Differential Regulation of Cytokine Gene Expression by Avian Heterophils During Receptor-Mediated Phagocytosis of Opsonized and Nonopsonized *Salmonella enteritidis*

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**ABSTRACT**

Internalization of pathogens by phagocytic cells triggers the innate immune response, which in turn regulates the acquired response. Phagocytes express a variety of receptors that are involved in recognition of pathogens, including (1) pattern recognition receptors (PRR), which recognize conserved motifs, (2) complement receptors (CR), which recognize complement-opsonized pathogens, and (3) Fc receptors (FcR), which recognize antibody-opsonized pathogens. Recognition of microbes is accompanied by the induction of multiple cell processes, including the production of proinflammatory and anti-inflammatory cytokines and chemokines. The objective of the present experiments was to use probes to known avian proinflammatory and anti-inflammatory cytokines and TaqMan technology to ascertain levels of cytokine gene expression in avian heterophils following receptor-mediated phagocytosis of either nonopsonized *Salmonella enteritidis* (SE), serum-opsonized SE, or IgG-opsonized SE. Expression of interleukin-6 (IL-6) and IL-8, considered in mammals as a proinflammatory chemokine, were upregulated following exposure to the nonopsonized or the opsonized SE. However, mRNA expression for IL-18 and interferon-γ (IFN-γ) was downregulated, and the expression of mRNA for the anti-inflammatory cytokine transforming growth factor-β (TGF-β) was upregulated. Interestingly, IL-1β mRNA expression was significantly upregulated in heterophils that phagocytized either the nonopsonized SE via PRRs or IgG-opsonized SE via FcRs, whereas serum-opsonized SE phagocytized by CRs induced a downregulation of IL-1β mRNA. These results suggest that signaling interactions initiated by receptor recognition of the microbe surface differentially regulate the induction of inflammatory cytokines in avian heterophils.

**INTRODUCTION**

Microbial invasion of the host is followed by a series of events designed to control (recognition, phagocytosis, microbicidal activities) and eventually resolve (inflammation and acquired immunity) the infection. The immediate response to invasive pathogens, clearance via the inflammatory response, and activation of the appropriate acquired responses are all coordinated and orchestrated by the innate host defenses. Recognition of potential pathogenic microbes by the innate immune system is the function of a restricted class of cellular receptors known as the nonclonal pattern recognition receptors (PRRs), such as the toll-like receptors (TLRs). The innate system uses these germ-line encoded receptors to recognize evolutionarily conserved molecular motifs (pathogen-associated molecular patterns [PAMPs]) of infectious microbes. The phagocytic process is triggered by two distinct cell surface receptors, one involving the recognition of the carboxy-terminus of immunoglobulin (Ig) molecules by the Fc receptor (FcR) and the other where complement receptors (CR) recognize complement fragments. Likewise, some PRRs, such as mannose receptors and scavenger receptors, have been shown to be involved in the phagocytosis process as well. The end product of microbial recognition and phagocytosis by cells of the innate response is the activation of intracellular signaling pathways that initiate cellular processes, such as activation of microbicidal killing mechanisms, the production of proinflammatory and anti-inflammatory cytokines, and the production of costimulatory molecules required for antigen presentation to the acquired immune system.

As the first cells to migrate to a site of infection, polymor-
Phagocytic leukocytes (PMNs) are vital cellular components of inflammatory and innate immune responses. The primary PMN in poultry is the heterophil, the avian equivalent of the mammalian neutrophil. Like neutrophils, avian heterophils are involved in the phagocytosis of invading microbes and foreign particles, the production of oxygen intermediates, and the release of proteolytic enzyme. As was formerly the case with neutrophils, heterophils have been regarded as terminally differentiated cells, devoid of transcriptional activity and with little protein synthesis. Neutrophil activation, following receptor-mediated phagocytosis, induces proinflammatory gene transcription that mediates the host innate responses and modulates receptor-mediated phagocytosis, induces proinflammatory gene transcription that mediates the host innate responses and modulates the acquired responses. However, unlike in neutrophils where various stimuli have been shown to induce gene transcription and translation of inflammatory mediators, heterophil responses at the level of gene transcription and expression are virtually unknown.

The recent cloning of chicken cytokines has driven the development of a more comprehensive panel of reagents for investigation of innate and acquired immune mechanisms at the cellular and molecular levels. Therefore, we have the opportunity to consider the role of heterophils and cytokines in the development of inflammatory and immune responses when stimulated with various phagocytic and inflammatory stimuli. Chicken orthologs of the Th1 cytokines interferon-γ (IFN-γ) and interleukin-18 (IL-18) and the proinflammatory cytokines IL-1β and IL-6 have been cloned and sequenced. The chicken chemokine IL-8 also has been cloned and sequenced. Genomic sequences and gene structures for IFN-γ, IL-1β, and IL-6 have been fully determined. Information now makes it possible to design probes and primers to specifically quantify cytokine mRNA using real-time quantitative fluorescent RT-PCR and to follow changes in cytokine transcription during heterophil phagocytosis.

Receptor activation in mammalian neutrophils and macrophages induces transcriptional events in these cells that mediate host cell responses. There is no information on regulation of the pattern of cytokine gene expression during receptor activation in heterophils. Therefore, we used real-time quantitative RT-PCR to determine gene expression in heterophils for proinflammatory and Th1 cytokines following receptor-mediated phagocytosis, particularly after the activation of PRRs, FcRs, and CRs. The results of these experiments should provide insights into the potential role of heterophils in the induction of an effector inflammatory response and also the development of the correct acquired response to a stimulus.

**MATERIALS AND METHODS**

**Experimental animals**

Outbred Rhode Island red (RIR) chickens were obtained from a local hatchery and reared and maintained at the Institute for Animal Health (IAH). Newly hatched chickens were reared in metal cages in rooms with temperatures of 31°C. The birds were provided with an animal protein-free diet (SDS Feeds, Manea, Cambridgeshire, U.K.) and water ad libitum.

**Isolation of peripheral blood heterophils**

Avian heterophils were isolated from the peripheral blood of day-old RIR chickens as described previously. Briefly, EDTA-anticoagulated blood was mixed with 3% methylcellulose (25 centipoise [cP]) (Sigma Chemical Co., St. Louis, MO) at a 1.5:1 ratio and centrifuged at 25g for 30 min. The serum and buffy coat layers were retained and suspended in Ca²⁺-Mg²⁺-free Hank's balanced salt solution (HBSS, 1:1) (Sigma Chemical Co.). This suspension was layered over a discontinuous Ficoll-Hypaque (Sigma Chemical Co.) gradient (specific gravity 1.077 over specific gravity 1.119). The gradient was then centrifuged at 250g for 60 min. After centrifugation, the 1.077/1.119 interfaces and 1.119 band containing the heterophils were collected, washed twice in RPMI 1640 medium (Sigma Chemical Co.), and resuspended in fresh RPMI 1640. Cell viability was determined by trypan blue exclusion. The purity of the heterophil suspensions was assessed by microscopic examination of Hema-3-stained (Curtin Mathison Scientific, Dallas, TX) cytopsin (Shandon Scientific, Pittsburgh, PA) smears. Heterophil preparations obtained by this method were typically >98% pure and >95% viable. On average, the other 2% comprised monocytes (at most 0.5%), lymphocytes (0.8%), and thrombocytes (at most 0.7%). The cell concentration was adjusted to 4 x 10⁶ heterophils/ml and stored on ice until used.

**Bacteria**

A poultry isolate of Salmonella enteritidis (SE) was obtained from the National Veterinary Services Laboratory (NVSL) (Ames, Iowa) and approved by the USDA Animal and Plant Health Inspection Service for use in our facilities. A carbomycin-resistant isolate was selected and stored in 75% TSB + 25% sterile glycerol in aliquots of 1 x 10⁷ colony-forming units (CFU) at —70°C until used. Tryptic soy broth (TSB) medium used to culture the resistant isolates in experimental studies contained 25 µg/ml novobiocin and 100 µg/ml carbomycin to inhibit the growth of other bacteria. SE cells used for opsonization were prepared in sterile phosphate-buffered saline (PBS), pH 7.2, and adjusted to a concentration of 10⁶ CFU/ml using a spectrophotometer with a 625-nm reference wavelength. The viable cell concentration of SE was determined by colony counts on brilliant green agar (BGA) + C-N (Oxoid Ltd., Basingstoke, Hampshire, U.K.) plates.

**Opsonization of SE**

Normal chicken serum (NCS) was used as a source of complement for opsonization of SE as described previously. The SE (10⁶ bacteria/ml) was suspended in pooled NCS, opsonized for 30 min at 39°C on a rotary shaker, washed twice with Ca²⁺-Mg²⁺-free HBSS, and stored at 4°C in HBSS (10⁸ bacteria/ml) until used.

A hyperimmune serum against the homologous serovar of SE used in the experiments was prepared in 42-week-old layer hens (Hy-Line W-36). The hyperimmune sera were collected from the blood of these hens and pooled. The pooled sera were octanoyl acid-fractionated, and the supernatant was submitted to DEAE chromatography (Bethyl Lab, Montgomery, TX). The predominantly IgG fractions were pooled and concentrated. SE
(10^8 bacteria/ml) were suspended in a subagglutinating concentration of IgG for 30 min at 39°C on a rotary shaker, washed twice with Ca^{2+}, Mg^{2+}-free HBSS, and stored at 4°C in HBSS (10^8 bacteria/ml) until used.

**Phagocytosis**

Phagocytosis of unopsonized SE by the heterophils was determined using duplicate, sterile 15-ml polypropylene screw-capped centrifuge tubes that contained 2 × 10^6 heterophils (500 μl) and 2 × 10^7 bacteria (200 μl) in a total volume of 1 ml RPMI 1640 as described. The tubes were centrifuged (450g for 15 min at ambient temperature) in order to maximize contact. The cells were then allowed to incubate at 39°C for 30–120 min on a rotary shaker to allow for phagocytosis. After each incubation period, the tubes were placed on ice to stop phagocytosis.

An individual unaware of the groups performed all counts. At least 100 heterophils on each slide were scored for the percentage of heterophils that contained bacteria and for the number of bacteria associated with each heterophil. The results are expressed as the percentage of heterophils that contain bacteria.

**Real-time quantitative RT-PCR**

Cytokine mRNA levels in control heterophils and heterophils following phagocytosis with opsonized or nonopsonized SE were quantitated using a method based on that of Kaiser et al. Total RNA was prepared from heterophils using the RNeasy minikit (Qiagen, Valencia, CA) following the manufacturer’s instructions. Purified RNA was eluted in 50 μl RNase-free water and stored at −70°C.

For both cytokine and 28S rRNA-specific amplification, primers and probes were designed using the Primer Express software program (PE Applied Biosystems, Foster City, CA). Details of the probes and primers are given in Table 1. All cytokine probes were designed, from the sequence of the relevant genes, to lie across intron/exon boundaries. Cytokine and 28S rRNA probes were labeled with the fluorescent reporter dye 5-carboxyfluorescein at the 5'-end and with the quencher N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3'-end.

RT-PCR was performed using the Reverse Transcriptase qPCR Master Mix RT-PCR kit (Eurogentec, Seraing, Belgium). Amplification and detection of specific products were performed using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems) with the following cycle profile: one cycle of 50°C for 2 min, 96°C for 5 min, 60°C for 30 min, and 95°C for 5 min and 40 cycles of 94°C for 20 sec, 59°C for 1 min.

Quantification was based on the increased fluorescence detected by the ABI PRISM 7700 Sequence Detection System caused by hydrolysis of the target-specific probes by the 5'-nuclease activity of the rTth DNA polymerase during PCR amplification. The passive reference dye 6-carboxy-X-rhodamine, which is not involved in amplification, was used to correct for fluorescent fluctuations, resulting from changes in the reaction conditions, for normalization of the reporter signal. Results are expressed in terms of the threshold cycle value (Ct), the cycle at which the change in the reporter dye passes a significance

<table>
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*Genomic DNA sequence.

*Forward.

*Reverse.

*5-Carboxyfluorescein.
threshold. In this work, the threshold values of the change in the reporter dye are given in Table 2 for all reactions described.

To generate standard curves for the cytokine and 28S rRNA-specific reactions, total RNA, extracted from control and appropriately stimulated heterophils, was serially diluted (10^{-10} - 10^{-5}) in sterile RNase-free water. Each RT-PCR experiment contained triplicate no-template controls and test samples and a log_{10} dilution series of standard RNA. Each experiment was performed in triplicate, with replicates performed on different days. Regression analysis of the mean values of six replicate RT-PCRs for the log_{10} diluted RNA was used to generate standard curves.

**Statistical analysis**

The anticoagulated blood from 50 chickens was pooled, and the heterophils were isolated from each treatment group as described. The RT-PCR assay was conducted three times over a 2-month period with pooled heterophils (heterophils pooled from 50 chickens for each preparation). At least three replicates were conducted for each assay with the heterophils from each pool of chickens. The data from these three repeated experiments were pooled for presentation and statistical analysis.

The mean and standard error of the mean (SEM) were calculated for each of the treatment groups. Differences between the nonstimulated heterophils and the phagocytic stimuli-activated heterophils were determined by analysis of variance (ANOVA). Significant differences were further separated using Duncan’s multiple range test. The data obtained using heterophils stimulated with the nonopsonized and opsonized SE were compared with nonstimulated control heterophils.

**RESULTS**

Phagocytosis was greatly increased by opsonization of SE with either complement or IgG (Fig. 1). Phagocytosis mediated by FcR occurred more rapidly than that mediated by CR (FcR > CR > PRR). By 120 min, all bacteria were similarly phagocytized (data not shown).

For the TaqMan experiments, replicate experiments on different days were highly repeatable. Regression analysis showed that four replicate standard curves were highly reproducible, as shown by the R^2 values shown in Table 2. There was a linear relationship between the amount of input RNA and the C_i values for the various reactions (Table 2). The increase in cycles per log_{10} decrease in input RNA for each specific reaction, as calculated from the slope of the respective regression line, is given in Table 2.

To account for the variation in sampling and RNA preparations, the C_i values for cytokine-specific product for each sample were standardized using the C_i value for 28S rRNA product for the same sample from the reaction run simultaneously. To normalize RNA levels between samples within an experiment, the mean C_i value for 28S rRNA-specific product was calculated by pooling values from all samples in that experiment. Tube-to-tube variations in 28S rRNA C_i values about the experimental mean were calculated. Using slopes of the respective cytokine and 28S rRNA log_{10} dilution series regression lines, the difference in input total RNA, as represented by the 28S rRNA, was then used to adjust cytokine-specific C_i values. Standardization does not dramatically alter the distribution of the results as a whole.

The adjusted cytokine-specific C_i values for the nonactivated heterophils for each of the cytokines were: IL-1β, 15.41 ± 0.48; IL-6, 13.94 ± 0.25; IL-8, 11.64 ± 1.1; IL-18, 15.97 ± 0.72; IFN-γ, 12.08 ± 0.54; and transforming growth factor-β4 (TGF-β4), 6.72 ± 0.82. The effect of receptor-mediated phagocytosis on the expression of each cytokine mRNA was compared with these control adjusted cytokine-specific C_i values by calculating the fold change in cytokine mRNA as described below. It is obvious that these cytokine mRNAs are constitutively expressed in the nonactivated heterophils.

**Proinflammatory cytokines**

Receptor-mediated phagocytosis of both the nonopsonized SE and IgG-opsonized SE induced a significant upregulation of expression of the proinflammatory cytokines, IL-1β (3-6-fold), IL-6 (11-fold), and IL-8 (12-fold) when compared with the nonstimulated control heterophils (Fig. 2). In contrast, NCS-opsonized SE induced a 7-fold downregulation of IL-1β mRNA expression, but a 13-fold and 10-fold increase in mRNA expression for IL-6 and IL-8, respectively, was measured in the heterophils from day-old chickens.

<table>
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<th>RNA</th>
<th>ΔRn^a</th>
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<th>C_i^b</th>
<th>R^2^c</th>
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<td>0.9891</td>
<td>3.1335</td>
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^aΔRn, change in the reporter dye.  
^bC_i, threshold cycle level, the cycle at which the change in the reporter dye levels detected passes the ΔR.  
^cR^2, coefficient of regression.
Th1/Th2 cytokines

Expression of mRNA of two Th1 cytokines, IL-18 and IFN-γ, was dramatically downregulated following the receptor-mediated phagocytosis of all three phagocytic stimuli when compared with the nonstimulated control heterophils (Fig. 3). Nonopsonized SE (4-fold), NCS-opsonized SE (7-fold), and IgG-opsonized SE (4-fold) all induced a significant upregulation in the expression of mRNA for the anti-inflammatory cytokine, TGF-β4, compared with the nonstimulated resting heterophils (Fig. 3).

DISCUSSION

The current study demonstrates that avian heterophils are capable of rapid changes in cytokine gene expression after receptor-mediated phagocytosis. As we have shown previously, the lipopolysaccharide (LPS) on nonopsonized SE, IgG on IgG-opsonized SE, and complement components on NCS-opsonized SE all interact with receptors on the surface of the heterophils. These results provide additional insights into how heterophils can respond to different phagocytic stimuli and how, by controlling cytokine gene expression, they can function in regulating inflammatory and subsequent acquired immune responses. The production of cytokines by heterophils suggests that these cells may be able to play a significant role in orchestrating not only proinflammatory or anti-inflammatory activities but also immunoregulatory actions. As heterophils represent the first cell type encountering and interacting with an inflammatory or etiologic agent, the ability to synthesize and release an array of cytokines provides evidence for their role in both immunoregulation and pathophysiology of disease.

The real-time quantitative RT-PCR results from the present studies demonstrate for the first time that chicken heterophils can be induced via receptor-mediated phagocytosis to express increased levels of cytokine mRNA. The cytokine mRNA appears to be differentially expressed depending on the receptor activated during phagocytosis. However, it should be pointed out that quantitative RT-PCR does not necessarily equate to bioactive protein. For example, to date only IFN-γ and IL-6 mRNA levels have been shown to correlate with protein levels. Only a limited number of reliable bioassays for chicken cytokines are available (IFN-γ, type I IFN, IL-1β, IL-2, IL-6, and IL-18) and even fewer for monoclonal antibodies (mAbs) (IL-2, IFN-γ, type I IFN). However, the bioassays for IFNs do not differentiate among each other without the availability of neutralizing antibodies. The bioassay for IL-1β is nonspecific in that it also measures IL-2. Lastly, we have not been able to effectively measure IL-18. Therefore, real-time quantitative PCR is the most highly sensitive method available to reliably quantify a wide variety of avian cytokines, particularly in the absence of suitable bioassays for most of them.

Resolution of infections in young immature avian hosts is initiated through phagocytosis and intracellular killing by heterophils and macrophages that are recruited to the site of infection. Phagocytosis is facilitated by opsonins (Fig. 1). Opsonins, such as complement components and acute-phase proteins, are induced early after infection, with antibodies produced later. These elements of innate antibacterial responses are induced, in part, by proinflammatory cytokines. For example, in poultry, invasion of avian cells by paratyphoid salmonellae stimulates a strong inflammatory response characterized by increased expression and release of the proinflammatory cytokine IL-6 and a massive infiltration of heterophils to the site. Based on the results from the present studies, the in-
FIG. 2. Quantitation of proinflammatory cytokines (IL-1β, IL-6, and IL-8) induced following receptor-mediated phagocytosis of nonopsonized SE and opsonized SE (NCS OpSE or IgG OpSE) by heterophils from day-old chickens expressed as fold change in cytokine mRNA levels, compared with those from age-matched control heterophils. Error bars show SEM from triplicate samples from four separate TaqMan experiments. Different lower case letters indicate significant difference (p < 0.01).
FIG. 3. Quantitation of Th1/Th2 cytokines (IL-18, IFN-γ, and TGF-β4) induced following receptor-mediated phagocytosis of nonopsonized SE and opsonized SE (NCS OpSE or IgG OpSE) by heterophils from day-old chickens expressed as fold change in cytokine mRNA levels, compared with those from age-matched control heterophils. Error bars show SEM from triplicate samples from four separate TaqMan experiments. Different lower case letters indicate significant difference (p < 0.01).
filariting heterophils could also be a further source of IL-6 after activation of cell surface receptors. The mRNAs encoding the proinflammatory cytokines IL-1β, IL-6, and IL-8 were upregulated following PRR-mediated and FcR-mediated phagocytosis by heterophils. These findings are consistent with the fact that receptor activation of PMNs induces transcriptional signals that subsequently mediate host responses. The results further demonstrate that as the first cells to a site of infection or inflammation, heterophils are capable of producing inflammatory mediators following activation. The fact that heterophils can serve as sources of cytokines suggests that these cells contribute to (1) the generation of conditions necessary for the recruitment and activation of other innate immune cells, (2) the expression of innate effector molecules that mediate the inflammatory response, and (3) the development of a protective immunogenic response.

Our data reveal that chicken heterophils also express IL-8 after receptor-mediated phagocytosis. All three phagocytic stimuli used here induced an upregulation of IL-8 mRNA in the chicken heterophils. The observation that heterophils, activated by receptor-mediated phagocytosis, are able to generate a chemotactic cytokine to which they can respond implies that these cells can promote their own recruitment to sites of inflammation. Heterophils play a vital role in early protection against SE infections in the chicken by rapidly migrating to the site of bacterial invasion. This heterophil influx was due to neither host-generated arachidonic acid metabolites nor bacterial LPS. Further experiments demonstrated that a host-derived IL-8-like cytokine was responsible for the recruitment of heterophils to the site of SE infection. The outcome of the present experiments indicates that heterophils could be a major autocrine source of this IL-8-like cytokine. Both Th1-inducing cytokine mRNAs that were quantitated in the present experiments, IL-18 and IFN-γ, were downregulated following receptor-mediated phagocytosis by the chicken heterophils. Our results suggest that this downregulation is the result of upregulation of mRNA expression for the anti-inflammatory cytokine TGF-β. It is reasonable to assume that early receptor-mediated phagocytosis by avian heterophils induces the secretion of TGF-β, which can then act in an autocrine or paracrine manner to inhibit the expression of the proinflammatory (IL-1β) and Th1 cytokines IL-18 and IFN-γ cytokines. In mammals, a somewhat similar mechanism has been described, where increased production of the anti-inflammatory cytokine, IL-10, following the ligation of the FcR exclusively, inhibited the specific production of IL-12. At present, no avian ortholog of IL-10 has been identified, but TGF-β has been found to have similar biologic activity.

In summary, the results presented here show that receptor-mediated phagocytosis of either nonopsonized or opsonized SE by avian heterophils induces a differential regulation of cytokine gene expression. Expression of the proinflammatory cytokines IL-6 and IL-8 was upregulated after exposure to nonopsonized or opsonized SE. However, mRNA expression for IL-18 and IFN-γ was downregulated, whereas the expression of mRNA for the anti-inflammatory cytokine TGF-β was upregulated. Interestingly, IL-1β mRNA expression was significantly upregulated in heterophils that phagocytosed either the nonopsonized SE via PRRs or IgG-opsonized SE phagocytized by FcR. In contrast, phagocytosis of serum-opsonized SE via CRs induced a downregulation of IL-1β mRNA. These studies provide the first description of expression of proinflammatory cytokine genes in chicken heterophils. These results suggest that signaling interactions initiated by receptor recognition of the microbe surface differentially regulate the induction of inflammatory cytokines in avian heterophils.

**REFERENCES**

CYTOKINE EXPRESSION IN AVIAN HETEROPHILS


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