

Doctoral Thesis

Studies on the innate immune functions and effects of
vaccination on it in the chicken ovary

(Summary)

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Chicken ovary is susceptible to bacterial and viral pathogenic microorganisms that may cause ovarian functional disorder and contamination in eggs. Therefore, the immune function in the ovary to prevent infection is essential for normal functions of the ovary and hygienic egg production. Toll-like receptors (TLRs) are pattern recognition receptors which recognize the microbe-associated molecular patterns (MAMPs) to initiate the innate immune response. Then the proinflammatory cytokines such as IL-1 β and IL-6, and antimicrobial peptides such as avian β -defensins (AvBDs) play roles in preventing the invasion of pathogens. In the chicken ovary, the information on the innate immune system is limited, although the expression of TLRs, cytokines and AvBDs in response to LPS has been reported. The concept of trained immunity with epigenetic reprogramming for innate immune memory induced by vaccination is proposed in mammals. However, the possible way to enhance the innate immune functions is not available in chickens till now. Therefore, the aim of this study was to determine the innate immune response to MAMPs, and the effects of vaccination on that innate immune function in the chicken ovary. The response of cytokines and AvBDs expression to different TLR ligands, and the responsible transcription factors in that process were examined in the chicken follicular theca. Then, the effects of vaccination on TLRs, cytokines and AvBDs expression and associations of histone modifications with that were also examined in the chick ovary and in follicular theca of laying hens.

1. Effects of TLR ligands on the expression of cytokines and avian β -defensins (AvBDs) and responsible transcription factors in its process in the theca of chicken follicles

The aim of this study was to determine whether the expression of proinflammatory cytokines and AvBDs was induced by different TLR ligands, and to identify the responsible transcription factors in that process. In Experiment 1, the theca tissues were cultured with or without Pam3CSK4, poly I:C, LPS, flagellin, R837 or CpG-ODN (TLR2, 3, 4, 5, 7 and 21 ligand, respectively), and the changes in the expression of cytokines (*IL-1 β* , *IL-6*, *TNFSF15*, *CXCLi2*, *IFN- α* and *IFN- β*) and AvBDs (*1*, *2*, *4*, *7* and *12*) were examined by real-time PCR. All TLRs, namely *TLR1* (type 1 and 2), *TLR2* (type 1 and 2), *3*, *4*, *5*, *7*, *15* and *21*, were detected in the follicular theca, although the PCR products of *TLR1* (type 2) and *TLR21* were faint. Pam3CSK4 and LPS up-regulated the expression of all detected cytokines, except for *IFN- α* by LPS. Poly I:C up-regulated the expression of *IL-6*, *CXCLi2* and *IFN- β* , while CpG-ODN up-regulated only *IL-1 β* . Flagellin and R837 did not significantly affect cytokines expression. The expression of *AvBD1* was down-regulated by poly I:C, and that of *AvBD4* was down-regulated by LPS and R837, although LPS up-regulated *AvBD1* and *7* expression. In Experiment 2, the theca tissues were cultured with or without inhibitors of NF κ B (BAY 11-7085) and AP-1 (Tanshinone \square A) in the medium containing Pam3CSK4, poly I:C, LPS or CpG-ODN, and the effects of those inhibitors on the expression of cytokines and AvBDs were examined. The expression of *IL-1 β* , *IL-6*, *CXCLi2* and *IFN- β* in the tissues incubated with LPS was down-regulated by BAY 11-7085. The expression of *AvBD7* in tissues incubated with poly I:C was up-regulated by BAY 11-7085, and that of *TNFSF15* incubated with Pam3CSK4 was up-regulated by Tanshinone \square A. These results suggest that the innate immune system, including pattern recognition by TLRs, and cytokines and AvBDs synthesis, is formed in the theca, whereas, the ability of these cells to recognize bacterial patterns is more developed than that for viruses.

2. Effects of vaccination on the expression of innate immune molecules and induction of histone modification in ovarian cells of chicks

In order to prevent and control infections, vaccination is used as one of the most important strategies which can be implemented to enhance immune functions. The aim of this study was to examine the effect of vaccination on the expression of innate immune molecules including TLRs, cytokines and AvBDs in the chick ovary challenged with or without LPS. Each ovarian tissue was cut into three specimens and cultured with LPS

at the concentration of 0, 100 ng/mL and 1000 ng/mL, and then the changes in the expression of immune molecules were examined by real-time PCR. The histone modification in chick ovary with or without vaccination was also examined by western blot. The expression of *TLR1-1* and *21* was up-regulated by vaccination with or without LPS challenge. The expression of *TLR2-1* and *2-2* was up-regulated only in vaccinated group cultured without LPS, whereas that of *TLR1-2*, *4* and *7* was down-regulated only in vaccinated group with LPS challenge. *TLR3* and *15* expression was up-regulated only in vaccinated group with LPS challenge. Both the vaccination and LPS treatment had no effect on the expression of *TLR5*. Among the examined cytokines, only *TNFSF15* expression was down-regulated in vaccinated chick ovary cultured with or without LPS, and no difference in the expression of *IL-1 β* , *IL-6*, *CXCLi2*, *IFN- α* and *IFN- β* was found between the vaccine and control groups regardless the treatment with LPS. The vaccination showed a tendency to down-regulate the expression of *AvBD1*, *2*, *4* and *7*. The histone modification of H3K9me2, H3K4me2/3, H3K9ac and H3K27ac were identified by western blot. The relative protein density of H3K9me2 and H3K9ac was significantly increased by vaccination, while that of H3K4me2/3 and H3K27ac showed no changes by vaccination. These results suggested that vaccination may affect the ability to recognize microbe pattern in chick ovary. The effect of vaccination on the cytokines expression was limited, whereas it is likely that *AvBDs* expression is down-regulated. These effects of vaccination on the immune molecules expression may associate with the epigenetic reprogramming by histone methylation and acetylation in chick ovarian cells.

3. Effects of SE vaccination on the expression of innate immune molecules and the induction of epigenetics in the follicular theca of laying hens

The routine vaccination program for chicken is performed in chick phase, and the efficiency that vaccination may not continue in the older laying hens. Although the laying hens are not usually vaccinated, if the vaccination positively works in them, it may be beneficial to extend the egg-laying cycle of aged hens. The aim of this study was to examine the effectiveness of single and repeated vaccination on the innate immune function in the ovarian follicular theca of laying hens. In Experiment 1, birds were injected with *SE* vaccine or PBS at 1 week before collection of the theca of the largest follicle (F1). The effects of single *SE* vaccination on the gene expression of immune molecules (*TLRs*, cytokines and *AvBDs*) and histone modification in the theca were examined. The expression of *TLR1-1*, *2-1*, *4* and *15* was up-regulated by single *SE* vaccination. Although no significant change in cytokines expression by single *SE* vaccination was found, the expression of *AvBD1*, *2*, *4* and *7* was up-regulated significantly by that vaccination. The H3K9me2 density was higher in single *SE* vaccination group compared with control group. In Experiment 2, birds were injected with *SE* vaccine or PBS for 5 times at every 1 week, and heat inactivated *SE* or PBS were injected to them on seven days after the final injection with vaccine or PBS. The expression of immune molecules in F2 follicles and histone modifications in F4 follicles were examined. The expression of *TLR1-1*, *2-1* and *7* was up-regulated by *SE* antigen challenge only in vaccinated group, while that of *TLR15* was elevated in non-vaccinated group. Only *TNFSF15* expression was down-regulated by repeated vaccination without *SE* antigen challenge, whereas the expression of *IL-1 β* , *IL-6*, *CXCLi2* and *IFN- α* was up-regulated by *SE* antigen challenge with or without repeated *SE* vaccination. The expression of *AvBD1* and *12* was lowered by the repeated vaccination. The expression of *AvBD1*, *2*, *4* and *7* was up-regulated by *SE* antigen challenge in both repeated vaccination and control groups. The H3K27ac relative density in repeated *SE* vaccination group was up-regulated by *SE* antigen challenge. These results suggest that the single *SE* vaccination up-regulates the innate immune functions, including *TLRs* and *AvBDs* expression, in the ovary of laying hens, which associates with the histone modification profile in the ovarian cells. The repeated *SE* vaccination may not be effective for up-regulation of potential ability to express innate immune molecules, although it increases H3K27ac in the theca.

Conclusion

In conclusion, the current study demonstrated that innate immune system including the expression of microbe-associated molecular pattern receptors, proinflammatory cytokines and antimicrobial peptides is formed in the ovary of chicks and laying hens. Routine vaccination in chicks may up-regulate *TLRs* expression, whereas suppress the *AvBDs* expression in the ovary. In laying hens, single *SE* vaccination may up-regulate the expression of *TLRs* and *AvBDs*, but repeated *SE* vaccination may down-regulate some *AvBDs* expression in the theca. Histone modification may associate with modulation of those innate immune functions by vaccination. Since it was found that vaccination affects the expression of innate immune molecules, further studies are expected to develop the new vaccines and vaccine program to enhance the innate immune functions in hen ovary.