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## Molecular cloning and characterization of chicken NK-lysin

Yeong Ho Hong<sup>a</sup>, Hyun S. Lillehoj<sup>a,\*</sup>, Rami A. Dalloul<sup>a</sup>, Wongi Min<sup>a,1</sup>,  
Kate B. Miska<sup>a</sup>, Wenbin Tuo<sup>a</sup>, Sung Hyen Lee<sup>a</sup>,  
Jae Yong Han<sup>b</sup>, Erik P. Lillehoj<sup>c</sup>

<sup>a</sup>Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute,  
United States Department of Agriculture, Beltsville, MD 20705, USA

<sup>b</sup>Division of Animal Genetic Engineering, Department of Food and Animal Biotechnology,  
Seoul National University, Seoul 151-921, South Korea

<sup>c</sup>Department of Pediatrics, School of Medicine, University of Maryland, Baltimore, MD 21201, USA

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### Abstract

NK-lysin is an anti-microbial and anti-tumor protein expressed by NK cells and T lymphocytes. In a previous report, we identified a set of overlapping expressed sequence tags constituting a contiguous sequence (contig 171) homologous to mammalian NK-lysins. In the current report, a cDNA encoding NK-lysin was isolated from a library prepared from chicken intestinal intraepithelial lymphocytes (IELs). It consisted of an 850 bp DNA sequence with an open reading frame of 140 amino acids and a predicted molecular mass of 15.2 kDa. Comparison of its deduced amino acid sequence showed less than 20% identity to mammalian NK-lysins. The tissue distribution of NK-lysin mRNA revealed highest levels in intestinal IELs, intermediate levels in splenic and peripheral blood lymphocytes, and lowest levels in thymic and bursa lymphocytes. Following intestinal infection of chickens with *Eimeria maxima*, one of seven *Eimeria* species causing avian coccidiosis, NK-lysin transcript levels increased 3–4-fold in CD4<sup>+</sup> and CD8<sup>+</sup> intestinal IELs. However, cell depletion experiments suggested other T lymphocyte subpopulations also expressed NK-lysin. The kinetics of NK-lysin mRNA expression indicated that, whereas infection with *E. acervulina* induced maximum expression only at 7–8 days post-infection, *E. maxima* and *E. tenella* elicited biphasic responses at 3–4 and 7–8 days post-infection. Finally, recombinant chicken NK-lysin expressed in COS7 cells exhibited anti-tumor cell activity against LSCC-RP9, a retrovirus-transformed B-cell line. We conclude that chicken NK-lysin plays important roles during anti-microbial and anti-tumor defenses.

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**Keywords:** NK-lysin; Chicken; Cloning; *Eimeria*; Intraepithelial lymphocytes

\* Corresponding author. Tel.: +1 301 504 8771;  
fax: +1 301 504 5103.

E-mail address: [hlilleho@anri.barc.usda.gov](mailto:hlilleho@anri.barc.usda.gov) (H.S. Lillehoj).

<sup>1</sup> Present address: Gyeongsang National University, Jinju,  
South Korea.

### 1. Introduction

NK-lysin and granulysin are homologous cationic peptides produced by natural killer (NK) cells and

cytotoxic T lymphocytes (CTLs). Both are members of a larger group of proteins, referred to as saposin-like proteins, that are found along with perforin in cytolytic lymphocyte granules (Munford et al., 1995; Liepinsh et al., 1997). These proteins share a common predicted structure and perform a variety of biological functions (Pena and Krensky, 1997). Anti-microbial and anti-tumor cell properties of NK-lysin and granulysin have been reported with targets including Gram-positive and Gram-negative bacteria, and protozoan parasites (Andersson et al., 1995; Tschopp and Hofmann, 1996; Stenger et al., 1998; Andreu et al., 1999; Hata et al., 2001; Jacobs et al., 2003). In particular, NK-lysin showed high anti-bacterial activity against *Escherichia coli* and *Bacillus megaterium*, and lytic activity against YAC-1, a NK cell sensitive tumor cell line (Andersson et al., 1996). The microbiocidal and tumor cytolytic activities of NK-lysin, like those of other saposin-like proteins, are believed to be due to its ability to form pores in the cell membrane due to its  $\alpha$ -helical structure (Ruyschaert et al., 1998; Zhang et al., 2000).

Avian coccidiosis is caused by several species of the genus *Eimeria* and is considered to be one of the most economically important diseases of domestic poultry. For many years, prophylactic use of anti-coccidial drugs as feed additives has been the primary means of controlling the disease. However, the use of coccidiostats has drawbacks because of the increasing emergence of drug resistant field strains of *Eimeria* (Allen and Fetterer, 2002). Vaccines may offer alternatives to drugs as a means of controlling coccidiosis since avian coccidia are highly immunogenic, and primary infections stimulate immunity to homologous parasite challenge. Although several promising vaccine candidates have been described (Lillehoj et al., 2000a, 2000b; Dalloul and Lillehoj, 2005), efforts to develop an effective, commercially feasible coccidiosis vaccine have been slow.

The putative chicken homologue of NK-lysin was previously reported in chicken splenocytes, thymus and intestinal intraepithelial lymphocytes (IELs) (Tirunagaru et al., 2000; Cui et al., 2004; Min et al., 2005). In prior work, we constructed a chicken cDNA library from *Eimeria*-infected intestinal IELs with the goal of identifying endogenous anti-microbial factors expressed by host cells during coccidiosis (Min et al., 2005). Contig 171, composed of 87 overlapping expressed sequence tags (ESTs), was provisionally

identified as a NK-lysin-like sequence based on its homology to mammalian NK-lysins and granulysin. Interestingly, ESTs derived from this gene occurred with the highest prevalence in the library, an indication that its encoded gene product was an important component of the intestinal immune response to coccidiosis. The current study was conducted to clone, express, and characterize contig 171 and confirm its identity as NK-lysin.

## 2. Materials and methods

### 2.1. Cloning of chicken NK-lysin

Construction of the IELs cDNA library in the pCMV-SPORT6 vector was described previously (Min and Lillehoj, 2004). Contig 171 (GenBank accession no. CD728315) was identified based on sequence homology to mammalian NK-lysin sequences available in GenBank using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>). The coding sequence was amplified from total RNA of IELs by RT-PCR using the following restriction enzyme-anchored primers: *Nco* I anchored forward primer, 5'-CCCCATGGGGGAAGGGA-CAGCATGGCCGCTGCTC TC-3; *Not* I anchored reverse primer, 5'-CGCGGCCCGGGGCTCTGGC CGTGCTCAG CCC-3' (restriction sites are underlined). PCR products were digested with *Nco* I and *Not* I (Roche, Indianapolis, IN), ligated into the corresponding restriction endonuclease sites of pET32a(+) (Novagen, Madison, WI), and transformed into BL21(DE3) competent cells (Invitrogen, Carlsbad, CA). For expression in COS7 cells, pET32-NK-lysin was digested with *Kpn* I and *Not* I, gel-purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA), subcloned into pTriEx-4 under control of the CMV immediate early promoter (Novagen), and transformed into TOP 10 competent cells (Invitrogen).

### 2.2. Real-time RT-PCR

Oligonucleotide primers for NK-lysin and GAPDH quantitative RT-PCR are listed in Table 1. The levels of NK-lysin transcripts were normalized to those of GAPDH using the Q-gene program (Muller et al., 2002). Amplification and detection were carried out

Table 1  
Real-time RT-PCR primers

Target	Primer sequences	Product size (bp)	Accession no.
Chicken NK-lysin	5'-GATGGTTCAGCTGCGTGGGATGC-3' 5'-CTGCCGGAGCTTCTTCAACA-3'	217	DQ186291
Chicken GAPDH	5'-GGTGGTGCTAAGCGTGTAT-3' 5'-ACCTCTGTC ATCTCTCCACA-3'	264	K01458

using equivalent amounts of total RNA isolated using TRIzol (Invitrogen) from lymphocytes of the bursa, thymus, spleen, peripheral blood, or intestine as described (Min et al., 2001), the Mx3000P system with Brilliant SYBR Green QPCR master mix (Stratagene, La Jolla, CA), and the following thermal profile: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 56 °C for 30 s, and extension at 72 °C for 1 min. The standard curve for chicken NK-lysin and GAPDH reactions were generated as follows: Each RT-PCR experiment contained two-fold dilutions of standard RNA in triplicate. To normalize RNA levels between samples within an experiment, the mean threshold cycle value ( $C_T$ ) for the NK-lysin and GAPDH products were calculated by pooling values from all samples (triplicate) in that experiment.

### 2.3. *Eimeria* infection

Fertilized eggs of specific pathogen-free chickens were obtained from SPAFAS (Charles River Laboratories, Wilmington, MA) and hatched at the Animal and Natural Resources Institute (Beltsville, MD). Chickens were provided free access to feed and water, and inoculated with  $1.0 \times 10^4$  sporulated oocysts of *E. maxima*, *E. tenella*, or *E. acervulina* at 3 weeks of age as described (Min et al., 2001). Chicken tissues and intestinal IELs were prepared from five birds per treatment as described (Lillehoj and Chai, 1988; Min et al., 2001).

### 2.4. Magnetic cell separation

Intestinal IELs were separated into CD4<sup>-</sup>, CD8<sup>-</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> subpopulations using the magnetic BD IMag Cell Separation System according to the manufacturer's instructions (BD Biosciences Pharmingen, San Jose, CA). Cell concentration was adjusted to  $2 \times 10^7$  ml<sup>-1</sup> and appropriate amounts

of anti-CD4 or anti-CD8 monoclonal antibodies (Lillehoj et al., 1988) were added as determined in preliminary titration experiments, the cells were incubated on ice for 45 min, and washed twice with Hank's balanced salt solution (HBSS) containing 3% FBS. 20 µg/ml (1 µg/10<sup>6</sup> cells) of biotinylated goat anti-mouse IgG (BD Biosciences) was added, and the cells were incubated for 15 min on ice, washed with an excess volume of 1 × BD IMag buffer, and 40 µl/ml of streptavidin-labeled magnetic particles (BD IMag Streptavidin Particles Plus-DM) were added on ice for 30 min. Magnetic particles were isolated and the positive and negative cell fractions were resuspended in serum-free HBSS.

### 2.5. Expression of recombinant NK-lysin protein

COS7 cells (ATCC, Manassas, VA) were cultured in Iscove's modified Dulbecco's medium (IMDM, Invitrogen) supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin in 75 cm<sup>2</sup> culture flasks and transiently transfected at 80–90% confluence with 24 µg of pTriEx4-NK-lysin plasmid DNA using 60 µl of lipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. At 18 h post-transfection, the cells were cultured in IMDM containing 10 mM insulin–transferring–sodium selenite supplement (Sigma, St. Louis, MO) and incubated for 24 h. Recombinant histidine-tagged NK-lysin was purified from cell-conditioned culture medium on a Ni<sup>2+</sup>-NTA His-bind resin column (Novagen) according to the manufacturer's protocol. The eluted protein was resolved on a 15% SDS-polyacrylamide gel, transferred to nitrocellulose membrane (Millipore, Bedford, MA), the membrane reacted with peroxidase-conjugated monoclonal anti-polyhistidine antibody (Sigma) for 1 h at room temperature, and developed with 4-chloro-1-naphthol substrate (Sigma).



reading frame of 420 nts encoding a protein of 140 amino acids with a predicted molecular mass of 15.2 kDa. Alignment of sequences for chicken, porcine, bovine, and equine NK-lysin, and human granulysin, revealed six conserved cysteine residues (except for the absence of the first cysteine in human granulysin) (Fig. 1). In addition, multiple conserved amino acid replacements were seen, particularly within the sequence bordered by the first and last conserved cysteines. Comparison of these amino acid sequences using the CLUSTAL W (1.82) program revealed 15% identity of chicken NK-lysin to the porcine and human proteins, 14% to bovine NK-lysin, and 17% to equine NK-lysin. By contrast, comparison of mammalian NK-lysins and human granulysin among themselves revealed levels of homology ranging from 36% (bovine NK-lysin versus human granulysin) to 67% (porcine versus equine NK-lysin).

### 3.2. Tissue distribution of NK-lysin expression

Because mammalian NK-lysins are expressed primarily by NK cells and CTLs, we investigated chicken NK-lysin mRNA expression in various lymphoid tissues by quantitative RT-PCR. NK-lysin was most highly expressed in intestinal IELs, with intermediate levels in splenic and peripheral blood lymphocytes (PBLs), and lowest levels in thymic and bursa lymphocytes (Fig. 2).

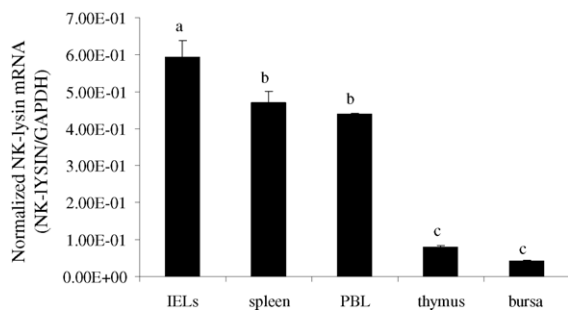


Fig. 2. Tissue distribution of NK-lysin mRNA. Total RNA was isolated from the indicated sources, analyzed for NK-lysin mRNA by real-time RT-PCR, and normalized to GAPDH mRNA. Each bar represents the mean  $\pm$  S.D. of five birds. Significant level was performed at  $p < 0.001$ .

### 3.3. Expression of NK-lysin in intestinal IELs

Our previous studies demonstrated that intestinal IELs contained a predominant subpopulation of NK cells that were major effectors in the host response to coccidiosis (Lillehoj and Chai, 1988; Lillehoj, 1989, 1998; Lillehoj and Chung, 1992). Therefore, we determined NK-lysin mRNA expression levels in intestinal IELs before and after *Eimeria* infection. In noninfected intestinal tissues, NK-lysin transcripts were expressed at highest levels in jejunum IELs, intermediate in the duodenum, and lowest in the caeca (Fig. 3A). Next, we isolated IELs from the jejunum of

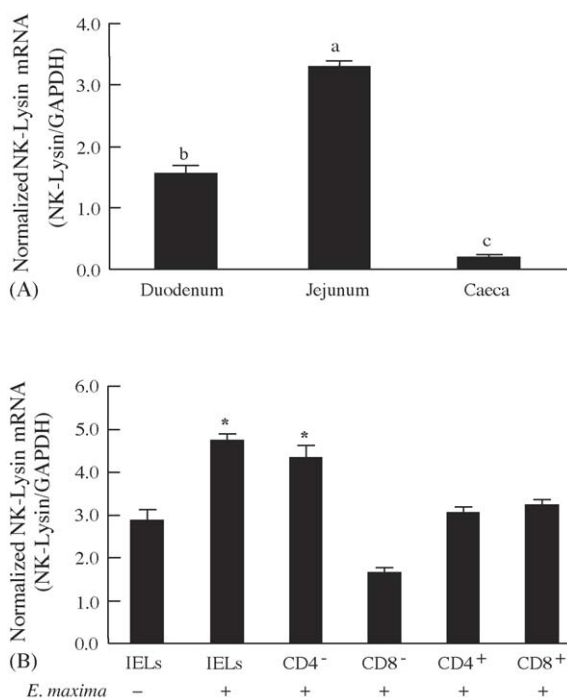


Fig. 3. NK-lysin expression profile in intestinal IELs. (A) IELs were isolated from the duodenum, jejunum, and caeca of noninfected chickens at 3 weeks of age, NK-lysin transcripts quantified by real-time RT-PCR, and normalized to GAPDH mRNA. Significant level considered at  $p < 0.001$ . (B) Chickens were noninfected or orally infected with  $1 \times 10^4$  oocysts of *E. maxima*, IELs were isolated from the jejunum on day 4 post-infection, separated into CD4<sup>-</sup>, CD4<sup>+</sup>, CD8<sup>-</sup> and CD8<sup>+</sup> subpopulations, NK-lysin transcripts quantified by real-time RT-PCR, and normalized to GAPDH mRNA. Each bar represents the mean  $\pm$  S.D. of five birds. \*  $p < 0.05$  compared with noninfected control. NK-lysin regression equation:  $C_1 = 3.25x + 30.6$ ,  $r^2 = 0.99$ ; GAPDH regression equation:  $C_1 = 3.42x + 32.2$ ,  $r^2 = 0.99$ .

either noninfected or orally infected chickens with  $1.0 \times 10^4$  oocysts of *E. maxima* at 4 days post-infection, and measured NK-lysin mRNA. As shown in Fig. 3B, infected animals exhibited approximately 60% increased NK-lysin transcripts in jejunum IELs compared with noninfected controls. This increase was mainly attributed to the CD4<sup>-</sup> subpopulation of IELs. Whereas CD4<sup>+</sup> and CD8<sup>+</sup> cells expressed equal amounts of NK-lysin, IELs depleted of CD8<sup>+</sup> cells exhibited reduced NK-lysin transcript levels.

### 3.4. Kinetics of NK-lysin expression in intestinal IELs

Different species of *Eimeria* infect different regions of the intestinal tract, with *E. acervulina* mainly localizing to the duodenum, *E. maxima* to the jejunum, and *E. tenella* to the caeca (Allen and Fetterer, 2002). To determine if NK-lysin transcripts were increased in these regions following infection with the various *Eimeria* species, chickens were orally infected with oocysts of *E. acervulina*, *E. maxima*, or *E. tenella* and NK-lysin transcripts quantified in intestinal IELs at day 0 (noninfected) and between days 1 and 10 post-infection. As illustrated in Fig. 4, NK-lysin mRNA levels in IELs from the duodenum of *E. acervulina*-infected animals reached maximum expression at 7–8 days post-infection, whereas *E. maxima* and *E. tenella* elicited biphasic responses at 3–4 and 7–8 days post-infection in the jejunum and caeca, respectively.

### 3.5. Cytotoxic activity of recombinant NK-lysin

In addition to mediating the host immune response to *Eimeria* infection, chicken intestinal IELs contain NK cell activity against tumor target cells, an activity likely mediated by NK-lysin (Chai and Lillehoj, 1988). To determine if NK-lysin was directly cytotoxic to tumor cells, its cDNA was subcloned into a mammalian expression vector, transfected into COS7 cells, and cell-conditioned medium was tested for its ability to inhibit uptake of [<sup>3</sup>H]thymidine by LSCC-RP9 tumor cells, a popular avian NK target cell (Sharma and Okazaki, 1981). Initially, we determined that COS7 cells transfected with the pTriEx-4 vector encoding a histidine-tagged NK-lysin protein synthesized NK-lysin.

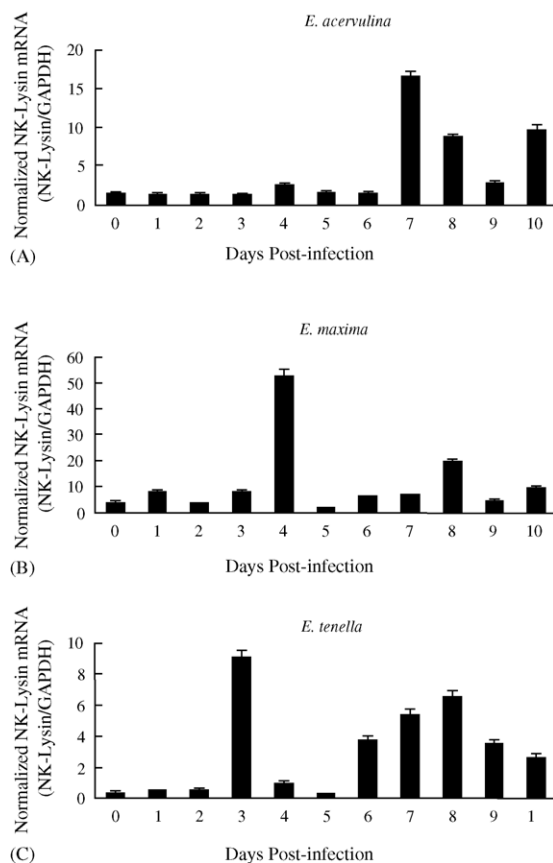


Fig. 4. Kinetics of NK-lysin expression following *Eimeria* infection. Chickens were noninfected (day 0) or orally infected with  $1 \times 10^4$  oocysts of *E. acervulina* (A), *E. maxima* (B), or *E. tenella* (C), IELs were isolated from the duodenum, jejunum, or caeca, respectively, at the indicated times post-infection, NK-lysin transcripts quantified by realtime RT-PCR in triplicates, and normalized to GAPDH mRNA. Each bar represents the mean  $\pm$  S.D. of five birds.

Western blot analysis of purified NK-lysin from cell-conditioned medium using anti-polyhistidine antibody revealed a prominent band indicative of NK-lysin expression (Fig. 5). The faster migrating band likely represented a minor proteolytic degradation fragment of the intact protein, as previously reported (Lillehoj et al., 2000a, 2000b). As shown in Fig. 6, treatment of LSCC-RP9 cells with cell-conditioned medium from NK-lysin-transfected COS7 cells for 4 h significantly inhibited [<sup>3</sup>H]thymidine uptake compared with cells treated with non-transfected cell-conditioned medium.

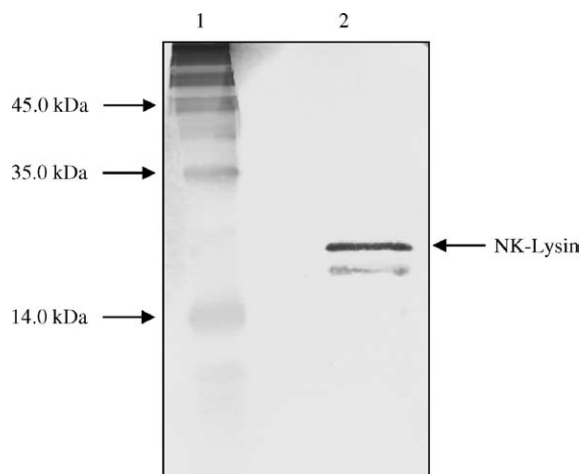


Fig. 5. Western blot analysis of recombinant NK-lysin. COS7 cells were transfected with pTriEx4-NK-lysin plasmid DNA, recombinant NK-lysin was purified from cell-conditioned culture medium on a  $\text{Ni}^{2+}$ -NTA His-bind resin column, and analyzed by Western blotting using anti-polyhistidine antibody. Stained NK-lysin band was higher than expected to 15.2 kDa because it included fusion proteins with enterokinase and thrombin. Lane 1, prestained protein markers with sizes indicated on the left in kiloDaltons (kDa). Lane 2, histidine-tagged NK-lysin.

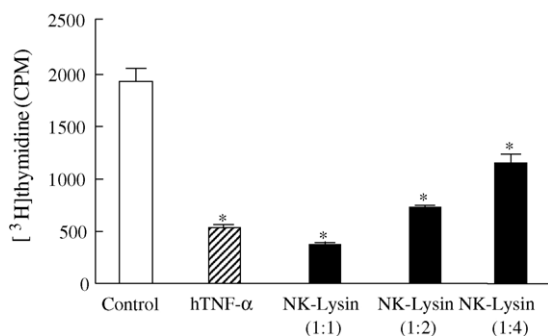


Fig. 6. Effect of NK-lysin on LSCC-RP9 cell viability. LSCC-RP9 cells were treated for 48 h with recombinant NK-lysin contained in supernatants of cell-conditioned medium of COS7 cells that were transfected with the pTriEx4-NK-lysin plasmid. Following treatment, the cells were labeled for 4 h with  $[^3\text{H}]$ thymidine, and radioactivity in the cells determined by liquid scintillation counting. NK-lysin expressed in COS7 cells significantly killed the RP9 cells as shown hTNF $\alpha$  (1  $\mu\text{g}/\text{ml}$ ) positive control. \* $p < 0.01$  compared with non-transfected COS7 cell-conditioned control media.

#### 4. Discussion

In a previous report, we described contig 171 which was composed of 87 individual ESTs, making it the most highly represented sequence in our *Eimeria*-induced intestinal cDNA library (Min et al., 2005). By BLAST search analysis, the DNA sequence of contig 171 was weakly homologous to porcine, bovine, and equine NK-lysin and human granulysin. In the current study, two approaches were used to confirm the identity of the putative chicken NK-lysin cDNA and characterize its encoded gene product. First, real-time RT-PCR primers based on the cDNA sequence were used to quantify NK-lysin transcripts in various chicken cells and tissues, particularly intestinal IELs. Highest levels of NK-lysin transcripts were detected in the jejunum following infection with *E. maxima*, a coccidia species localizing to this region of the intestine. Jejunum IELs, particularly  $\text{CD4}^-$ , were mainly responsible for the infection-induced mRNA synthesis. Two additional species, *E. acervulina* and *E. tenella*, also induced NK-lysin mRNA in IELs isolated from the duodenum and caeca, respectively. When normalized to GAPDH mRNA, maximum NK-lysin transcript levels stimulated by *E. maxima* were 3–6-fold greater compared with those induced by infection with *E. acervulina* or *E. tenella*. Second, expression of the NK-lysin cDNA in COS7 cells led to production of a recombinant protein with an apparent molecular weight predicted from its deduced amino acid sequence, and the isolated protein exhibited NK cell-like anti-tumor activity.

NK cells are phenotypically characterized as non-T, non-B, non-macrophage mononuclear cells that lack immunological memory and possess MHC-unrestricted cytotoxic activity. Our prior study demonstrated that chicken NK cells were present in intestinal IELs, and the greatest ability to lyse tumor targets occurred with IELs isolated from the jejunum (Chai and Lillehoj, 1988). Here, we reported that NK-lysin transcript levels were highest in this region of the intestine of noninfected chickens, indicating a positive correlation between NK cell activity and NK-lysin expression. Furthermore, the kinetics of NK-lysin expression following *Eimeria* infection paralleled those of parasite oocyst production, as shown in previous work (Lillehoj, 1989). This further implies that the peak in NK activity and, by extension, innate

host immunity, occurs during the time of maximum parasite infectivity. Taken together, these results support the role of NK-lysin as an important mediator of NK cell activity during avian coccidiosis.

Chicken IELs are composed of 21% CD3<sup>+</sup>, 70% CD8<sup>+</sup>, and 9% CD4<sup>+</sup> T lymphocytes with about 30% of the CD8<sup>+</sup> subset possessing NK cell activity (Gobel et al., 2001; Min et al., 2005). However, because no appropriate surface markers efficiently discriminate chicken NK cells from T lymphocytes, we sorted total intestinal IELs into four subpopulations (CD4<sup>-</sup>, CD4<sup>+</sup>, CD8<sup>-</sup>, and CD8<sup>+</sup>) and quantified NK-lysin expression in each. The results indicated that whereas CD4<sup>+</sup> and CD8<sup>+</sup> cells expressed equal amounts of NK-lysin, IELs depleted of CD8<sup>+</sup> cells, but not CD4<sup>+</sup> cells, exhibited reduced NK-lysin transcript levels. We interpreted these results to indicate that NK-lysin was expressed in all subsets of T lymphocytes as well as NK cells. This pattern of NK-lysin expression is consistent with that reported for the bovine and equine NK-lysin gene, which were detected in CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, and  $\gamma\delta$  T cells (Endsley et al., 2004; Davis et al., 2005). Similarly, porcine NK-lysin was identified in CD2<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells (Andersson et al., 1996).

Intestinal NK cells are known to serve an important role in controlling avian coccidiosis, as exemplified by the fact that strain differences in susceptibility to *Eimeria* infection are mirrored by NK activity. Thus, the IELs of SC strain chickens revealed significantly higher levels of NK cells compared with FP-strain chickens, and the SC strain is known to be less susceptible to experimental coccidiosis than the FP strain (Lillehoj, 1989). Additional factors controlling NK cell activity of IELs include the type of tumor target cells and NK-tumor cell incubation parameters. For example, LSCC-RP9 (avian leukosis virus-transformed B-cell line), MDCC-MSB-1 (Marek's disease virus-transformed), and MDCC-CU36 tumor cells were susceptible to intestinal NK cells, while LSCC-RP12 was resistant to lysis even after a prolonged incubation (Chai and Lillehoj, 1988). In kinetic studies, NK cell-mediated cytotoxicity was detectable from 2 h of incubation and progressively increased up to 18 h (Lillehoj and Chai, 1988).

In conclusion, a cDNA encoding chicken NK-lysin was identified, cloned, and characterized. Its deduced amino acid sequence, while showing the

presence of the characteristic cysteine residues present in mammalian NK-lysin and granulysin, demonstrated less than 20% overall sequence identity to the mammalian proteins. NK-lysin transcript levels in intestinal IELs increased following *Eimeria* infection and maximum levels were detected at days 3–4 (*E. maxima*, *E. tenella*) and 7–8 (*E. acervulina*, *E. maxima*, *E. tenella*) post-infection. Finally, bioactivity of recombinant NK-lysin protein against avian leukosis virus-transformed tumor cells was demonstrated. Current studies are directed at further defining the role of NK-lysin in immunity to avian coccidiosis.

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