification of NO production. Untreated HD11 cells were stimulated with recombinant chicken IFN-γ were given 200 μl of RPMI 1640 plus supplements alone and incubated under identical conditions. A total of four replicate experiments, each consisting of six identical wells per each of 8 treatments (unstimulated and untransfected with siRNA, IFN-γ-stimulated and untransfected with siRNA, stimulated and transfected with siRNA #1, 2, 3, 4, or a non-sense negative control, and stimulated and treated with a combination of siRNA #1, 2, 3, and 4) were performed.

2.2. iNOS siRNA construction and sequence

Four siRNA targeted towards different regions of the chicken iNOS mRNA sequence (GenBank Accession # MN 204961) and one non-sense negative control were designed with siRNA Target Finder software (Ambion, Austin, TX) and were custom synthesized and HPLC purified by Ambion. The iNOS siRNA sequences used are as follows:

siRNA #1 5′ GUGUGGAGUUCACAAAGUUtt 3′
siRNA #2 5′ GAUUCUGUGCAUGGAUGAGtt 3′
siRNA #3 5′ UUCCCAUGAAGCUGAAAUUtt 3′
siRNA #4 5′ GCCGUGCAUUCUUAUUGGCtt 3′
siRNA neg. 5′ CUUGAUGACUAUAGAUGCGtt 3′

The non-sense negative control siRNA sequence has a similar percentage of each nucleotide as represented in the four iNOS siRNAs and shows no homology to the chicken genome (Ensembl release 48, December 2007, www.ensembl.org/Gallus_gallus/index.html).

2.3. Quantification of nitric oxide

Thawed aliquots of 50 μl culture supernatants mixed with 50 μl Griess reagent: 5% phosphoric acid (Fisher Scientific, Fair Lawn, NJ), 1% sulfanilamide and 0.1% *N*-naphthylethylenediamine (Sigma Aldrich, St. Louis, MO) were incubated at room temperature for approximately 10 min and then were read on an ELISA Microplate plate reader (Bio-Rad, Hercules, CA) at 570 nm (Green et al., 1982). A standard curve produced from 0 to 50 nmoles of $NaNO_2$ was prepared for calculation of NO production in test samples.

2.4. RNA isolation and gene expression

Total RNA was isolated from pooled samples (6 individual wells per treatment, 4 replicates per each treatment performed on different days) using RNAqueous© (Ambion, Austin, TX) according to manufacturer's instructions. Gene expression levels of mRNA transcripts were analyzed by quantitative real-time RT-PCR using QuantiTect SYBR Green RT-PCR (Qiagen, Waltham, MA) as previously reported (Cheeseman et al., 2007). Primer sequences for 28S, IL-1β, IL-6, IFN-γ, and TGF-β4 have been previously reported and do not amplify genomic DNA as they span an intron–exon boundary (Kogut et al., 2003). Because the primers for iNOS (Xing and Schat, 2000) have been reported to amplify both RNA and genomic DNA, samples were DNase treated with DNA-Free (Ambion, Austin, TX) according to manufacturer's instructions before amplification. Chicken TGF-β4 is generally recognized as the avian counterpart of mammalian TGF-β1, having similar functions in immunity in birds (Jakowlew et al., 1997). The SOCS-3 primer sequences are as follows:

Forward 5′ GCCCCAGGTGATGGTGTA 3′
Reverse 5′ CTTAGAGCTGAACGTCTTGAGG 3′

Quantitative real-time RT-PCR reactions, run in triplicate for each sample and gene, were performed as previously described (Cheeseman et al., 2007). Briefly, the q-RT-PCR reactions were performed on an Opticon 2 (Bio-Rad, Hercules, CA) with the following program: 1 cycle at 50 °C for 30 min, 95 °C for 15 min followed by 45 cycles of 94 °C for 15 s, 59 °C for 30 s, and 72 °C for 30 s followed by reading of the plate. Additionally, a melting curve from 60 to 90 °C with a reading every 1 °C was generated on all RT-PCR 96-well plates. Data were transformed and expressed as the adjusted Ct (cycle threshold) value using the following formula:

$$40 - \left[(\text{mean test gene Ct}) + (\text{median 28S Ct} - \text{mean 28S Ct}) \times \left(\frac{\text{test gene slope}}{\text{28S slope}}\right)\right]$$

Slopes were determined with a series of 10-fold dilutions of plasmids encoding each target gene to determine PCR efficiency, and median 28S Ct represents the median Ct value of all

individual samples for this housekeeping reference gene.

Additionally, for differentially expressed genes, the fold change in mRNA expression of the untransfected HD-11 cells compared to iNOS siRNA transfected cells was calculated using the $2_{T}^{-\Delta\Delta C}$ method (Livak and Schmittgen, 2001).

2.5. Statistical analysis

Individual mRNA levels for each treatment and the four replicates are analyzed as the mean of triplicate well measurements. Analysis of gene mRNA expression and analyzed NO production levels was performed with an ANOVA model using JMP software (JMP) (SAS Institute, 2004).

3. Results and discussion

HD11 chicken macrophages produce nitric oxide when stimulated with recombinant chicken IFN-γ (Lillehoj and Li, 2004). To determine the role of iNOS expression in NO production in chicken macrophages, HD11 cells were cultured with one of four iNOS siRNAs, a combination of the four iNOS siRNAs, or a non-sense (negative) siRNA, then stimulated with chicken IFN-γ. After 48 h of IFN-γ stimulation, NO was determined using the Griess assay. Using a standard curve produced from 0 to 50 nmoles of $NaNO_2$, we determined the amount of NO (in μM) produced in HD11 macrophages.

HD11 cells transfected with iNOS siRNAs and stimulated with IFN-γ produced significantly lower levels of NO than those transfected with a non-sense iNOS siRNA and stimulated with IFN-γ, or HD11 cells stimulated with IFN-γ alone (Table 1). The non-sense (negative) iNOS siRNA induced a modest but significant decrease in NO production by the HD11 macrophages. Compared to IFN-γ-stimulated macrophages, treatment with the non-sense siRNA resulted in a reduction of only 4.5% while the other iNOS specific siRNAs showed significantly lower levels of NO production compared to the non-sense siRNA or no siRNA transfected macrophages.

All iNOS siRNAs significantly decreased NO production in the HD11 chicken macrophages to varied degrees (Table 1). Macrophages treated with iNOS siRNA #1 showed the largest decrease in NO production (28.6%) compared to non-transfected IFN-γ-stimulated macrophages. The least reduction of NO production was observed in siRNA #2 treated macrophages, corresponding to a 14.5% reduction in NO production compared to non-transfected IFN-γ-stimulated chicken macrophages.

Approximately 30% of macrophages were successfully transfected with iNOS siRNAs as assessed by flow cytometry using a FAM labeled GAPDH siRNA (Ambion, Austin TX). Therefore, the decreased NO production observed (22%) at the whole culture level was due to only the fraction of cells which exhibited the effects of gene knock-down. Increasing the efficiency of siRNA transfection in future experiments could abate this ''diluting'' effect and demonstrate a more robust interference of the iNOS gene.

To better verify that the IFN-γ-iNOS-NO pathway was directly affected by iNOS knock-down and not the result of changes in expression of related genes, we determined mRNA levels for several genes known to be involved in IFN-γ-induced iNOS biological pathways. Transfection with iNOS siRNAs did not alter mRNA expression levels for IFN-γ, IL-1β, IL-6, TGF-β4, or SOCS-3 in HD11 chicken macrophages (Fig. 1). However, we observed a significant decrease or ''knock-down'' of iNOS mRNA expression in macrophages treated with all iNOS siRNAs compared to HD11 cells stimulated with IFN-γ alone (Fig. 1). Transfection with iNOS siRNAs induced significantly lower iNOS mRNA levels corresponding to 15 to 50-

Table 1
Nitric oxide production in HD-11 chicken macrophages treated with IFN-γ and siRNAs

Treatment	OD 570 nm	μM $NaNO_2$ *	% reduction compared to IFN-γ alone
IFN-γ	0.1032[a]	9.05	0
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

OD readings not sharing a letter are significantly different by LS Means Student's *t*-test ($P < 0.05$). A standard curve was used to calculate μM concentration of $NaNO_2$ (*) (μM NO = 142.06 (OD 570 nm)−5.09).

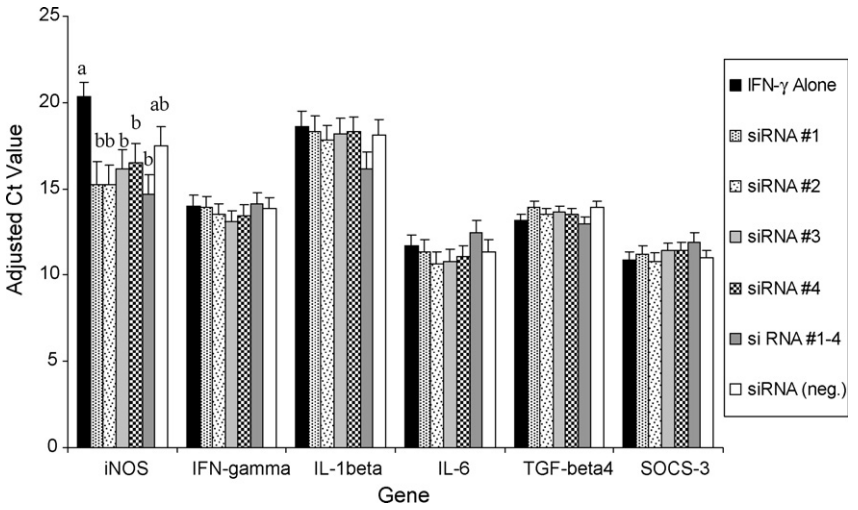

Fig. 1. mRNA expression of iNOS, IFN-γ, IL-1β, IL-6, TGF-β4, and SOCS-3 in HD-11 chicken macrophages treated with iNOS siRNAs and stimulated with IFN-γ. Reduction of iNOS mRNA expression in HD-11 chicken macrophages treated with iNOS siRNAs and IFN-γ. Bars not sharing a letter are significantly different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

fold less iNOS mRNA expression compared to untreated HD11 cells (not shown). No difference in iNOS mRNA expression was observed for HD11 macrophages treated with a non-sense or negative siRNA compared to those stimulated with only IFN-γ.

In this paper we report the usage of siRNAs to knock-down gene expression in avian macrophages. Specifically, we demonstrate that HD11 chicken macrophages when treated with iNOS siRNAs and stimulated with recombinant chicken IFN-γ produce significantly less NO and have lower iNOS mRNA levels compared to IFN-γ-stimulated HD11 cells untreated with siRNAs. 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.

Recently, inhibition of the cytokine genes IL-10 and IFN-γ with siRNAs was reported in the pig (Sidahmed and Wilkie, 2007) demonstrating the feasibility of RNA interference in studies involving agricultural animal species. 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 responses to infection.

Our novel demonstration of siRNA-mediated knock-down of iNOS mRNA and nitric oxide production in HD11 macrophages enhances the validity and feasibility of using RNAi technology in the avian immune system and provides a foundation for future investigations in avian immune function and chicken immune responses to disease (Benes et al., 2006).

Acknowledgements

The authors thank Natalie Smith (Iowa State University) for her literature research and Pete Kaiser (Institute for Animal Health, Compton, UK) for information on quantitative real-time RT-PCR primers and data analysis. This study was supported by the Animal Health, Hatch Act, State of Iowa and Iowa State University Center for Integrated Animal Genomics Funds, National Research Initiative Grant no. 2004-35205-14234 from the USDA Cooperative State Research, Education, and Extension Service, and Research Grant US-3408-03 from BARD, the Binational Agriculture Research and Development Fund.

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