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Synergistic effect of co-stimulation of membrane and endosomal TLRs on chicken innate immune responses

Sungwon Kim, Pete Kaiser[1], Dominika Borowska, Lonneke Vervelde*

The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, EH25 9RG, UK

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ABSTRACT

Toll-like receptor (TLR) ligands (TLR-Ls) are critical activators of immunity and are successfully being developed as vaccine adjuvants in both mammals and birds. In this study, we investigated the synergistic effect of co-stimulation of membrane and endosomal TLRs on the innate immune responses using chicken bone marrow-derived macrophages (BMMs), and studied the effect of age on the induction of innate responses. BMMs from 1 and 4-week-old birds were stimulated with Pam3Cys-SK4 (PCSK; TLR2), synthetic monophosphoryl lipid A (MPLA), Di[3-deoxy-D-manno-octulosonyl]-lipid A ammonium salt (KLA; TLR4), Gardiquimod, Resiquimod (R848; TLR7), CpG class B and C (TLR21). Nitric oxide (NO) production and mRNA levels of IL-1β, IL-10 and IL-12p40 showed macrophages from 4-week-old birds showed more sensitive responses compared to 1-week-old birds. The most potent TLR-Ls, PCSK, MPLA and CpG B were used to study the effect of co-stimulation on macrophages. Co-stimulation with TLR21 and TLR4 synergistically up-regulated inflammatory-related genes, as well as NO production. However, incubation of splenocytes with PCSK, MPLA and CpG B did not induce cell proliferation. Moreover, treatment with CpG B led to significant cell death.

1. Introduction

Toll-like receptor ligands (TLR-Ls) are potent immunostimulants with profound effects on the generation of adaptive immune responses. These properties are being exploited in TLR-based vaccines and therapeutic agents in mammalian species and chickens (St Paul et al., 2013; Steinhagen et al., 2011; Wilson-Welder et al., 2009). In the poultry industry, most vaccinations are achieved either *in ovo* or within 1 week post-hatch. The immune function of chickens is limited in the first 2 weeks post-hatch (Bar-Shira et al., 2003; Mast and Goddeeris, 1999; Reemers et al., 2010b), but adjuvants and the use of potent antigen-delivery systems may counteract age-related defects in immune responses to vaccination.

Multiple studies have shown that TLR-Ls increase vaccine efficacy in chickens. Stimulation of TLRs with TLR-Ls leads to the activation of different transcription factors (nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), interferon regulatory factor 3 (IRF3)), resulting in the production of cytokines and chemokines that enhance proliferation, and increase the capacity of innate immune cells to present antigen. Administering the TLR2 ligand Pam3Cys-SK4 (PCSK) as a vaccine adjuvant significantly enhanced antibody-mediated immune responses by up-regulating antigen-specific IgY (Erhard et al., 2000). Additionally, PCSK up-regulates the Th1 associated cytokines IFN-γ and IL-12, along with the Th2 associated cytokine IL-4 (St Paul et al., 2012). Administration of TLR4 ligands via the respiratory routes or *in ovo* (Barjesteh et al., 2015) increased protective responses to avian respiratory virus infections, through increased type I IFNs (Parvizi et al., 2014) or higher antibody titres (Tseng et al., 2009). Cytosine-phosphate-guanosine (CpG) oligodeoxynucleotides (CpG ODN) has been the most widely used TLR-L in chickens to enhance the immunogenicity of vaccines against bacterial (Gomis et al., 2003; Taghavi et al., 2008), protozoal (Dalloul et al., 2005) and viral (Dar et al., 2014; Singh et al., 2015) infections. Like LPS, CpG ODN is a strong inducer of nitric oxide (NO) production and type I and II IFNs (Barjesteh et al., 2014), but the type and dose of CpG ODN used has an effect on the immune responses (Reemers et al., 2010a; Singh et al., 2015). Synergistic effects of co-stimulation of TLRs on chicken immune responses have been reported using TLR3 and TLR21, leading to enhanced NO production (He et al., 2007), and up-regulation of IFN-γ and IL-10 (He et al., 2012) in PBMC compared to treatment with poly I:C and CpG

Abbreviations: TLR, toll like receptor; TLR-Ls, toll-like receptor ligands; BMMs, bone marrow-derived macrophages; PCSK, pam3Cys-SK4; MPLA, synthetic monophosphoryl lipid A; KLA, Di[3-deoxy-D-manno-octulosonyl]-lipid A ammonium salt; R848, resiquimod; CpG, cytosine-phosphate-guanosine; CpG ODN, CpG oligodeoxynucleotides; CpG B, class B CpG ODN; CpG C, class C CpG ODN

* Corresponding author.
E-mail address: Lonneke.Vervelde@roslin.ed.ac.uk (L. Vervelde).
[1] Deceased

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Table 1
Primer sequences for qRT-PCR analyses of cytokine transcripts.

Gene	Forward primer	Reverse primer	Reference/Accession No.
28S	GGCGAAGCCAGAGGAAACT	GACGACCGATTTGCACGTC	(Kaiser et al. (2000)
IL-1β	CAGCAGCCTCAGCGAAGAG	CTGTGGTGTGCTCAGAATCCA	NM_204524
IL-10	GAGTTTAAGGGGACCTTTGGCT	ATGACTGGTGCTGGTCTGCA	NM_001004414
IL-12p40	TGGGCAAATGATACGGTCAA	CAGAGTAGTTCTTTGCCTCACATTTT	NM_213571

alone.

In this study, we investigated the synergistic effect of co-stimulation of membrane and endosomal TLRs on the innate immune responses using chicken bone marrow-derived macrophages (BMMs). We examined the innate immune responses to various TLR-Ls in BMMs derived from two different ages: 1-week-old or 4-week-old birds to determine the effect of age on the induction of innate responses. The most potent TLR-Ls, class B CpG (ODN (CpG B), PCSK and synthetic monophosphoryl lipid A (MPLA) were then tested for potential synergistic effects in 4-week-old birds.

2. Materials and methods

2.1. TLR ligands

The synthetic lipoprotein PCSK (EMC microcollections, Germany) for TLR2, MPLA, Di[3-deoxy-D-manno-octulosonyl]-lipid A ammonium salt (KLA; Avanti Polar Lipids, US) for TLR4 (Braun et al., 2017), CpG B (Chrząstek et al., 2014) and CpG C (Invivogen, US) for TLR21 were reconstituted with sterile PBS following manufacturer's instructions. Gardiquimod and Resiquimod (R848) (Chemdea, US) for TLR7 (Braun et al., 2017) were resuspended with DMSO (Sigma-Aldrich, US) following manufacturer's instructions.

2.2. Isolation and culture of chicken BMMs

Commercial Novogen Brown layers were hatched and housed in premises licensed under a UK Home Office Establishment License in full compliance with the Animals (Scientific Procedures) Act 1986 and the Code of Practice for Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Requests for animals were approved by the local Animal Welfare and Ethical Review Board and animals were humanely culled in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986.

BMMs from 1-week or 4-week-old birds were cultured as described previously (Kim et al., 2018). Purified cells were seeded in 24-well (Griess test) and 6-well plates (gene expression), at 0.5×10^6 cells/well and 2×10^6 cells/well respectively, and differentiated in the presence of recombinant chicken CSF1 for 6 days, followed by stimulation with TLR-Ls.

2.3. Stimulation of BMMs with TLR-Ls

To test the optimal concentration, as well as the effect of age on the immune responses, TLR-Ls were used at the following concentrations for 6 and 48 h (hr): PCSK, MPLA and KLA (0.01, 0.1, 1, 10 μg/mL), Gardiquimod, R848, CpG B and CpG C (0.05, 0.5, 5 μg/mL). Ultra-pure LPS (Kogut et al., 2005) was used as a positive control and a medium control with either PBS or DMSO was used as a control for the TLR-Ls solvents. Combinations of PCSK (0.01 and 0.1 μg/mL), MPLA (0.1 and 1 μg/mL), and CpG B (0.5 and 5 μg/mL) were used in co-stimulation and cell proliferation assays. BMMs were stimulated for 6 h to measure gene expression and for 48 h to measure produced nitrite (NO_2^-) in the media by the Griess test (Kim et al., 2009).

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the stimulated BMMs using the RNeasy Mini Spin Column (Qiagen, US) and 1 μg of RNA was reverse transcribed with iScript™ cDNA synthesis kit (Bio-Rad, US), followed by 1:5 dilution for IL-1β, IL-12p40 and IFN-γ and 1: 100 dilution for 28S ribosomal RNA. For IL-10 mRNA levels, undiluted cDNA was used as a template. To measure mRNA levels from the BMMs, 1 μL of diluted cDNA was mixed with 5 μL of SsoFast™ EvaGreen® Supermix with Low ROX (Bio-Rad) and forward/reverse primer (500 nM final concentration) (Table 1) in nuclease-free water (final volume 10 μL), followed by qRT-PCR reaction described previously (Kim et al., 2009). Target gene expression was normalised against the expression of 28S rRNA, and the relative gene expression was compared to a medium (PBS or DMSO-treated) control sample (Kaiser et al., 2000).

2.5. Stimulation and cell proliferation of splenocytes by TLR-Ls

Splenocytes from 4-week-old birds were purified as described before (Sutton et al., 2015), resuspended in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS, 2% L-glutamine, 1 U/mL penicillin and 1 μg/mL streptomycin (Gibco, US).

Splenocytes were stimulated with two concentrations of TLR2, TLR4 and TLR21 ligands, followed by measuring cell viability and cell proliferation using flow cytometric analysis. Splenocytes were seeded in a 96-well plate at 5.0×10^5 cells/well, and an equal volume of the TLR-Ls was added. ConA (5 μg/mL final concentration) was used as a positive control and medium with PBS as a negative control. After 48 h, the splenocytes were incubated with 2 mM EDTA, and harvested by centrifugation at $300 \times g$ for 5 min (Dalgaard et al., 2016). The cells were washed twice and resuspended with cold FACS buffer (0.5% BSA and 0.05% Sodium Azide (Sigma-Aldrich) in PBS) containing SYTOX Blue (Invitrogen, US). Cells were analysed by flow cytometry using a BD LSR Fortessa (BD Biosciences) and the data were analysed with FlowJo (Three Star, USA). Samples were recorded for 10,000 events in the leukocyte gate based on SSC-FSC pattern, followed by gating for single cells. Cell viability was determined by SYTOX Blue staining and the proliferation by forward light scatter (Gaines et al., 1996; MacDonald and Zaech, 1982).

2.6. Statistical analysis

All data were analysed by either the Student's *t*-test or one-way analysis of variance using program R (R Development Core Team, 2015), and significant differences between groups were considered significant by Tukey's honest significant difference test at $P < 0.05$ (confidence level = 95%). Synergistic effect was determined if the NO production and mRNA expression value from the co-stimulatory treatment was significantly higher than the combined value from both treatments with each agonist alone (He et al., 2007).

3. Results and discussion

In the poultry industry, most vaccinations are achieved either *in ovo* or within 1 week post-hatch. Since the immune function of birds is limited in the first 2 weeks post-hatch (Bar-Shira et al., 2003; Mast and