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Potential directions for chicken immunology research

Cameron R. Stewart [a,*,1], Anthony L. Keyburn [a,1], Celine Deffrasnes [a,1], S. Mark Tompkins [b]

[a] CSIRO Biosecurity Flagship, Australian Animal Health Laboratory, Geelong, Victoria 3220, Australia
[b] Department of Infectious Diseases, University of Georgia, Athens, GA 30602, USA

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ABSTRACT

The importance of poultry, particularly chicken, as a food source continues to increase globally. Moreover, zoonotic infectious diseases such as avian influenza virus not only continue to impact poultry production, but also pose an increasing threat to public health. This review discusses the importance of poultry in both agricultural and public health arenas. Recent developments in avian immunology are described, with an emphasis on host–pathogen interactions and noting differences from mammalian systems. Next generation technologies including functional genomics and targeted gene disruption (e.g. zinc finger nucleases and meganucleases) are discussed as new approaches for not only understanding immune responses in poultry, but also as novel disease intervention strategies.

1. Poultry production and disease

The poultry sector continues to grow and industrialize in many parts of the world. Increases in population, purchasing power, and urbanization have been strong drivers of growth. It was estimated that in 2013 the global chicken and turkey meat production reached over 105 million metric tons second only to pork and egg production reached 70 million tons in 2012 (USDA, 2012).

In addition to their importance as a food source, poultry are susceptible to a range of bacterial, fungal, protozoan and viral pathogens, a sub-set of which are transmissible to humans. Of significant importance are bacteria of the genera *Salmonella* and *Campylobacter*, a major source of food poisoning in humans. Newcastle disease virus can cause conjunctivitis and an influenza-like illness in humans, but is generally considered non-pathogenic. Arguably the most important human pathogen derived from poultry is highly pathogenic avian influenza virus subtype H5N1, which has resulted in over 370 reported human fatalities (2012), and the culling of tens of millions of poultry to halt the spread of the virus. In addition to zoonotic pathogens there are many diseases of poultry that have serious detrimental effects leading to significant loss of production and can even lead to 100% mortality. These include but are not limited to fowl cholera, necrotic enteritis, infectious laryngotracheitis, Marek's disease, infectious bursal disease, infectious bronchitis, coccidiosis and fowl pox.

2. Improving understanding of chicken immune responses

We rationalise that a greater understanding of the chicken immune response to pathogens would increase preparedness to respond to new infections emerging from poultry. For example, the recent emergence of an H7N9 influenza virus in China, which has infected and killed a number of people in a short time, and currently shows no disease in poultry; yet data suggests the virus is emerging from birds (Chen et al., 2013; Gao et al., 2013; Kageyama et al., 2013; Li et al., 2013; Uyeki and Cox, 2013). This is in stark contrast to H5N1 and H7N7 human infections where disease in humans was associated with a highly pathogenic phenotype in poultry (Perdue and Swayne, 2005). Such events highlight the need to study the immunological responses to these emerging viruses in poultry and other bird species. To achieve this there needs to be a focus on developing chicken specific reagents for immunological studies. Antibodies represent one of the most widely used tools to study the immune response. Several techniques such as flow cytometry, immunofluorescence, ELISA and western blot, employ monoclonal and polyclonal antibodies and these therefore represent a priority in terms of reagents to develop to increase our understanding of the avian immune system. Flow cytometry and immunofluorescence are commonly used to identify and characterise immune cell populations present in organs during infection or disease compared to homeostasis. Until recently few chicken specific antibodies were available and although more reagents are becoming commercially available, more efforts should be put into increasing the pool of antibodies.

The chicken has similar immune cell populations to mammalians (macrophages, NK cells, B cells, T cells, DC) but also unique cell types such as the heterophils and thrombocytes. Although these cell populations can share common surface markers with mammals, low

* Corresponding author. Address: CSIRO Australian Animal Health Laboratory, Geelong, Victoria 3220, Australia. Tel.: +61 352275601.
E-mail address: cameron.stewart@csiro.au (C.R. Stewart).
[1] These authors contributed equally.

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conservation between mammalian and chicken genes and proteins results in little cross-reactivity of anti-human or anti-mouse monoclonal antibodies on chicken cells (Viertlboeck and Gobel, 2007). Moreover, the vast majority of currently available chicken antibodies target lymphocytes and there is currently a lack of specific reagents targeting the innate immune cells such as macrophages, DC and NK cells to allow characterisation of their different subsets. Chicken T lymphocytes populations are defined by markers such as CD3, CD4, CD8, CD25, CD28, CD44, CD45 and TCR 1/2/3 (Beirao et al., 2012; Dalgaard et al., 2010; Fair et al., 2008; Lee et al., 2011a; Seliger et al., 2012; Shanmugasundaram and Selvaraj, 2011; Viertlboeck and Gobel, 2007) while B cell subsets can express markers such as Bu-1, CD1-1, CD25, CD44 and IgM (Beattie et al., 2001; Lee et al., 2011a; Ly et al., 2010; Seliger et al., 2012; Viertlboeck and Gobel, 2007). Although a number of antibodies are available to study T helper cell subsets in chicken, only the Th1, Th2 and Treg subsets have been described so far (Kaiser, 2010) and therefore more antibodies need to be developed to investigate the existence, role played and cytokines secreted by other subsets such as Th3, Th9, Th17 and Th22 in the chicken immune response to infections and diseases. Chicken monocytes/macrophages subsets can be identified by their expression of Bu-1, CD25, CD44, CD45, CD80, KUL01 and MHC-II (Lee et al., 2011a; Seliger et al., 2012) while DC can express markers such as CD11c, CD1-1, CD40, CD80, CD83, CD86, MHC-II, and DEC-205 (Hansell et al., 2007; Igyarto et al., 2006; Lee et al., 2011b, 2012; Ly et al., 2010; Wu et al., 2010). A recent study suggested an heterogeneity of NK cell subsets based on previously unrecognised markers for NK cells such as Bu-1, CD1-1, CD5, CD8α, CD44, CD56 (NCAM) and KUL01 (Zhang et al., 2012), however additional reagents are still needed to better define these subsets and unravel their functions. A group also recently described a 3–4 colour flow cytometry assay to distinguish monocytes, thrombocytes, B cells, T cells and heterophils from blood samples, nevertheless, this assay cannot detect eosinophils, basophils, and NK cells (Seliger et al., 2012) illustrating the need to extend the range of existing antibodies for chicken immunology.

Avian thrombocytes, which are large nucleated cells, are most frequently regarded as contaminating cells in peripheral blood monocuclear cell preparations. Few markers are available to exclude these cells and thrombocytes are often detected based on positive staining with the K1 marker which is also shared with macrophages, combined with absence of staining with pan-leukocyte markers such as CD45 and/or K55 (Bohls et al., 2006; Seliger et al., 2012). Table 1 lists commercially available monoclonal antibodies that have successfully been used to identify immune cell populations in the chicken.

Similarly, few antibodies have been developed to study the role of cytokines in the avian immune response to pathogens by flow cytometry or ELISA. Most studies rely on mRNA expression analysis to measure differences in cytokine expression and while this approach is useful, protein analysis is critical for accurate understanding of cytokine responses. Recent studies detected IL-1β, Il-2, IL-4, IL-6, IL-10, IFN-γ in organs such as cecal tonsil, intestine, peripheral blood lymphocytes or spleen by simplex ELISA (Deng et al., 2012a,b), however, further reagent development and research is greatly needed. Luminex assays are another valuable tool for immune response analysis, but none are currently available for chicken cytokines and should be another focus of reagent development.

3. New technologies to further host–virus studies in the chicken

3.1. Comparative functional genomics

It has been suggested that chickens may be susceptible to select pathogens due to irregularities in their immune system. One piece of evidence supporting this hypothesis is the apparent absence of the RNA helicase RIG-I in the chicken genome, which may underlie the susceptibility of chickens to avianinfluenza virus compared to other poultry species that express RIG-I (Barber et al., 2010). Technologies that identify differences in the host–virus interaction across species are emerging – such as genome-wide RNA interference (RNAi) screening. This technology involves the RNAi-mediated suppression of ~18,000 host genes, and measuring the impact of specific gene loss-of-function on the replication of pathogens of interest. This technology has been employed particularly to decipher virus life cycle events (Ang et al., 2010; Berger et al., 2009; Brass et al., 2008, 2009; Hao et al., 2008; Karlas et al., 2010; Konig et al., 2008, 2009; Krishnan et al., 2008; Ng et al., 2007; Panda et al., 2011; Randall et al., 2007; Sessions et al., 2009; Shapira et al., 2009; Supekova et al., 2008; Tai et al., 2009; Zhou et al., 2008). As all viruses, especially RNA viruses with small and limited genomes, lack the complement of proteins required for the production of infectious virus, they are dependent on hundreds of host molecules for replication. Information from RNAi screens of host–virus interactions can be combined with analogous technologies such as transcriptomics, 2-hybrid screens and chemical compound screens to compile a comprehensive view of the virus-host "interactome". RNAi screens of host–virus interactions have thus far been limited largely to human systems due to the availability of RNAi reagents at a genome-wide level. However, as genome sequencing and RNAi technologies develop, it will soon be possible to compare host genes required for virus replication across various species. The ability to translate findings from RNAi screens *in vivo*, in the form of transgenic animals, could potentially rectify deficiencies in host responses to pathogens.

3.2. RNAi-based therapeutics

Current limitations associated with vaccine production underscores the need for novel virus intervention strategies. Short interfering (si)RNAs have been administered to protect from viral infection both *in vitro, in vivo* and *in ovo*. siRNAs targeting conserved regions of the influenza PB1 and PA gene segments inhibit low pathogenic (PR8) influenza growth in embryonated chicken eggs (Ge et al., 2003), while similar siRNAs inhibited HPAI in a mouse model of infection (Tompkins et al., 2004). Furthermore, several groups have demonstrated that the immunostimulatory properties of siRNAs can be exploited to enhance antiviral properties of siRNAs targeting viral genes. After differences in serum IFN-α levels were observed in mice 6 h post-intravenous administration with siRNAs with distinct sequences (but with identical siRNA dosage and synthesis methods), systematic alteration of nucleotide sequences demonstrated that siRNAs containing specific motifs rich in UG content were particularly adept at inducing pro-inflammatory cytokines in human PBMCs, including IFN-α, IL-6 and TNF (Judge et al., 2005). Numerous groups, took advantage of this to rationally design immunostimulatory antiviral siRNAs for use in the chicken. Labelling the siRNA targeting influenza PB1, PB1-2257, with a 5 bp 5′-UGUGU-3′ motif, selectively at the 5′ end, improved the inhibition of H5N1 influenza virus growth in chicken macrophages (Stewart et al., 2011). Interestingly, a 21 base pair (bp) siRNA duplex consisting entirely of poly-UG induces type I IFNs in chicken splenocytes more effectively than siRNAs lacking poly-UG repeats (Villanueva et al., 2011).

RNAi therapies offer several appealing features as antiviral therapies. Therapies targeting conserved viral genomes would be expected to be efficacious in both humans and animals against a wide distribution of virus sub-types. In anticipation of a virus pandemic, siRNAs could be stockpiled for ready use. However, in the context of managing viral disease outbreaks in poultry, several hurdles exist, including cost, successful delivery of RNAi *in vivo*

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