Oxidative burst mediated by toll like receptors (TLR) and CD14 on avian heterophils stimulated with bacterial toll agonists

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Abstract

Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA), which are found in the cell walls of gram-negative and gram-positive bacteria, respectively. This study was conducted to determine if TLRs are present on chicken heterophils and if these receptors mediate oxidative burst. Heterophils isolated from neonatal chicks were exposed to gram-negative Salmonella enteritidis (SE), gram-positive Staphylococcus aureus (SA), SE-LPS, and SA-LTA and the oxidative burst quantitated by luminol-dependent chemiluminescence. SE, SA, SE-LPS, and SA-LTA stimulated a significant increase in oxidative burst from heterophils. Furthermore, we measured the inhibitory effects of polyclonal antibodies on rat CD14, human TLR2 and TLR4 on the oxidative burst of heterophils when stimulated with LPS and LTA. The data suggest that TLR2 and TLR4 mediate LPS-stimulated oxidative burst while CD14 and TLR2 mediate LTA-stimulated oxidative burst in heterophils. This is the first report of PAMPs from gram-positive and gram-negative bacteria interacting with TLRs of avian heterophils.

Keywords: Toll like receptors; Pattern recognition receptors; Pathogen associated molecular patterns; Heterophils; Oxidative burst; Salmonella enteritidis; Staphylococcus aureus

1. Introduction

Cells of the mammalian innate immune system have been shown to recognize pathogens by pattern recognition receptors (PRRs) that interact via pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), found in the cell wall of gram-negative bacteria and lipoteichoic acid (LTA), found in the cell wall of gram-positive bacteria [1–5].
Toll like receptors (TLRs) have been shown to be functionally and structurally conserved among species, such as *Drosophila*, humans, plants, and chickens [6–8]. Insights may be gained into the mechanisms of the avian innate immune system through comparative studies with mammalian species. For example, recent findings identified regions of chicken DNA from the bursa of Fabricius with 80.7 and 72% amino acid sequence homology to mammalian TLR2 [7,8].

Heterophils are essential cellular components of the avian innate immune system. These granulocytic phagocytes kill pathogens by the release of toxic oxygen metabolites (oxidative burst) and the release of lytic enzymes and antimicrobial peptides (degranulation) [9]. Heterophils, the second most numerous cells in the peripheral blood, can respond to invading microorganisms within 30 min of infection and are more efficient at phagocytizing and killing than macrophages [10].

The objective of the present study was to determine if PRRs were present on avian heterophils. Additionally, experiments were designed to determine if these receptors mediate heterophil oxidative burst. For these studies, we examined the effects of various TLR agonists including formalin-killed *Salmonella enteritidis* (FKSE), formalin-killed *Staphylococcus aureus* (FKSA), LPS from SE, and LTA from SA on the oxidative burst of neonatal chicken heterophils. To confirm the role of TLRs in heterophil function, we studied the effects of polyclonal antibodies against rat CD14, human TLR2, and human TLR4 on the oxidative burst of heterophils following stimulation with LPS and LTA.

2. Materials and methods

2.1. Chickens

Single-comb white Leghorn male chicks (Hy-Line W-36) were obtained on the day-of-hatch from a commercial hatchery (Hy-Line International, Bryan, TX) and placed in floor pens with supplemental heat. Chicks were provided water and a balanced, unmedicated, corn–soybean ration ad libitum that met or exceeded the National Research Council guidelines for chicken nutrition [11].

2.2. Isolation of heterophils

Blood was collected by decapitation and EDTA was used as an anticoagulant. Blood was pooled from 50–100 neonates and peripheral heterophils isolated as previously described [10]. Briefly, blood was mixed with 1% methylcellulose (Sigma, St Louis, MO) in a 1.5:1 ratio and centrifuged at 25 × g for 15 min. The supernatant was removed and resuspended in Hanks balanced salt solution without calcium and magnesium in a 1:1 ratio. The suspension was then layered over a 1.077/1.119 Ficoll-Hypaque gradient (Sigma, St Louis, MO) and centrifuged at 250 × g for 60 min. Following a RPMI wash, the cells were quantitated using a Neubauer hemacytometer and the concentration was adjusted to 4 × 10⁶ heterophils/ml. Cell viability was routinely >95% as determined by trypan blue exclusion. The purity of the heterophil suspensions was assessed by microscopic examination of Diff-Quick-stained cytopsin (Shandon Scientific, Pittsburgh, PA) smears. Heterophil preparations obtained by this method were typically >95% pure.

2.3. Bacterial preparation

A primary poultry isolate of *S. enteritidis* (NVSL# 97-11771), isolated from the heart of a chicken was obtained from the National Veterinary Services Laboratory (NVSL; Ames, IA) for use in our laboratory. The *S. enteritidis* was selected for resistance to carbenicillin. The brilliant green agar plates used to culture the SE contained 25 μg/ml of novobiocin and 100 μg/ml of carbenicillin to inhibit the growth of other aerobic bacteria.

A primary poultry isolate of *S. aureus* (ATCC # 12600) was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Mannitol salt agar plates were the selective media used to culture the *S. aureus*.

Bacteria were incubated at 41 °C for 8 h in tryptic soy broth and passaged two additional times. Bacteria were concentrated by centrifugation and washed in phosphate-buffered saline (PBS) twice. Dilutions of the bacteria were made and spread plated to determine the concentration.
2.4. TLR agonists

2.4.1. Killed bacteria
Both SE and SA were killed by a 24-hour incubation in 1% formalin in PBS at 4 °C. The cells were washed four times in cold PBS to remove the formalin. The bacteria were resuspended in PBS to a stock concentration of 10^9 cfu/ml and stored at 4 °C until used.

2.4.2. SE-LPS and SA-LTA
SE-LPS and SA-LTA (Sigma, St Louis, MO) were dissolved in DMSO and diluted in RPMI to stock concentrations of 1 mg/ml and stored at 20 °C until used. The final concentration of DMSO in the experiments was less than 0.5%.

2.5. Polyclonal antibodies to CD14, TLR2, and TLR4
Goat polyclonal antibodies raised against peptides near the amino terminus of rat CD14, the carboxy terminus of human TLR2 (TLR2C), the amino terminus of human TLR2 (TLR2N), and the carboxy terminus of human TLR4 were purchased from Santa Cruz Biotechnology Lab (Santa Cruz, CA). Pre-immune isotype matched goat IgG was used as a negative control (Santa Cruz Biotechnology Lab, Santa Cruz, CA). Antibodies were used at concentrations of 2.0 and 0.2 μg/ml as suggested by the manufacturer and verified by dose response. The heterophils and antibodies were incubated, prior to stimulation with the agonists; at 39 °C for one hour in 15 ml screw capped polypropylene conical tubes on a rocker plate.

2.6. Luminol-dependent chemiluminescence (LDCL) assay
Oxidative burst of heterophils was measured by use of LDCL assay adapted from a previously described procedure [12]. Briefly, heterophils (8 × 10^5) and luminol (0.1 M, Sigma, St Louis, MO) in 0.5 ml of RPMI were placed in polypropylene scintillation vials and stimulated with FKSE, FKSA, LPS, or LTA for 30 min. LDCL was measured in a 1219 RackBeta liquid scintillation counter (LKB Wallac, Turku, Finland), using the tritium channel and in the coincidence mode. All samples were assayed in replicates of five tubes per treatment.

2.7. Experimental design

2.7.1. Agonist dose response
Heterophils were stimulated with several concentrations of FKSE, FKSA, LPS, or LTA to determine the optimal concentration of agonist. The samples were placed into scintillation vials and incubated with luminol for 30 min at room temperature in the dark before LDCL measurement. Each dose response was performed in two identical experiments.

2.7.2. Polyclonal neutralization
To confirm whether TLRs mediate oxidative burst by avian heterophils, cells were incubated for one hour at 39 °C in a 15 ml conical tube with 0.2 or 2.0 μg/ml of TLR antibody, isotype matched pre-immune goat IgG, or untreated. The unwashed samples were incubated in the dark for 30 min at room temperature prior to LDCL measurement. Each trial was repeated in four identical experiments.

2.8. Statistical analysis
Data was pooled for each group and compared to a stimulated control using a paired T test from Sigma Stat 2.0 statistical software (Jaundel Corporation, Chicago, Illinois).

3. Results

3.1. Agonist dose response
These experiments were designed to determine if SE, SA, LPS and LTA could stimulate an oxidative burst from avian heterophils. A dose response was performed in order to determine the most efficacious concentration of FKSE and FKSA (Fig. 1) or LPS and LTA (Fig. 2).

FKSE, at concentrations of 10^7, 10^8, and 2 × 10^8 cfu/ml, significantly (p < 0.04) increased oxidative burst in heterophils when compared to non-stimulated controls (169, 151, and 133% of the unstimulated control, respectively). Lower concentrations of FKSE (10^1–10^6 cfu/ml) did not stimulate
Fig. 1. LCDL of avian heterophils stimulated with FKSE or FKSA. Heterophils were incubated with formalin-killed bacteria and luminol for 30 min and LCDL was measured as an indicator of oxidative burst. Data shown as the mean ± SEM of two repetitions (N = 30). Significant differences were determined using a paired T test (* represents p < 0.01 compared to heterophils unexposed to bacteria). Unstimulated controls are represented as 0 CFU on the X-axis.

Fig. 2. LCDL of avian heterophils stimulated with SE-LPS or SA-LTA. Heterophils were incubated with SE-LPS or SA-LTA and luminol for 30 min and LCDL was measured as an indicator of oxidative burst. Data shown as the mean ± SEM of two repetitions (N = 30). Significant differences were determined using a paired T test (* represents p < 0.01 compared to heterophils unexposed to stimulant). Unstimulated controls are represented as 0 µg/ml on the X-axis.
a significant increase in oxidative burst. Likewise, FKSA at concentrations of $10^7$, $10^8$, and $10^9$ cfu/ml significantly ($p < 0.03$) increased oxidative burst in chicken heterophils when compared to non-stimulated controls (140, 416, and 314% of the control, respectively) (Fig. 1). Lower concentrations of FKSA ($10^1 – 10^6$ cfu/ml) did not stimulate a significant increase in oxidative burst above the non-stimulated controls. FKSE and FKSA, at the optimum concentrations of $10^7$ and $10^9$ cfu/ml respectively, were used in subsequent experiments.

LPS concentrations of $10$, $20$, and $50$ mg/ml significantly ($p < 0.001$) increased oxidative burst of heterophils by 159, 167, and 178% of the control, respectively (Fig. 2). LPS at concentrations below $10$ mg/ml had no significant increase in heterophil oxidative burst when compared to controls. LTA at a concentration of 10 mg/ml significantly ($p < 0.001$) increased heterophil oxidative burst by 126% of the control (Fig. 2). LTA at concentrations below or above 10 mg/ml did not significantly increase oxidative burst. LPS and LTA were used, in subsequent experiments, at the optimum concentration of 10 mg/ml.

### 3.2. Neutralization of oxidative burst by TLR and CD14 polyclonal antibodies

#### 3.2.1. Heterophils stimulated with SE-LPS

Heterophils were pre-incubated with CD14 Ab, TLR2C Ab, TLR2N Ab, TLR4 Ab, isotype control IgG, or RPMI only (Table 1). All groups were then stimulated with 10 μg/ml of SE-LPS and LCDL was measured.

Isotype matched pre-immune goat IgG (2.0 and 0.2 μg/ml) nonspecifically inhibited heterophil oxidative burst by 36.1 and 24.5%, respectively, when stimulated with 10 μg/ml of LPS (Table 1). Heterophils treated with goat anti-human CD14 antibody had no significant effect on oxidative burst when stimulated with LPS as compared to the IgG control treated cells. Treatment of heterophils with the goat anti-human TLR2C (2.0 μg/ml) antibody significantly ($p < 0.001$) decreased oxidative burst when stimulated with LPS when compared to the IgG control. The anti-human TLR2N antibody at a concentration of 2.0 μg/ml significantly ($p < 0.001$) decreased the oxidative burst generated by heterophils when stimulated with 10 μg/ml of LPS as compared to the IgG control. Treatment of the chicken heterophils with the anti-human TLR4 antibody at 2.0 μg/ml significantly ($p < 0.001$) decreased heterophil oxidative burst when stimulated with LPS, as compared to the LPS stimulated control.

#### 3.2.2. Heterophils stimulated with SA-LTA

Heterophils were pre-incubated with CD14 Ab, TLR2C Ab, TLR2N Ab, TLR4 Ab, isotype control IgG, or RPMI only (Table 2). All groups were then stimulated with 10 μg/ml of SA-LTA and LCDL was measured.

The isotype matched goat IgG had no nonspecific inhibitory activity on heterophil oxidative burst when stimulated with 10 μg/ml of LTA. Goat anti-human CD14 antibody at 2.0 μg/ml significantly ($p < 0.001$) decreased oxidative burst by 36.6% when the heterophils were stimulated with LTA. The anti-human TLR2C antibody at 2.0 and 0.2 μg/ml significantly ($p < 0.001$) decreased oxidative burst by 35.2 and 26.1%, respectively, when compared to

<table>
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<tr>
<th>Group</th>
<th>Peak cpm × 10^5 (% decrease)</th>
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<tr>
<td>SE-LPS only (10 μg/ml)</td>
<td>11.40 ± 1.45</td>
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<tr>
<td>IgG (μg/ml) + SE-LPS</td>
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<tr>
<td>2.0</td>
<td>7.29 ± 0.77 (36.1)a</td>
</tr>
<tr>
<td>0.2</td>
<td>8.60 ± 1.35 (24.5)a</td>
</tr>
<tr>
<td>CD14 (μg/ml) + SE-LPS</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>6.34 ± 0.74 (44.4)a</td>
</tr>
<tr>
<td>0.2</td>
<td>6.79 ± 0.94 (40.5)a</td>
</tr>
<tr>
<td>TLR2C (μg/ml) + SE-LPS</td>
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</tr>
<tr>
<td>2.0</td>
<td>4.82 ± 0.54 (57.7)a,b</td>
</tr>
<tr>
<td>0.2</td>
<td>7.59 ± 0.98 (33.4)a</td>
</tr>
<tr>
<td>TLR2N (μg/ml) SE-LPS</td>
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</tr>
<tr>
<td>2.0</td>
<td>4.52 ± 0.77 (60.3)a,b</td>
</tr>
<tr>
<td>0.2</td>
<td>7.81 ± 0.77 (31.4)a</td>
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<tr>
<td>TLR4 (μg/ml) + SE-LPS</td>
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<tr>
<td>2.0</td>
<td>5.46 ± 0.56 (52.1)a,b</td>
</tr>
<tr>
<td>0.2</td>
<td>7.12 ± 0.60 (37.5)a</td>
</tr>
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Peak counts per minute (cpm) after 30 min for SE-LPS groups. Data are given as mean ± SEM.

* Significantly ($p < 0.001$) different from SE-LPS control.

b Significantly ($p < 0.014$) different from IgG control.
the non-antibody treated, LTA stimulated heterophils. Anti-human TLR2N and anti-human TLR4 antibody had no effect on oxidative burst in heterophils stimulated with LTA.

4. Discussion

PAMPs, like LPS and LTA, have been previously shown to be potent activators of mammalian monocytes, macrophages, dendritic cells, endothelial cells, and neutrophils [6,13,14]. In the present study, we demonstrated that FKSE, FKSA, purified SE-LPS, and purified SA-LTA also activate chicken heterophils, the avian equivalent to the mammalian neutrophil. The difference in LPS and LTA dose response curves is to be expected, as the agonists are PAMPs that bind to different PRRs. The concentration of LPS used was higher than what is seen in mammalian studies due to the fact that chickens are refractory to LPS. The sudden drop in oxidative burst of heterophils stimulated with LTA is due to the toxic nature of increased levels of LTA to the cells. These data suggest that these PAMPs stimulate oxidative burst in chicken heterophils via PRRs.

We hypothesized that these PRRs are in fact TLRs, which have been shown to be an important component of the innate immune system [6,15]. Numerous papers have been published about the interactions of TLRs on mammalian macrophages and dendritic cells. However, little emphasis has been placed on TLRs on polymorphonuclear cells such as the mammalian neutrophil or the avian heterophil, which have been demonstrated to be an extremely important effector cell in neonatal chicks and mice [10,14,16]. While, TLRs have been found on mammalian polymorphonuclear leukocytes they have not been previously reported on avian heterophils or shown, in either species, to mediate oxidative burst. However, it has been recently reported that TLRs are present in several chicken tissues and on macrophages [8,17]. These developments led us to hypothesize that that chicken heterophils may also possess TLRs and that these TLRs may mediate oxidative burst.

TLRs have been previously reported to be evolutionarily conserved between Drosophila, humans, plants and chickens [6–8]. Recent findings identified regions of chicken DNA from the bursa of Fabricius with 80.7 and 72% amino acid sequence homology to mammalian TLR2 [7,8]. Chicken TLR antibodies are not yet commercially available, but mammalian TLR antibodies are readily available from several commercial sources. These highly conserved proteins allow researchers to utilize mammalian reagents for avian studies. For example, Dil and Qureshi used mammalian CD14 and TLR4 antibodies to inhibit nitric oxide synthase in chicken macrophages. They further illustrated the presence of the PRRs on chicken macrophages by flow cytometry. Similarly, we confirmed that TLRs mediate oxidative burst of chicken heterophils by using antibodies to mammalian TLRs to block chicken TLRs. Based on the data presented here, neutralizing antibodies to mammalian TLRs also neutralize chicken TLRs.

Isotype matched goat IgG was used as a negative control in these experiments to account for nonspecific binding of chicken TLRs. This goat IgG did in fact decrease oxidative burst in LPS stimulated heterophils suggesting the binding of LPS to heterophil receptors was nonspecifically blocked. However,

<table>
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<tr>
<th>Group</th>
<th>Peak cpm × 10^5 (%) decrease</th>
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<tr>
<td>SA-LTA Only (10 μg/ml)</td>
<td>9.45 ± 0.58</td>
</tr>
<tr>
<td>IgG (μg/ml) + SA-LTA 2.0</td>
<td>8.67 ± 1.05 (8.3)</td>
</tr>
<tr>
<td>0.2</td>
<td>10.83 ± 1.65 (0)</td>
</tr>
<tr>
<td>CD14 (μg/ml) + SA-LTA 2.0</td>
<td>5.99 ± 0.55 (36.6)a,b</td>
</tr>
<tr>
<td>0.2</td>
<td>10.36 ± 2.15 (0)</td>
</tr>
<tr>
<td>TLR2C (μg/ml) + SA-LTA 2.0</td>
<td>6.13 ± 0.63 (35.2)a,b</td>
</tr>
<tr>
<td>0.2</td>
<td>6.99 ± 0.71 (26.1)a,b</td>
</tr>
<tr>
<td>TLR2N (μg/ml) + SA-LTA 2.0</td>
<td>7.45 ± 1.19 (21.2)b</td>
</tr>
<tr>
<td>0.2</td>
<td>7.84 ± 0.99 (17.1)b</td>
</tr>
<tr>
<td>TLR4 (μg/ml) + SA-LTA 2.0</td>
<td>10.28 ± 2.28 (0)</td>
</tr>
<tr>
<td>0.2</td>
<td>8.59 ± 1.31 (9.1)b</td>
</tr>
</tbody>
</table>

Peak counts per minute (cpm) after 30 min for SA-LTA groups. Data are given as mean ± SEM.

a Significantly (p < 0.001) different from SA-LTA control.
b Significantly (p < 0.003) different from IgG control.
the TLR antibodies further significantly decreased oxidative burst above the IgG control suggesting that specific blocking did occur. The isotype matched IgG had no effect on LTA stimulated heterophils. The difference in nonspecific binding between LPS and LTA is probably attributed to the difference in the receptors that are involved.

Recently, TLR-1, -2, and -4 mRNA were reported to be expressed in human neutrophils and were shown to be regulated in neutrophils stimulated with LPS [14]. Comparatively, our research demonstrates that TLR2 and TLR4 mediate LPS stimulated oxidative burst in heterophils, while CD14 and TLR2 mediate LTA stimulated oxidative burst in heterophils. We believe this to be the first report of the presence of TLRs on chicken heterophils and the first report of TLR functional activity in heterophils.

Neonatal chicks are highly susceptible to infection during the first four days of life [10]. The innate immune system could be primed through these TLRs to activate the required immune system thereby protecting susceptible neonates against infection by both arms of the immune system [4]. As antibiotics become less effective and available for agricultural needs, other means of preventing disease such as stimulating the innate immune system via TLRs should become more desirable. Further experiments are required and planned to characterize the exact signaling cascade that regulates this mechanism of bacterial killing in chicken heterophils.

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References