In vivo lipopolysaccharide injection alters CD4⁺CD25⁺ cell properties in chickens

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ABSTRACT: In chickens, thymic CD4⁺CD25⁺ cells are characterized as regulatory T cells. The objectives of this experiment were to study the effects of an in vivo lipopolysaccharide (LPS) injection on the percentage of CD4⁺CD25⁺ cells in peripheral organs and the suppressive properties of splenic CD4⁺CD25⁺ cells in chickens. Chickens were injected with LPS and CD4⁺CD25⁺ cells were analyzed at 1, 2, 3, 5, and 10 d post LPS injection. The LPS injection increased CD4⁺CD25⁺ cell percentage approximately 5-fold in the blood at 1 d post LPS injection (P < 0.001), 3-fold in the thymus at 3 d post LPS injection (P = 0.001), and 2.5-fold in the spleen at 2 d post LPS injection (P = 0.001) compared with the no-LPS-injected group. The LPS injection did not alter the CD4⁺CD25⁺ cell percentage in the cecal tonsil (P = 0.162), lung (P = 0.098), or bone marrow (P = 0.071) at any time point measured. At 2 d post LPS injection, splenic CD4⁺CD25⁺ cells lost their suppressive ability (P < 0.001). At 5 d post LPS injection, splenic CD4⁺CD25⁺ cells not only regained their suppressive ability, but also became supersuppressive (P < 0.001). Splenic CD4⁺CD25⁺ cells at 5 d post LPS injection produced 5.5-fold more (P = 0.005) IL-10 mRNA than splenic CD4⁺CD25⁺ cells at 0 and 2 d post LPS injection. In conclusion, chicken regulatory T cells are differentially activated to facilitate immune response during the early stage of inflammation and to facilitate immune suppression at a later stage of inflammation.

Key words: chicken, inflammation, lipopolysaccharide, T cell, toll-like receptor

INTRODUCTION

Natural T regulatory cells (Treg), a subset of T cells, specialize in immunosuppression. In chickens, thymic CD4⁺CD25⁺ cells are characterized as Treg (Shanmugasundaram and Selvaraj, 2011b). Uncontrolled inflammatory mediators can cause undesirable host damage such as muscle wasting and autoimmune diseases (Belkaid and Rouse, 2005). Depending on the amount of damage, Treg will be stimulated to prevent local or systemic immune damage at a later stage of infection (Sakaguchi, 2003). But hyperactive Treg can impair immune cell activity and therefore are implicated in impaired microbial defense and pathogen persistence (Li et al., 2008). Thus, the balance between Treg and other immune cells is essential for the proper control of an immune response.

The host maintains balance between effector cell and Treg responses through toll-like receptor (TLR) signaling. Activation of Treg through lipopolysaccharide (LPS), a TLR ligand, abrogates the suppressive properties of mammalian Treg (Liu et al., 2006; Sutmuller et al., 2006). Abrogation of Treg suppressive functions will facilitate efficient effector cell functions. In mammals, the negating of Treg function by TLR ligands is temporary as the suppressive functions of LPS-treated Treg are markedly enhanced at a later time point. Thus, Treg actually become supersuppressive post LPS treatment (Caramalho et al., 2003). Supersuppressive properties of Treg during later stages of inflammation can protect against immune cell-mediated host damage. In chickens, in vitro LPS treatment increases Treg cell proliferation but abrogates Treg suppressive functions at 2 d post in vitro LPS treatment. At 4 d post in vitro LPS treatment, Treg become supersuppressive and have 7-fold greater...
The percentage of CD4+CD25+ cells in different or-

IL-10 mRNA expression than T_{reg} at 0 d post LPS treat-

This experiment was conducted to study effects of an in vivo LPS injection on chicken CD4+CD25+ cell characteristics in peripheral organs.

**MATERIALS AND METHODS**

All animal protocols were approved by the Ohio Agricultural Research and Development Center Animal Care and Use Committee.

**Birds and Mouse Anti-chicken CD25**

All experiments used UCD 003, white leghorn inbred (99.9%) chickens of a defined MHC haplotype (B^{17}/B^{17}; Abplanalp, 1992) maintained in specific pathogen-free conditions. Production of primary mouse anti-chicken CD25 was described previously (Shanmugasundaram and Selvaraj, 2011b). The primary monoclonal anti-chicken CD25 antibody was conjugated to phycoerythrin (PE) using the R-PE conjugation kit (Prozyme, Hayward, CA) following the manufacturer’s instructions.

**CD4^+CD25^+ Cell Percentage in Different Organs Post Lipopolysaccharide Injection**

Fifteen 8-wk-old chickens were injected with *Salmonella typhimurium* LPS (No. L9516, Sigma, St. Louis, MO) at 500 μg/kg BW (Selvaraj et al., 2010). As a no-LPS-injection control, tissues from 3 chickens that were not injected with LPS were collected. Chickens for each time point (n = 3) were selected randomly at 1, 2, 3, 5, and 10 d post LPS injection, and blood, thymus, spleen, lung, cecal tonsils, and bone marrow were collected. Approximately 3 mL of blood were collected from the heart with 100 μL of 2% EDTA (Sigma, St. Louis, MO) immediately post CO2 asphyxiation. At each time point, single-cell suspensions of blood, thymus, spleen, lung, cecal tonsils, and bone marrow were concentrated for lymphocytes by density centrifugation over Histopaque (1.077 g/mL, Sigma, St. Louis, MO) at 400 × g for 20 min at 25°C. Cells (1 × 10^6) were incubated with 10 μg/mL of primary PE-linked mouse anti-chicken CD25, 1:200 fluorescein isothiocyanate-conjugated mouse anti-chicken CD4 (Southern Biotech, Birmingham, AL), and 1:200 dilution of unlabelled mouse IgG (Abcam, Cambridge, MA) for 45 min. Unbound antibodies were removed by centrifugation at 400 × g for 15 min at 25°C. The percentage of CD4^+CD25^+ cells in different organs was analyzed in a flow cytometer (Guava Easycyte, Millipore, Billerica, MA) and expressed as the percent-age of CD4^+ cells to facilitate comparison among samples.

**Suppressive Properties of Splenic CD4^+CD25^+ Cells Post Lipopolysaccharide Injection**

A suppression of T cell proliferation assay was employed to assess the suppressive properties of splenic CD4^+CD25^+ cells. This assay is a co-culture assay between CD4^+CD25^+ T_{reg} (effector cells) and carboxyfluorescein succinimidyl ester (CFSE)-labeled CD4^+CD25^- cells (responder cells). This experiment was designed so that 1 batch of responder cells was used to evaluate the suppressive properties of T_{reg} post LPS injection. Three chickens were injected with LPS at 500 μg/kg BW. Three days after the first injection, another set of 3 chickens was injected with LPS at 500 μg/kg BW. Two days later, CD4^+CD25^+ cells from the spleen of chickens at 2 and 5 d post LPS injection were flow sorted using a reflection cell sorter (iCyt, Chapmain, IL; ~99% pure). As a no-LPS-injection control, CD4^+CD25^+ cells from 3 chickens that were not injected with LPS were collected and processed similarly.

Naïve CD4^+CD25^- T cells (responder cells) were collected from the spleen of 1 chicken, labeled with CFSE, and stimulated with a 1:750 dilution of soluble anti-chicken CD3 and CD28 as described previously (Shanmugasundaram and Selvaraj, 2010, 2011b). A co-culture assay was performed by co-incubating 5 × 10^4 CFSE-labeled responder cells with effector cells (CD4^+CD25^-) at an effector:responder cell ratio of 1:1, 0.25:1, or 0:1 at 37°C in the presence of 5% CO2. The CFSE dilution of CFSE-labeled responder cells was measured at 72 h of co-culture as an indicator of cell proliferation (Guava Easycyte, Millipore). Unproliferated cell percentage in the co-culture group was determined after gating on the CFSE-positive responder cells.

**Interleukin-10 mRNA Analysis of Splenic CD4^+CD25^+ Cells Post Lipopolysaccharide Injection**

Total RNA from in vivo studies were extracted from splenic CD4^+CD25^+ cells at 0, 2, and 5 d post LPS injection and analyzed for the relative expression of *IL-10* mRNA (5'-cagctgctgggctgaa-3' and 5'-ctgtctcgtgtctgag-3') after normalizing for β-actin (5'-accggactgt-agctggtc-3') and 5'-cagctgctgggctgaa-3' after normalizing for β-actin (5'-accggactgt-agctggtc-3') and 5'-cagctgctgggctgaa-3' after normalizing for β-actin (5'-accggactgt-agctggtc-3'). The annealing temperature for *IL-10* mRNA was 55°C and for β-actin was 57°C. Fold change from the reference was calculated as 2^{ΔΔCt} (where Ct is the threshold cycle (Selvaraj et al., 2010). The reference group was CD4^+CD25^+ cells from the no-LPS-injected group.
Interleukin-2 mRNA Analysis Post Lipopolysaccharide Injection

Total RNA was extracted from splenic CD4+CD25+ cells at 0, 2, and 5 d post LPS injection and analyzed for IL-2 mRNA (5’-ttggctgtatttcggtagca-3’ and 5’-cctgggtct-cagttgtgt-3’) content. The annealing temperature for IL-2 mRNA expression analysis was 58.4°C. Data were reported as 40 – Ct value. To determine the approximate contamination percentage of splenic CD4+CD25+ cells with non-T_reg, thymic CD4+CD25+ cells (1 × 10^6 cells) from chickens (n = 3) were replaced with either 0, 20, 40,
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60, 80, or 100% of CD4+CD25− cells. Total mRNA was extracted from the cells and analyzed for IL-2 mRNA expression as described earlier. A standard curve was drawn based on the 40 – Ct value.

**Statistical Analysis**

A mixed-model ANOVA (JMP software, Cary, NC) was used to examine the main effects of time after LPS injection on the dependent variables. The model included the fixed effect of time and random effect of chicken. When main effects were significant (P < 0.05), differences between means were analyzed by Tukey’s least squares means comparison.

**RESULTS**

**CD4+CD25+ Cell Percentage in Different Organs Post Lipopolysaccharide Injection**

The LPS injection increased (P < 0.001) CD4+CD25+ cell percentage in the blood approximately 5-fold at 1 d post LPS injection compared with the no-LPS-injected group (Figure 1). The LPS injection increased (P = 0.001) CD4+CD25+ cell percentage approximately 3-fold in the thymus at 3 d post LPS injection compared with the no-LPS-injected group. The LPS injection increased (P = 0.001) CD4+CD25+ cell percentage approximately 2.5-fold in the spleen at 2 d post LPS injection compared with the no-LPS-injected group. The LPS injection did not alter the CD4+CD25+ cell percentage in the cecal tonsil (P = 0.162), lung (P = 0.098), or bone marrow (P = 0.071) at any time point measured.

**Suppressive Properties of Splenic CD4+CD25+ Cells Post Lipopolysaccharide Injection**

In no-LPS-injected chickens, splenic CD4+CD25+ cells were suppressive at an effector:responder cell ratio of 1:1 (P < 0.001; Figure 2). The unproliferated cell percentage was comparable (P = 0.126) among groups that had no effector cells and the group with an effector:responder cell ratio of 0.25:1, which shows that CD4+CD25+ cells were not suppressive at an effector:responder cell ratio of 0.25:1 in no-LPS-injected chickens. At 2 d post LPS injection, CD4+CD25+ cells were not suppressive (P = 0.168) at an effector:responder cell ratio of 1:1; thus splenic CD4+CD25+ cells lost their suppressive ability at 2 d post LPS injection. At 5 d post LPS injection, CD4+CD25+ cells became supersuppressive in that CD4+CD25+ cells suppressed responder cell proliferation at an effector:responder cell ratio of 1:1 (P < 0.001), as well as at a ratio of 0.25:1 (P < 0.001).

**Interleukin-10 mRNA Content of Splenic CD4+CD25+ Cells Post Lipopolysaccharide Injection**

Splenic CD4+CD25+ cells from chickens at 5 d post LPS injection produced a greater (P = 0.005) amount of IL-10 mRNA than CD4+CD25+ cells from chickens at 0 and 2 d post LPS injection (Figure 3). At 5 d post LPS injection, Treg had 5.5-fold greater IL-10 mRNA expression than Treg from no-LPS-injected chickens.
Interleukin-2 mRNA Analysis
Post Lipopolysaccharide Injection

Chicken \( T_{\text{reg}} \) had no detectable IL-2 mRNA. The 40 – Ct value of IL-2 mRNA analysis of chicken \( T_{\text{reg}} \) was dependent on the amount of contamination with CD4\(^+\)CD25\(^-\) cells and the curve was linear (\( r = 0.99 \); Figure 4A). The mean 40 – Ct value of CD4\(^+\)CD25\(^+\) cells from spleen at 0, 2, or 5 d post LPS injection was lower (\( P < 0.001 \)) than that of CD4\(^+\)CD25\(^-\) cells from spleen. When the 40 – Ct value was analyzed using the equation derived from Figure 4A, the maximal percentage of CD4\(^+\)CD25\(^+\) non-\( T_{\text{reg}} \) contaminating the splenic CD4\(^+\)CD25\(^+\) cell population was suggested to be approximate 7% in no-LPS injected groups, 29% at 2 d post LPS injection, and 17% at 5 d post LPS injection.

DISCUSSION

Regulatory T cells originate in the thymus, a primary lymphoid organ, and migrate to several secondary lymphoid organs. This article describes the effects of an inflammatory stimulus on chicken CD4\(^+\)CD25\(^+\) cells in peripheral organs, which are the sites of action for \( T_{\text{reg}} \).

In chickens, thymic CD4\(^+\)CD25\(^+\) cells are characterized as \( T_{\text{reg}} \) even though chickens lack transcription factor FoxP3, a commonly used \( T_{\text{reg}} \)-specific marker in mammals (Shanmugasundaram and Selvaraj, 2011b). In chickens, CD25 is not a \( T_{\text{reg}} \)-specific marker in the periphery. Conventional T cells (non-\( T_{\text{reg}} \)) transiently upregulate CD25 in the periphery during inflammation in chickens (Hala et al., 1986; Kim et al., 2000; Teng et al., 2006). Thus, activated non-\( T_{\text{reg}} \) with transient CD25 expression could contribute to the CD25\(^+\) population in the periphery and confound the effect of LPS on CD4\(^+\)CD25\(^+\) \( T_{\text{reg}} \). Identifying markers unique to chicken \( T_{\text{reg}} \) will differentiate \( T_{\text{reg}} \) from non-\( T_{\text{reg}} \) with transient CD25 upregulation. Unfortunately markers unique to chicken \( T_{\text{reg}} \) have yet to be identified.

To overcome and minimize the drawbacks of studying \( T_{\text{reg}} \) in vivo in a species with no \( T_{\text{reg}} \)-specific markers, certain assumptions were made to interpret the data. This included comparing the IL-2 mRNA content of the splenic CD4\(^+\)CD25\(^+\) cells from chickens injected with LPS with the IL-2 mRNA content of splenic and thymic CD4\(^+\)CD25\(^+\) cells from chickens not injected with LPS. Decreased IL-2 production by T regulatory cells is a unique feature to \( T_{\text{reg}} \) (Scheffold et al., 2007). Chicken thymic CD4\(^+\)CD25\(^+\) cells (\( T_{\text{reg}} \)) have no detectable IL-2 mRNA whereas chicken CD4\(^+\)CD25\(^-\) cells have increased IL-2 mRNA (Shanmugasundaram and Selvaraj, 2011b). The 40 – Ct value of IL-2 mRNA analysis had a linear relationship with the percentage of CD4\(^+\)CD25\(^-\) cell contamination. Therefore, we assumed that comparing the 40 – Ct value of IL-2 mRNA analysis will identify the probable amount of non-\( T_{\text{reg}} \) contaminating the \( T_{\text{reg}} \) population. If non-\( T_{\text{reg}} \) with transient CD25 upregulation do not contaminate the CD4\(^+\)CD25\(^+\) cells, the IL-2 mRNA will be non-detectable in the CD4\(^+\)CD25\(^+\) cells. If non-\( T_{\text{reg}} \) with transient CD25 upregulation contaminate the CD4\(^+\)CD25\(^+\) cells, the abundance of IL-2 mRNA will depend on the amount of contamination. These assumptions are pointed out to ensure that the conclusions reached were not beyond the findings of the data.

Regulatory T cells originate in the thymus (Fontenot et al., 2005). Increased CD4\(^+\)CD25\(^+\) cell output from thymus 3 d post LPS injection might be an adaptive response to downregulate an immune response after in-
flammation. Bone marrow is the major organ of storage for T<sub>reg</sub> (Zou et al., 2004). Chicken CD4<sup>+</sup>CD25<sup>+</sup> cell numbers did not change significantly in the bone marrow post LPS injection. The LPS injection increased CD4<sup>+</sup>CD25<sup>+</sup> cell percentage in the spleen and blood at 2 d post LPS injection. Proliferation of CD4<sup>+</sup>CD25<sup>+</sup> cells and upregulation of CD25 by CD4<sup>+</sup>CD25<sup>+</sup> cells post LPS injection could contribute to the observed increase in CD4<sup>+</sup>CD25<sup>+</sup> cell percentage. In vitro LPS treatment stimulates CD4<sup>+</sup>CD25<sup>+</sup> cell proliferation (Shanmugasundaram and Selvaraj, 2011a). In vitro LPS treatment also increases the upregulation of CD25 by CD4<sup>+</sup>CD25<sup>+</sup> cells (Shanmugasundaram and Selvaraj, 2011b). The relative contribution by each of the above populations to the total increase in CD4<sup>+</sup>CD25<sup>+</sup> cell population in different organs is not known. The IL-2 mRNA analysis suggests that at 2 d post LPS injection, the maximal contamination can be up to 30%. In thymus, spleen, and blood of LPS-injected birds, the increase in CD4<sup>+</sup>CD25<sup>+</sup> cell percentage is greater than 30% than that in birds with no-LPS-injection, and it is likely that LPS-induced T<sub>reg</sub> proliferation contributed to the increase in CD4<sup>+</sup>CD25<sup>+</sup> cell numbers. In both chickens (Shanmugasundaram and Selvaraj, 2011a) and mammals (Caramalho et al., 2003), in vitro LPS treatment induces T<sub>reg</sub> to proliferate. Identification of unique markers specific for chicken T<sub>reg</sub> will help to quantify the percentage increase in the chicken T<sub>reg</sub> population after an inflammatory challenge with greater accuracy.

Although the CD4<sup>+</sup>CD25<sup>+</sup> cell numbers increased in spleen at 2 d post LPS injection, they lost their suppressive ability. The ability of LPS to abrogate the suppressive properties of mammalian T<sub>reg</sub> has been reviewed extensively (Sutmuller et al., 2007; van Maren et al., 2008). In mammals, LPS abrogates the suppressive properties of T<sub>reg</sub> through the TLR pathway. Chicken T<sub>reg</sub> express approximately 30-fold greater TLR 2-type and 6-fold greater TLR-4 mRNA content than CD4<sup>+</sup>CD25<sup>+</sup> cells (Shanmugasundaram and Selvaraj, 2011a). The loss of CD4<sup>+</sup>CD25<sup>+</sup> cell suppressive properties was temporary, and CD4<sup>+</sup>CD25<sup>+</sup> cells regained their suppressive properties and became supersuppressive at 5 d post LPS injection. Non-T<sub>reg</sub> which have upregulated CD25, are not suppressive (Shanmugasundaram and Selvaraj, 2011b), which eliminates the possibility of non-T<sub>reg</sub> contributing to the supersuppressive properties observed at 5 d post LPS injection. In addition, the IL-2 mRNA content of CD4<sup>+</sup>CD25<sup>+</sup> cells at 5 d was almost at the quantities of CD4<sup>+</sup>CD25<sup>+</sup> cells in birds with no-LPS-injection, which eliminates the possibility of T<sub>reg</sub> contamination with non-T<sub>reg</sub>. In mammals, the transient CD25 upregulation post inflammation disappears at 2 d of CD25 upregulation (Couper et al., 2007).

The supersuppressive ability of CD4<sup>+</sup>CD25<sup>+</sup> cells at 5 d post LPS injection can be explained by the 5-fold increase in their IL-10 mRNA production. Interleukin-10 is an anti-inflammatory cytokine that acts to suppress T cell proliferation (Bettini and Vignali, 2009) and is essential for T<sub>reg</sub> functions in humans (Sun et al., 2010). Chicken T<sub>reg</sub> produce approximately 29-fold more IL-10 mRNA than CD4<sup>+</sup>CD25<sup>+</sup> T cells (Shanmugasundaram and Selvaraj, 2011b).

During pathogen infections, an efficient T cell response results in pathogen clearance. However, many pathogens induce persistent infections despite continuous measurable T-cell responses (Rehermann et al., 1996), a situation in which T<sub>reg</sub> may be involved. In mammals, T<sub>reg</sub> dysregulation is present during many persistent viral infections (Li et al., 2008). Chicken CD4<sup>+</sup>CD25<sup>+</sup> cell supersuppressive properties during the late phase of inflammation might explain why chickens are unable to clear a secondary respiratory bacterial infection after a viral infection (Glisson, 1998) or why some avian species are not able to clear certain persistent viral infections like avian influenza (Kuchipudi et al., 2012). In chickens, CD4<sup>+</sup>CD25<sup>+</sup> cell percentages increase post avian influenza infections (Teng et al., 2006), suggesting the role of T<sub>reg</sub> in avian influenza persistence.

Activated CD4<sup>+</sup>CD25<sup>+</sup> cells temporarily lose their suppressive properties, possibly to permit T effector-cell activation and pathogen clearance. At late-phase inflammation, however, CD4<sup>+</sup>CD25<sup>+</sup> cells are differentially activated to suppress the activity of immune cells, perhaps to prevent immune damage. In conclusion, it is likely that chicken CD4<sup>+</sup>CD25<sup>+</sup> cell functions are modified to permit effective immune responses during early stages of inflammation and to facilitate immune suppression at later stages of inflammation.

**LITERATURE CITED**


