High-throughput Gene Expression Analysis to Investigate Host-pathogen Interaction in Avian Coccidiosis

Hyun S. Lillehoj

Animal Parasitic Disease laboratory
Animal and Natural Resources Institute
Beltsville Agricultural Research Center
United States Department of Agriculture
Agricultural Research Service
Beltsville, MD 20705

ABSTRACT Poultry products including meat and eggs constitute a major protein source in the American diet and disease-causing pathogens represent major challenges to the poultry industry. More than 95% of pathogens enter the host through the mucosal surfaces of the respiratory, digestive and reproductive tracts and over the past few decades, the two main mechanisms used to control diseases have been the use of vaccines and antibiotics. However, in the poultry industry, there are mounting concerns over the ability of current vaccines to adequately protect against emerging hyper-virulent strains of pathogens and a lack of suitable, cost effective adjuvants. Thorough investigation of the immunogenetic responses involved in host-pathogen interactions will lead to the development of new and effective strategies for improving poultry health, food safety and the economic viability of the US poultry industry. In this paper, I describe the development of immunogenomic and proteomic tools to fundamentally determine and characterize the immunological mechanisms of the avian host to economically significant mucosal pathogens such as *Eimeria*. Recent completion of poultry genome sequencing and the development of several tissue-specific cDNA libraries in chickens are facilitating the rapid application of functional immunogenomics in the poultry disease research. Furthermore, research involving functional genomics, immunology and bioinformatics is providing novel insights into the processes of disease and immunity to microbial pathogens at mucosal surfaces. In this presentation, a new strategy of global gene expression using avian macrophage (AMM) to characterize the multiple pathways related to the variable immune responses of the host to *Eimeria* is described. This functional immunogenomics approach will increase current understanding of how mucosal immunity to infectious agents operates, and how it may be enhanced to enable the rational development of new and effective strategies against coccidiosis and other mucosal pathogens.

(Key word : high-throughput, host-pathogen interaction, avian coccidiosis, host immune response)

Host Immune Response to *Eimeria* Parasites

Chicken meat is a major protein source in the American diet (Delmarva Poultry Industry, http://www.dpichickn.org). Poultry meat also represents a major U.S. export particularly to developing countries where increasing meat consumption parallels national economic growth. Thus, improving the efficiency of U.S. poultry production will have a positive impact on our national broiler industry profitability and trade competitiveness in international markets both now and in the future. Infectious diseases are one of the greatest threats to the viability of the food animal industry. Commercial broilers, in particular, have a higher risk

of acquiring contagious diseases than other sectors of the food industry due to intensive collective farming practices (Gavora, 1990). Coccidiosis is a poultry disease of substantial economic importance, estimated to cost the U.S. industry greater than $700 million annually. In the absence of efficient vaccines to control this disease and the emergence of new antigenic variants of *Eimeria*, the broiler industry has relied upon prophylactic medication. However, anti-coccidial drugs are expensive and their effectiveness is hindered by widespread parasite drug resistance and the high cost of new drug development (Chapman, 1998). Moreover, consumer concern about drug residues in the food supply may eventually force the industry to eliminate this

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[†] To whom correspondence should be addressed : HLILLEHO@anri.barc.usda.gov

practice. Therefore, there has been increasing interest to develop novel strategies for coccidiosis control. In this regard, understanding various factors which control host protective immunity and genetic resistance against coccidiosis is critical (Lillehoj et al., 2004; Dalloul and Lillehoj, 2005). Examination of the patterns of disease resistance following experimental infection has suggested 2 separate mechanisms of genetic control of protective immunity to coccidiosis, an innate mechanism following primary infection and acquired immunity following secondary infection (Lillehoj et al., 1999). In immune hosts, parasites enter the gut early after infection but are prevented from further development indicating that acquired immunity to coccidiosis may involve mechanisms that inhibit the natural progression of parasite development (Trout and Lillehoj, 1996). Although a direct role of immune effector lymphocytes in inhibiting parasite development has not been proven, CD8+ cytotoxic T cells and interferon-γ (IFN-γ) have been identified as important components of host protection (Lillehoj and Choi, 1998). At the genetic level, both MHC-linked genes and non-MHC genes have been implicated in controlling host immune responses to coccidiosis (Lillehoj et al., 1989) and a recent study from our laboratory identified QTLs affecting disease resistance to coccidiosis (Zhu et al. 2003).

There is a timely need for the development of novel strategies to control coccidiosis, but this will only be realized after a systematic and detailed analysis of host-parasite interactions at the molecular and cellular levels are completed. In particular, there is a need to increase fundamental knowledge on the basic immunobiology of the events associated with parasite invasion and intracellular development as well as parasite biology and metabolism. Immune responses to coccidia are extremely complex and different effector mechanisms may be involved depending on the species of Eimeria, stage of parasite development, prior host exposure, the nutritional status of infected chickens and the genetic makeup of the host (Lillehoj et al., 2004; Dalloul and Lillehoj, 2005). Additional basic research is needed to ascertain the detailed immunological and physiological processes mediating protective immunity.

Lymphokines mediate intercellular signals during normal immune responses and have thus been investigated as vaccine immunopotentiators for avian coccidiosis (Lillehoj et al., 2000). Few chicken lymphokines homologous to their mammalian coun-

terparts have thus far been described (IFN-γ, interleukin-2, interleukin-15, transforming growth factor and tumor necrosis factor) of which IFN-γ has received the most attention as an immunomodulator (Yun et al., 2000). The chicken IFN-γ gene was cloned (Song et al., 2000), transfected into chicken fibroblast cells and inhibited intracellular development of E. tenella following in vitro infection (Lillehoj and Choi, 1998). An identical effect on E. tenella development in vivo was observed after administration of recombinant IFN-γ protein to chickens prior to challenge with virulent parasites. Recently, a 19-kDa recombinant E. acervulina protein (3-1E) stimulating native IFN-γ production by chicken spleen cells was expressed in bacterial and eukaryotic vectors and the recombinant protein purified to homogeneity (Lillehoj et al., 2000). One-day-old chicks intramuscularly immunized twice with 1.0 ug of purified 3-1E protein in incomplete Freund's adjuvant were significantly protected from oral challenge with E. acervulina oocysts as measured by subsequent fecal oocyst shedding. Co administration of 3-1E recombinant protein with cDNAs encoding chicken IFN-γ or IL-2/15 led to further enhancement of Eimeria specific immunity. These results raise the exciting possibility of using IFN-γ immunoprophylactically to control coccidiosis on commercial poultry flocks.

Functional Genomics and Immunogenomics

The recent improvements of sequencing technologies, and in particular the publication of the initial version of the chicken genome sequence together with the large-scale sequencing of transcribed sequences, have opened the field of large-scale functional approaches of biological systems: functional genomics. Among these approaches, DNA microarray technology which allows a simultaneous measurement of gene transcription on a whole-genome or tissue-specific basis, enable us to describe the complex transcription status of any biological sample. A major application of transcriptome is notably the ability to classify biological samples according to their transcriptional similarity, opening new way to understand complex biological systems. The combination of genomic and immunological technologies is defined as immunogenomics which allows studying complex immunological processes based on large scale genomic approaches.

DNA arrays have become prominent because they are easier to use than other gene expression profiling methods, do not require large-scale DNA sequencing, and allow the parallel quantification of thousands of genes from multiple samples. As such, the microarray has become a powerful tool for the study of immune system function. Although a variety of large-scale commercial arrays are available for human and other mammalian species, there are few such tools available for agricultural species. In the avian, a small number of low-density and high-density cDNA based microarrays have been developed (Cogburn et al., 2004; Min et al., 2003; Morgan et al., 2001; Neiman et al., 2003). More recently, a consortium of research groups has developed a comprehensive 13,000 element chicken cDNA microarray and a commercially available whole genome chicken oligonucleotide array (Affymetrix Corp., Sunnyvale, CA) has been developed for use by the avian research community. Conceptually, DNA array technology relies on nucleic acid hybridization between labeled free targets derived from a biologic sample and an array of many DNA fragments (the probes, representing genes of interest), tethered to a solid surface. The targets, produced by reverse transcription and simultaneous labeling of RNA molecules, are part of a complex mixture of distinct cDNA fragments that hybridize with their cognate probes during the assay. The signal generated on each probe reflects the mRNA expression level of the corresponding gene in the sample. After detection, quantification, and integration of signals with specialized software, intensities are normalized for technical deviations, providing a "gene expression profile" for each sample, comparable to profiles from other samples.

Application of Avian Macrophage Microarray to Coccidiosis Immunity Research

To aid in studies of the avian innate immune response, we have recently constructed a 4,906 element (14,718 spot) avian macrophage-specific cDNA microarray (AMM). This array has been used to examine the transcriptional response of avian macrophages to gram-negative bacteria (*Escherichia coli*) and their cell wall components (LPS) and has specifically been used to evaluate the contribution of the TLR pathway to this response (Bliss et al., 2005). This approach has enabled us to signifi-

cantly enhance our understanding of the innate immune response mediated by the avian macrophage in response to bacteria. In a subsequent study, the AMM was used to elucidate the avian macrophage's transcriptional response to three related but biologically distinct avian protozoan pathogens: *E. acervulina*, *E. maxima*, and *E. tenella* (Dalloul et al., 2007). This approach enabled us to identify common genetic elements whose transcriptional expression is induced by exposure to *Eimeria* sporozoites and to identify transcription patterns unique to an individual *Eimeria* species. Analysis of avian chemokine and cytokine expression patterns unveiled new insight into the avian immunological responses to these related but biologically unique pathogens.

More recently, a 5K avian innate immunity microarray (AIIM) has been created by modification of the first generation AMM (Bliss et al., 2005). This array consists of approximately 5,000 elements that were obtained from EST libraries of stimulated avian macrophages and supplemented by genes of interest from several specific innate immune pathways. The elements are spotted in triplicate on the slide, giving approximately 15,000 total spots per slide. The array contains 13 interleukin, 7 chemokine, and 5 cytokine elements. The AIIM also contains elements for several nearly complete innate immune pathways, including 42 of the 45 avian genes involved in the toll-like receptor (TLR) pathway (including all 7 of the currently known receptors), 22 out of the 23 known avian interferon/antiviral response pathway genes, and 9 of 11 genes involved in the oxidative burst. In addition, the array contains apoptosis marker genes (including perforin and granzyme B) and 29 cell surface marker genes for monocytes, macrophages, heterophils, dendritic cells, and T-cells.

The AIIM has been successfully used in experiments using several avian cell or tissue types as well as a variety of pathogens and has proven to be a useful tool in identifying gene expression changes that take place globally and in specific pathways. Innate immune gene expression changes have been observed *in vivo* using intestinal epithelial and splenic tissues and *in vitro* using peripheral blood monocytes, heterophils, nonadherent blood lymphocytes, and multiple avian macrophage cell lines (HD11, HTC). In addition, innate immune responses have been elucidated following stimulation with bacteria (*Salmonella*, *E. coli*, *Mycoplasma*), viruses (influenza), parasites (*Eimeria*), cell components (LPS), and immune modulators (in-

terferon- γ).

Eimeria parasites are ubiquitous pathogens and the causative agents of poultry coccidiosis, one of the highest costly endemic diseases to the poultry industry worldwide. The three species, *E. acervulina*, *E. maxima* and *E. tenella*, are the most commonly encountered in the field with each infecting a specific intestinal site. Infections, when not deadly, induce protective immunity against subsequent challenges; however, such immunity remains confined to homologous species with no cross species protection (Dalloul and Lillehoj, 2007). Among the three, *E. maxima* are characterized by high immunogenicity where priming infection with few oocysts induces full protective immunity to subsequent homologous challenge. Conversely, far more *E. acervulina* and *E. tenella* oocysts are required to induce comparable protective immunity within species limits. For these reasons, there is a pressing need to elucidate the fundamental similarities as well as the differences in immune responses induced by these three related but distinctly unique pathogens. Identification of the early host responses at the gene transcription level provides a molecular immune profile of the events that occur during and immediately following infection with *Eimeria* sp.

Determining the macrophage genes that are transcribed during the early stages of each *Eimeria* sp. infection discerns the molecular pathogenesis of coccidiosis. Using the HTC macrophage cell line, common as well as species-specific host responses were identified. This study also shows that the extent of early macrophage activation events induced by individual species of *Eimeria* appears to correlate with the quantity of genes and the overall magnitude of gene regulation elicited by each individual species. Indeed, almost the same number of elements changed in response to the three species and individual species uniquely induced expression changes in a similar number of elements. A set of core response elements has been identified comprising 25 genes including many immune-related genes, while 60~67 elements were uniquely induced or repressed by individual species. Such differential responses may be attributed to the species-specific immunity induced by each *Eimeria* sp. and a deeper look into the functional aspects of those elements could prove critical in shedding some light on the lack of cross-species protection. Further characterization of both sets of elements would help elucidate the pathogenicity and/or immunogenicity of each species leading to better recombinant vaccine design and control strategies.

Total number of unique elements exhibiting significant expression changes using this AMM is shown in Table 1. The majority of the 25 core response elements were induced by all three treatments and they included several important immune effector genes, such as chemokines ah221 and MIP-1 β, and osteopontin. While many elements were highly induced following two of the three treatments, none of the 25 core elements fell among the top ten induced genes but the quiescence specific protein precursor CH21 was among the most highly repressed in all three treatments. As measured by Q-RT-PCR, the proinflammatory cytokine IL-1 β was highly induced (> 5-fold) by the three species IL-1 β is secreted by macrophages and other cells upon activation by stimuli (Rodenburg et al., 1998), which in turn upregulates the production of other chemokines like MIP-1 β, K203, and ah221, and cytokines like osteopontin, thereby amplifying the immune response. MIP-1 β and K203 belong to the CC chemokine family, normally involved in the recruitment of macrophages and they were both upregulated in all three treatments (Table 2). Using IFN- γ stimulated macrophages (HD11), Laurent et al. (2001) observed similar results suggesting that macrophages are the main effector inflammatory cells at *Eimeria* infection sites. Osteopontin has been described as an important component of early cellular immune responses (Patarca et al., 1993). It is known to directly induce chemotaxis and indirectly facilitate macrophage migration to other chemoattractants, and characterized as an early protein expressed by

Table 1. Statistical analysis of AMM data quality and elements displaying significant (> 2-fold) changes in expression

Elements on the AMM	4,906
Elements with high-quality replicate data	3,140
Elements exhibiting >2-fold change in EA treatment	288
Statistically significant >2-fold changes in EA treatment	111
Elements exhibiting >2-fold change in EM treatment	262
Statistically significant >2-fold changes in EM treatment	134
Elements exhibiting >2-fold change in ET treatment	282
Statistically significant >2-fold changes in ET treatment	122
Total number of unique elements exhibiting significant expression changes	265

Table 2. Expression changes of selected genes in response to three species of *Eimeria*

Gene	Sporozoite treatment			Measured by
	E. acervulina	*E. maxima*	*E. tenella*	
Interleukin-1 β	++	++	++	Q-PCR
Interleukin-6	+	++	+	QPCR
Interleukin-18	++	+	+	Array & Q-PCR
Chemokine K60	–	–	++	Array & Q-PCR
Chemokine K203	++	++	++	Array & Q-PCR
Chemokine ah221	+	+	+	Array
MIP-1 β	+	+	++	Array
Interferon- γ	–	–	++	Q-PCR
iNOS	+	++	++	Q-PCR

+ = induction, − = repression.
2~5 fold = 1 signal, >5 fold = 2 signals.

activated macrophages and natural killer cells (O'Regan et al., 2000). Osteopontin enhances T helper 1 (Th1) and inhibits Th2 cytokine expression. In mice, it directly induces macrophages to produce IL-12, and inhibits IL-10 expression by lipopolysaccharide (LPS)-stimulated macrophages (Ashkar et al., 2000). In chickens, *Eimeria* infections induce Th1 immune responses (Dalloul and Lillehoj, 2005) and this observation further indicates such a premise. The paradigm of Th1/Th2 cytokine polarization suggests that early expression of Th1 cytokines is critical to a protective host response against intracellular infection (Abbas et al., 1996) like coccidiosis. Therefore, factors (including antigens) augmenting Th1, and inhibiting Th2, cytokine expression might function as powerful modulators of cell-mediated immunity (CMI), the main effector branch of the bird's immune system against coccidiosis (Dalloul and Lillehoj, 2006; Lillehoj et al., 2004).

Conversely, other elements like IL-16 and quiescence-specific protein were consistently repressed in all three treatments. Quiescence-specific protein is a secreted 20-kDa molecule belonging to the Lipocalin protein family and among the most prevalent proteins present in quiescent chicken heart mesenchymal (CHM) cells (Bédard et al., 1987) and chick embryo

fibroblasts (CEF) (Mao et al., 1993). By contrast, this protein is virtually absent in actively dividing cells, the case in discussion here. However, during intracellular infection (herpesvirus) of CEF, Morgan et al. (2001) observed a high expression of the protein, suggesting that the virus inhibits cell cycle progression while allowing those cells to accumulate factors needed for its own replication. Interestingly, some cytokine/ chemokine elements were differentially expressed following treatment with different *Eimeria* species, including K60 and IFN- γ. K60 was highly induced at 48 hr in *E. tenella* exposed macrophages but repressed at 48 hr in *E. acervulina* and *E. maxima* treated macrophages. IFN- γ was also highly expressed in *E. tenella* treatment but not in the other two. *In vivo* K60 transcripts levels have been shown to remain unchanged or increase slightly compared to levels of other chemokines (MIP-1 β and K203) following *E. tenella* or *E. maxima* infections (Laurent et al., 2001). Increased IFN- γ levels in response to such infections are well documented both *in vitro* (Lillehoj and Choi, 1998) and *in vivo* (Dalloul et al., 2003; Laurent et al., 2001; Min et al., 2003) especially in early response to *E. tenella* infection (Yun et al., 2000).

Other cytokine/chemokine elements also show differential expression following treatments with different *Eimeria* species like IL-18, a typically Th1 type cytokine. IL-18 was induced at 18 hr in *E. acervulina* and *E. tenella* treated macrophages but only after 48 hr in response to *E. maxima* exposure. Also, the CC chemokine MIP-1 β up regulation peaked at 18 hr in response to *E. acervulina* and *E. tenella* sporozoites but it was induced the highest at only 4 hr in *E. maxima*. Further, although little is known about the chemokine ah221, it is noteworthy that it was upregulated very early on during all three *Eimeria* infections, albeit at a much higher level in *E. acervulina*, and the transcript levels came down progressively with time. While a shared similarity in transcript quality exists among the three species, a difference remains obvious in the magnitude, direction, and timing of the immune responses to each individual species. In addition, many elements of unknown functions were observed to be highly induced or repressed in both the core group and within unique responses to individual species. Therefore, more questions remain to be answered and investigations are underway to characterize the *in vivo* immune responses

using this macrophage array as well as a new 10 K intestinal array derived from intraepithelial lymphocytes.

Conclusions

In the poultry industry, there are mounting concerns over the ability of current vaccines to adequately protect against emerging hyper-virulent strains of pathogens and a lack of suitable, cost effective adjuvants. Comprehensive investigation of the immunogenetic responses involved in host-pathogen interactions will lead to the development of new and effective strategies for improving poultry health, food safety and the economic viability of the U.S. poultry industry. Application of the immunogenomic and proteomic tools to disease research is critical to study immunological mechanisms of the avian host to the mucosal pathogens including *Eimeria*. Futuristically, this work will also aid our understanding of the roles that different cell types play in the avian mucosal immune response of the host defense mechanisms against infectious diseases thus providing opportunities to develop more effective methods of disease control.

Acknowlegements

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