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Contribution of leukocytes to the induction and resolution of the acute inflammatory response in chickens

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ABSTRACT

A successful immune response against invading pathogens relies on the efficient activation of host defense mechanisms and a timely return to immune homeostasis. Despite their importance, these mechanisms remain ill-defined in most animal groups. This study focuses on the acute inflammatory response of chickens, important both as an avian model with a unique position in evolution as well as an increasingly notable target of infectious zoonotic diseases. We took advantage of an *in vivo* self-resolving intra-abdominal challenge model to provide an integrative view of leukocyte responses during the induction and resolution phases of acute inflammation. Our results showed rapid leukocyte infiltration into the abdominal cavity post zymosan challenge (significant increase as early as 4 h), which was dominated by heterophils. Peak leukocyte infiltration and ROS production reached maximum levels at 12 h post challenge, which was significantly earlier than comparative studies in teleost fish and mice. Both heterophils and monocyte/macrophages contributed to ROS production. Local leukocyte infiltration was preceded by an increase in peripheral leukocytes and a drop in the number of bone marrow leukocytes. The proportion of apoptotic leukocytes increased following peak of acute inflammation, rising to significant levels within the abdominal cavity by 48 h, consistent with other indicators for the resolution of inflammation. Importantly, comparison of chicken phagocytic responses with those previously shown in agnathan, teleost and murine models suggested a progressive evolutionary shift towards an increased sensitivity to pro-inflammatory pathogen-derived particles and decreased sensitivity towards homeostatic stimuli. Thus, while significant conservation can be noted across the immune systems of endotherms, this study highlights additional unique features that govern the induction and resolution of acute inflammation in the avian system, which may be relevant to disease susceptibility and performance.
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1. Introduction

Innate immune responses are pivotal to restrict pathogen dissemination following entry into a host. Pathogen invasion triggers a series of acute inflammatory processes which are followed by a timely return to immune homeostasis (Kogut et al., 2005). Assessment of the evolution of these responses based on agnathan, teleost and mammalian models indicates conservation of key players as well as divergence in select molecular and cellular mechanisms (Chadzinska et al., 2008, 1999; Copeland et al., 2005; Havixbeck et al., 2016, 2014; Fujieda et al., 2013; Rao et al., 1994; Rieger et al., 2012; Rieger and Barreda, 2011). Despite the absence of myeloperoxidase expression, L-arginine biosynthesis, and RIG-1 gene expression, birds remain among the most successful of animal groups suggesting that they may possess unique strategies governing effector and regulatory mechanisms of inflammation (Barber et al., 2010; Farnell et al., 2006; He et al., 2003; Magor et al., 2013; Penniall and Spitznagel, 1975; Tamir and Ratner, 1963).

The abdominal cavity provides a useful environment to study the contributions of migrating leukocytes to the induction and regulation of acute antimicrobial responses (Zarember and Kuhns, 2011). Unlike circulating blood leukocytes, migrating leukocytes are recruited to the inflammatory site following adhesion and diapedesis through endothelial walls, and are further exposed to the inflammatory milieu at the challenge site. Under these conditions, infiltrating leukocytes can also be easily harvested and examined for changes in gene expression and functional

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antimicrobial responses. This offers several advantages for characterization of sterile and pathogen driven inflammatory events.

Pathogen-recognition receptors (PRRs) provide a long-standing strategy for recognition of invading pathogens. They can be found from basal multicellular organisms to highly complex vertebrates (Gordon, 2002; Zhang et al., 2010). As in other vertebrates, the avian genome has conserved genes encoding major PRRs (Cormican et al., 2009). Among these, the toll-like receptor 2/dectin-1 complex represents one of the most important tools to sense and combat fungal infections in mammals (Brown, 2006; Megías et al., 2015; Nakamura et al., 2008) and avian systems (Genovese et al., 2013; Magor et al., 2013).

Our goal for the present study was to examine the functional contributions of avian leukocytes during the acute inflammatory response. We considered the kinetics for induction and resolution of acute inflammation and the participation of distinct leukocyte subsets as they migrated from the hematopoietic compartment to the immune challenge site. Further, we examined changes in the immune effector mechanisms at this challenge site and the contribution that apoptosis has in the transition from induction to resolution phases of acute inflammation. Overall, we find that birds produce a significantly faster cellular acute inflammatory response when compared to murine and teleost models (Havixbeck et al., 2014; Rieger et al., 2012; Rieger and Barreda, 2011). Massive heterophil infiltration was detected as early as 4 h post challenge. Kinetics of leukocyte infiltration and reactive oxygen species (ROS) production displayed similar patterns, however, the latter revealed lower levels compared to those found in mice and fish reported in previous studies. *Ex vivo* analysis showed that chicken activated leukocytes have a higher sensitivity to pathogen-derived particles than other animal models when co-incubated with homeostatic particles. Apoptotic cells, however, remain as effective contributors to down-regulation of inflammatory responses *in vivo*.

2. Materials and methods

2.1. Chickens

Three to six-week old chickens (*Gallus gallus*) Ross 708 broilers were used. All birds were housed in the Poultry Research Facility of the Department of Agricultural, Food and Nutritional Sciences at the University of Alberta. All animals were maintained according to the guidelines specified by the Canadian Council on Animal Care, and protocols were approved by the University of Alberta Animal Care and Use Committee. Chickens were terminated by cervical dislocation and exsanguination; all efforts were made to minimize animal stress.

2.2. Intra-abdominal injections

Zymosan A (2.5 mg, Sigma-Aldrich) was resuspended in 500 μl of $PBS^{-/-}$ (no calcium/no magnesium) and administered through injection into the abdominal cavity to induce inflammation. Our primary goal was to select experimental conditions that effectively triggered an acute inflammatory response *in vivo* which, subsequently, were also effectively controlled. *In vivo* stimulation of our birds with 2.5 mg of zymosan intra-abdominally effectively induced this self-resolving process (both induction and resolution clearly observed). Given that the leukocyte responses observed were already significantly faster than those previously identified in mice and fish (e.g. rates of leukocyte recruitment, initiation of cytokine gene expression, activation of antimicrobial responses) we chose to remain at this zymosan dose rather than increase the dose to normalize conditions based on overall body weight. Apoptotic cells were prepared as described in section 2.6. For *in vivo* administration of apoptotic cells, 1.5×10^7 cells were injected into the abdominal cavity in 500 μl of $PBS^{-/-}$ at different time points prior or together with zymosan injections (−4, −2 and 0 h before zymosan injections).

2.3. Isolation of blood, bone marrow and intra-abdominal leukocytes

Blood samples, bone marrow and intra-abdominal leukocytes were measured at 4, 8, 12, 18, 24 and 48 h after zymosan intra-abdominal injections in order to determine the dynamics of total leukocytes in the hematopoietic, circulatory and inflammatory compartments after zymosan challenge. Briefly, 2 ml of blood samples were taken in EDTA tubes, and processed for Hema3 staining. Bone marrow leukocytes were obtained after femur collection and bones were flushed out with 10 ml of $PBS^{-/-}$, followed by a red blood cell lysis step. Leukocytes were subsequently counted under light microscopy. Intra-abdominal leukocytes were recovered by injecting 20 ml of 1× $PBS^{-/-}$ in the lower left quadrant at the abdominal site and gently massaged to distribute the solution homogeneously throughout the abdominal cavity. Cells were harvested and maintained at 4 °C. Non-injected chickens were used as a negative control (0 h group). For *ex vivo* functional assays, 12 and 48 h samples post zymosan injections were used as representative periods of proinflammatory and proresolving stages of acute inflammation, respectively.

2.4. Cell viability

After intra-abdominal leukocyte collection, annexinV/propidium iodide (PI) staining was performed in order to determine cell viability, as previously described (Havixbeck et al., 2015; Rieger et al., 2010). One hundred microliters of intra-abdominal lavages at different points were washed twice with Annexin V binding buffer (BD pharmigen) and resuspended in 100 μl of AnnexinV binding buffer. AnnexinV (eBioscience) was added according to the manufacturer. Propidium iodide (PI, Sigma) was added at a final concentration of 4 μg/ml, followed by 30 min incubation at 41 °C. Ten thousand events were acquired using conventional flow cytometer (BD Canto II) and an imaging flow cytometer (Imagestream MK II, Amnis, EMD Millipore).

2.5. Reactive oxygen species production

2.5.1. Dihydrorhodamine (DHR)

Intra-abdominal leukocytes were incubated for 5 min with dihydrorhodamine (DHR, Molecular probes) at a final concentration of 10 μM. Phorbol 12-myristate 13-acetate (PMA, Sigma) was added to a final concentration of 100 ng/ml. Cells were incubated for 30 min at 41 °C to allow oxidation of DHR. Cells were analysed in conventional flow cytometer BD Canto II. At least 10,000 events were recorded.

2.5.2. Nitroblue tetrazolium assay (NBT)

The NBT assay was used as previously described (Neumann and Belosevic, 1996). Briefly, 2.5×10^5 intra-abdominal leukocytes were placed in 96-well microplates. NBT (100 mg/ml, Sigma Aldrich) was diluted at a final concentration of 2 mg/ml in 1× $PBS^{-/-}$. PMA was added to the NBT at 100 ng/ml final concentration. Fifty μl of NBT/PMA solution was added to the cell suspension and incubated for 25 min at 41 °C. The microplate was spun down and fixed with 200 μl of 70% methanol for 1 min and washed twice with the same solution to remove unreduced NBT. Potassium hydroxide (2 M) was used to dissolve reduced NBT by pipetting vigorously. Seventy microliters of DMSO was added and optical densities were read at

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