Doctoral Thesis

STUDIES ON THE ROLE OF CYTOKINES AND ANTIMICROBIAL PEPTIDES IN THE INNATE IMMUNE SYSTEM IN THE OVARY OF LAYING HENS

Mohamed Abdallah Mohamed Abdelsalam

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ABBREVIATIONS

avβD: avian β-defensin

BSA: bovine serum albumin

cDNA: complementary deoxyribonucleic acid

DAB: 3, 3-diaminobenzidine tetrahydrochloride

dNTP: deoxyribonucleotide triphosphate

EDTA: ethylenediamine tetra-acetic acid

F1: the largest follicle

F3: the third largest follicle

F5: the fifth largest follicle

H_2O_2: hydrogen peroxide

IL-1β: interleukin-1β

LPS: lipopolysaccharide

MHC: major histocompatibility complex

POF: postovulatory follicle

RNA: ribonucleic acid

RT-PCR: reverse transcriptase polymerase chain reaction

SE: *Salmonella enteritidis*

SEM: standard error of mean

TLR: toll-like receptor

v/v: volume/volume

w/v: weight/ volume

WF: white follicle

Chapter 1

General Introduction

Poultry eggs and meat are highly nutritious. Eggs are rich in proteins, vitamins, phosphorus and other minerals. The egg contains the proteins (egg white and yolk), the lipids (yolk), and all vitamins and minerals necessary for human health. Because of its importance in human food and in avian reproduction as well as its application in pharmaceuticals, cosmetics, and food industries, the egg has attracted research interests for decades. A typical chicken layer produces 280-300 eggs/year. Chicken eggs represents 92% of global production of eggs for human consumption (FAO, 2009). This indicates the importance of poultry as one of the major sources for human food. Production of safe eggs and meat is a major concern for many scientists and poultry producers. The contamination of this source of food will transfer infection to human making the human health in danger. Also eggs contamination will cause the vertical transmission of infection to the offspring that affects poultry production.

Chicken ovary and follicular growth

In the embryonic stages chicken has a pair of ovary and oviduct primordia; but by the day 10 of incubation the right ovary and oviduct start regression by the effect of Müllerian inhibiting substance (Hustson *et al.*, 1985). That is why the reproductive system of chicken has only the left ovary and oviduct (Romanoff and Romanoff, 1949; Kinsky, 1971). The chicken ovary locates on the backside at the

middle part of the body cavity and attached by the mesovarian ligament at the cephalic end of the left kidney. The immature ovary is a mass of tissue containing numerous small ova. Many of these ova reach to maturity and ovulated through the life span of the chicken. The number of ovulated eggs produced in the year varies greatly between different chicken breeds. The ovary of mature laying hen consists of an ovary stroma, a mass of large number of small follicles in the stroma (stromal follicles), and many prehierarchal (white) follicles, and several hierarchal (yellow) follicles. Commonly, there are 4-6 large yolk-filled hierarchal follicles (preovulatory follicles) which are going to be ovulated, and the biggest one will be the first to be ovulated. The size of yellow follicles ranges from approximately 5-35 mm in diameter, whereas white follicles are lesser than 5 mm in diameter (Fig.1). Ovarian follicle consists of the oocyte containing yolk and follicular wall surrounding it. The follicular wall consists of different tissue layers. These layers from inside towards the outside are (1) perivitelline layer, (2) granulosa layer, (3) basal lamina (basement membrane), (4), theca layer (interna and externa), and (5) superficial connective tissue layer and (6) superfacial epithelium (Fig.2). The theca externa is a dense fibrous connective tissue, whereas the theca externa, which is thin compared to the theca externa, is rich in cellular components including thecal interstitial cells that are steroidogenic cells. The preovulatory follicles are highly vascularized except at the stigma (the rupture point during ovulation) (Nalbandov and James, 1949). The theca interna contains well-developed blood capillaries (Dahl, 1970). Granulosa layer of the white follicle consists of closely packed cuboidal cells, which may be several cell layers. In the yellow follicles, it becomes a single squamous cell layer. There are spaces and cell junctions between granulosa cells allowing the transport of yolk from blood capillaries in the theca to the inside of the follicle. Stromal follicles undergo growth in two stages, several-month growth period that consists mainly of deposition of yolk proteins and rapid growth

phase during final 6-11 days prior to ovulation. Yolk precursors formation takes place in the liver under stimulation of estrogen, and then they are transported through blood circulation to the theca. Then, they cross the basement membrane and the granulosa layer to inside of the follicle. Follicle stimulating hormone (FSH) stimulates granulosa cell differentiation and steriodogensis in the granulosa cells of periherarchal follicles. Luteinizing hormone (LH) surge appears 4-6 h prior to ovulation in hens. LH stimulates steroidogensis in the granulosa and theca cells, and also, stimulates the germinal vesicle breakdown and ovulation. After ovulation, follicular tissues remain attached to the ovarian stroma and they are known as postovulatory follicle (POF). The POF undergoes regression and disappear within a period of 6-10 days via the process of apoptosis (Tilly *et al*., 1991).

Salmonellosis in poultry

Salmonella is one of the major causes of food-born bacterial gastroenteritis worldwide. Up to 30,000 and an estimated 1.4 million cases of human Salmonellosis are reported each year in the United Kingdom and United States, respectively (Mead *et al.,* 1999). Approximately 550 deaths annually recorded in USA because of food-borne disease with the majority of cases because of Samonellosis (Thorns, 2000). Since the mid to the late of 1980 the number of *Salmonella enteritidis* (SE) outbreaks in human increased dramatically (Hogue *et al*., 1997). In USA, the latest outbreak of human SE infections which was associated with eggs contamination occurred in 2010. According to Centers of Disease Control and Prevention (CDC) in USA, approximately 1,939 illnesses were reported that are likely to be associated with this outbreak. Contamination of poultry meat and eggs is a big challenge for poultry production and human health. Colonization of *Salmonella* in the reproductive organs of laying hens

leads to the production of contaminated eggs (De Buck *et al.*, 2004). Eggs and egg products are the major risk factor for SE infection in human (Hedberge *et al.*, 1993; St. Louis *et al.,* 1998). Meanwhile, the incidences of other salmonella serovars infections in human have declined or remain same (Cogan and Humphrey, 2003). There may be two pathways of egg contamination by SE. First, it may penetrate the eggshell after ovipoition (Gast and Beard, 1990; Barrow and Lovell 1991). Secondly, eggs may be contaminated during egg formation; SE may be transmitted from infected reproductive organs directly to the yolk, albumen, and eggshell membranes before oviposition (Timoney *et al.*, 1989; Shivaprasad *et al.,* 1990). The injection of laying hens with SE resulted in the colonization of the ovary and oviduct (Keller *et al.*, 1995; Okamura *et al.*, 2001; De Buck *et al.*, 2004). The SE has been isolated from the tissues surrounding the yolk of preovulatory follicles after oral inoculation of laying hens with SE bacteria (Thiagarajan *et al.,* 1994). Also, SE could interact with granulosa cells of preovulatory follicle and was able to invade and multiply in these cells (Thiagarajan *et al.*, 1994, 1996). After SE oral inoculation, it was able to penetrate the periviteline membrane and multiply in the yolk (Gast and Holt, 2001). These reports suggest that preovulatory follicles are the preferred sites for SE colonization in the ovary.

The immune system in birds

The immune system consists of two main branches; the adaptive and innate immune systems. Pattern of antigen recognition, specificity of receptors, and speed of immune response for infections are the main differences between the adaptive and innate immune responses. Antigen presenting cells, B and T cells are the main cells of the adaptive immune system. Major histocompatibility comples (MHC) class II are expressed in the antigen presenting cells such as macrophages, dendritic cells, and B

and T cells. Antigens are presented by MHC class II and class I to helper and cytotoxic T cells, respectively. Helper T cells stimulate phagocytosis of macrophages and antibody synthesis of B cells. Cytotoxic T cells kill the cells infected by viruses. Each T cell and B cell has a structurally unique single receptor that can recognize a particular antigen. Such diversity of receptors increases the probability that every antigen will be encountered by a lymphocyte that has a specific receptor for this antigen. Once, the antigen is presented by MHC class II to a lymphocyte, the activation and proliferation of that lymphocyte will be triggered producing a huge number of cells with the same receptor to encounter infection (clonal expansion). Clonal expansion is very important for efficient immune response. However, it takes three to five days for sufficient number of clones to be produced and differentiated into effective cells, and even for antibody production. This leaves a good chance for the pathogen to replicate in the body and destroy the host during that period. On the contrast, the innate immunity effector mechanisms are activated immediately after infection to prevent the infecting pathogen (Mackay and Rosen, 2000).

The innate immune responses are also initiated through recognition of pathogens by pattern recognition receptors. The structures recognized by the innate immune system on the surface of pathogens are known as pathogen-associated molecular patterns (PAMPs). The best-known examples are LPS, peptidoglycan, lipoteichoic acid, Bacterial DNA, double-standed RNA, and CpG DNA (Medzhitov and Janeway, 1997a).

Toll-like receptors

Toll receptor was first discovered in drosophila (Hashimoto *et al.*, 1988). Similar receptors were discovered in animals and called Toll-like receptors (TLRs) (Medzhitov and Janeway, 1997b; Rock *et al.*, 1998). TLRs are pattern recognition receptors that recognize a variety of bacterial and viral PAMPs (Anders *et al.*, 2004). Till now, ten types of TLRs have been discovered in chicken (Table 1) (Hoshino *et al.*, 1999; Iqbal *et al.*, 2005; Keestra *et al.*, 2007). The interaction of TLRs with corresponding PAMPs ligands is known to induce, through the NFκB pathway, the expression of a variety of cytokines and chemokines that are crucial to the innate and adaptive immune systems (Medzhitov *et al.*, 1997). Namely, IL-1, IL-6 and CXCli2 were upregulated in chicken heterophels by activation of TLRs using their specific agonists (Kogut *et al.*, 2005). TLRs activation may also induce the expression of antimicrobial genes (Pasare and Medzhitov, 2004). Thus, TLRs signal the presence of infection inducing the recognition of PAMPs in the innate immune system (Medzhitov and Janeway, 1997b), which in turn control the activation of the adaptive immune responses (Macky *et al.*, 2000).

Chicken cytokines and chemokines

ytokines are a group of soluble peptide mediators that are secreted by some cells and play an important role in initiation and regulation of the immune response and inflammation. They are classified according to the source of product or target cells or according to the function of cytokine. One of the most important groups of cytokines is the proinflammatory cytokines. These cytokines are responsible for induction of the innate immune responses and inflammation (Staeheli *et al.*, 2001; Ferro *et al.*, 2004; Huges *et al.*, 2007). The IL-1β, IL-6 and IL-8 (CXCLi2) are important members of this group. They are produced in response to bacterial

infection. IL-1β has pleiotropic activities including T cell proliferation, fever induction, secretion of glucocorticoids, and acute-phase protein induction. These activities may be achieved through the ability of IL-1β to induce other cytokines and chemokines (Staeheli *et al.*, 2001). IL-6 is a multifunctional cytokine able to regulate Ig production (Kishimoto and Hirano, 1988), T cell activation (Lotz *et al.*, 1988; Rincon *et al.*, 1997; Diehl *et al.*, 2000) and induction of dendritic cells differentiation (Chomarat *et al.*, 2000). Recombinant chicken IL-6 was able to induce cell proliferation of murine hybirdoma cell line (van Sinck *et al.*, 1986) and increase of serum corticosterone (Kaiser *et al.*, 2004). CXCLi2 is a member of CXC chemokines that are known to be chemoattractant for polymorphonuclear cells (Zlotnik and Yoshie, 2000).

Avian anti-microbial peptides

Antimicrobial peptides (AMPs) are small molecular weight proteins (less than 100 amino acids) with broad spectrum antimicrobial activity against bacteria, viruses and fungi (Zasloff, 2002; Izadpanah and Gallo, 2005). They are cationic molecules rich in histidine, lysine and arginine. They are also amphipathic with both hydrophobic and hydrophilic regions (Wu *et al.*, 2003; Selsted and Ouellette, 2005). Defensins are the members of antimicrobial peptides that have been isolated from vertebrates. They are classified into three sub-families, α-defensins, β-defensins, and θ-defensins. Only β defensins have been found in birds, and they are called avian β-defensins (avβD) (Semple *et al.*, 2003). They are considered essential parts of innate immunity in many animal species. β-defensins, the biggest subset of antimicrobial peptides, are cysteine-rich peptides with small molecular weight with hydrophobic and cationic residues (Selsted and Quellette, 2005). β-defensins have not only antimicrobial activity but also immnomodulatory properties. β-defensens can promote

the adaptive immune system by recruitment of adaptive immune cell by chemotaxis of monocytes (Territo *et al.,* 1989), T lymphocytes (Chertov *et al.,* 1996), dendritic cells (Yang *et al.,* 1999) and mast cells (Niyonsaba *et al.,* 2002) to site of inflammation. Also, macrophages phagocytosis can be enhanced by defensins (Ichinose *et al.*, 1996).

Till now, 14 types of avβDs have been discovered by different groups in chicken, namely avβD1 (Evans *et al.*, 1994; Harwing *et al.*, 1994), avβD2 (Harwing *et al.,* 1994), avβD3 (Zhao *et al.,* 2001), avβD4, 5, 6, 7, 8, 9, 10, and 12 (Lynn *et al.*, 2004; Xiao *et al.*, 2004), avβ8 and 13 (Xaio *et al.*, 2004; Higgs *et al.,* 2005), avβD11 (Xiao *et al.,* 2004) and avβD14 (Lynn *et al.,* 2007). The 14 avβD genes are located in ~ 86.0 kb single β-defensin cluster on chromosome *3q3.5- q3.7* (Xiao *et al.,* 2004; Lynn *et al.,* 2007) (Table 2).

avβDs have been isolated from blood cells of chicken, turkeys and ostrich heterophils (Harwig *et al.,* 1994; Evans *et al.,* 1995; Sugiarto and Yu, 2006), and also they are found to be expressed in the epithelial tissues (Zhao *et al.,* 2001). The expression of avβDs showed some of tissue specificity. avβD1 and 2 were isolated from peripheral leukocytes (Harwig *et al.,* 1994). In the bone marrow, avβD4-7 were strongly expressed but they showed weak or no expression in the heterophils (Lynn *et al.*, 2004; Xiao *et al.*, 2004). avβD1 and 2 has a moderate to strong level of expression in the lung tissue (Zhao *et al.*, 2001; Lynn *et al.*, 2004). avβD3 and avβD9 was strongly expressed in the respiratory tract (Zhao *et al.,* 2001; van Dijk *et al.*, 2007). Mean while, most of the other avβDs are weakly or moderately expressed in these tissues (Harwig *et al.,* 1994; Zhao *et al.,* 2001; Lynn *et al.*, 2004; Xiao *et al.*, 2004). avβDs shows different expression patterns in different tissues, namely they are expressed not only in the blood cells and respiratory tract but also in the skin and digestive tract (Xaio *et al.*, 2004; van Dijk *et al.*, 2007). Many of avβDs genes are also expressed in the male

reproductive tract (Yamamoto and Matsui, 2002; Sang *et al.*, 2006; Das *et al.*, 2011) and female reproductive tract (Aono *et al.*, 2006; Subedi *et al.*, 2007b). Comparing the data published by different research groups, avβDs expression level could be sometimes variable in the same tissue. It may be possible that some differences in the expression profiles occur depending on differences in the used breeds, animal age and immune status (van Dijk *et al.*, 2008).

Few reports studied the antimicrobial activity of avβDs. avβD1 that was isolated from chicken and turkey heterophils showed a bactericidal and fungicidal activity against avian pathogens (Evans and Harmon., 1995). Recombinant chicken avβD9 showed a strong microbicidal activity against Gram negative bacteria, Gram positive bacteria and yeast but not E. coli or S. Typhimurium (van Dijk *et al.*, 2007). The actual mechanisms by which β-defensins kill microorganisms are not fully understood. However, many researches believe that cationic peptides of β-defensins are able to interact with the negatively charged bacterial cell membrane (Hancock, 1997). After that, the negative electrostatic charge may pull β-defensins molecules towards the membrane to allow penetrate it. The β-defensins form dimmers with the hydrophobic sites of the peptide facing the inner bacterial membrane. The formed dimmers create channels in the cell membrane. These channels change the bacterial cell membrane permeability and disrupt the membrane in detergent-like way (Evans and Harmon, 1995; Oren and Shai, 1998; Powers and Hancock, 2003).

Goal of study

The innate immune system is able to respond directly in a short time for attacking pathogens and protect the host from destruction before the adaptive

immune system, which takes longer time for eliciting enough immune response is activated. The understanding of the local immune system in the chicken ovary is necessary to ensure and strengthen the defense function against pathogen, leading to suppress infection in this organ and eggs.

The goal of this study was to determine the innate immune function mediated by avβDs in chicken ovary. Specifically, it was examined whether avβDs proteins were expressed in the ovary, and then whether Lipopolysaccharide (LPS), or cytokines induced by LPS enhanced the expression of avβDs. It was also examined whether LPS enhanced T cell influx in association with the changes in cytokine expression to know the presence of linkage between innate and adaptive immunity in the ovary.

In Chapter 2, changes in the localization of immunoreactive avβD8, 10, and 12 with follicular growth were examined. In Chapters 3 and 4, it was examined whether the avβDs expression in the follicles was affected directly by LPS or indirectly by cytokines induced by LPS. Experiments in Chapter 3 confirmed that LPS upregulated the expression of cytokines (IL-1β and IL-6) and chemokine (CXCLi2) in the preovulatory follicles. The increase of T cell influx in the follicles in association with those cytokines was also confirmed in this experiment. The Chapter 4 examined the effects of LPS or cytokine (IL-1β) on the expression of avβDs in the cultured theca tissue. This experiment may show the direct effect of LPS or indirect effect of LPS mediated by cytokines on the avβDs expression in the follicles. Finally, Chapter 5 describes the general discussion on the immune function mediated by avβDs and the mechanism by which the avβD expression was regulated in the chicken ovarian follicles.

Table 1. Chicken Toll-like receptors

Name	References	Function
TLR1 type1	Keestra *et al.,* 2007	Lipoproteins and peptidoglycans
TLR1 type2	Keestra *et al.,* 2007	Lipoproteins and peptidoglycans
TLR2 type1	Keestra *et al.,* 2007	Lipoproteins and peptidoglycans
TLR2 type2	Keestra *et al.,* 2007	Lipoproteins and peptidoglycans
TLR3	Iqbal *et al.,* 2005	Double stranded RNA
TLR4	Hoshino *et al.,* 1999	LPS
TLR5	Iqbal *et al.,* 2005	Bacterial flagellin
TLR7	Iqbal *et al.*, 2005	Single stranded RNA
TLR15	-	-
TLR21	Keestra *et al.,* 2010	CpG DNA

Table 2. Avain beta-defensins

Name	Genbank accession number	References
avβ1	AAB30584	Evans *et al.*,1994; Harwig *et al.*,1994
avβD2	AAB30585	Harwig *et al.*,1994
avβD3	Q9DG58	Zhao *et al.,* 2001
avβD4	AAS99318	Lynn *et al.,* 2004; Xaio *et al.*, 2004
avβD5	AAS99320	Lynn *et al.*, 2004; Xaio *et al.,* 2004
avβD6	AAS99315	Lynn *et al.,* 2004; Xaio *et al.*, 2004
avβD7	AAS99316	Lynn *et al.,* 2004; Xaio *et al.,* 2004
avβD8	AAU07922	Higgs *et al.*, 2005; Xaio *et al.,* 2004
avβD9	AAS99317	Lynn *et al.,* 2004; Xaio *et al.,* 2004
avβD10	AAS99319	Lynn *et al.,* 2004; Xaio *et al.,* 2004
avβD11	AAT45551	Xiao *et al.,* 2004
avβD12	AAS99321	Lynn *et al.,* 2004; Xaio *et al.,* 2004
avβD13	AAT48937	Xaio *et al.,* 2004
avβD14	AM402954	Lynn *et al.,* 2007

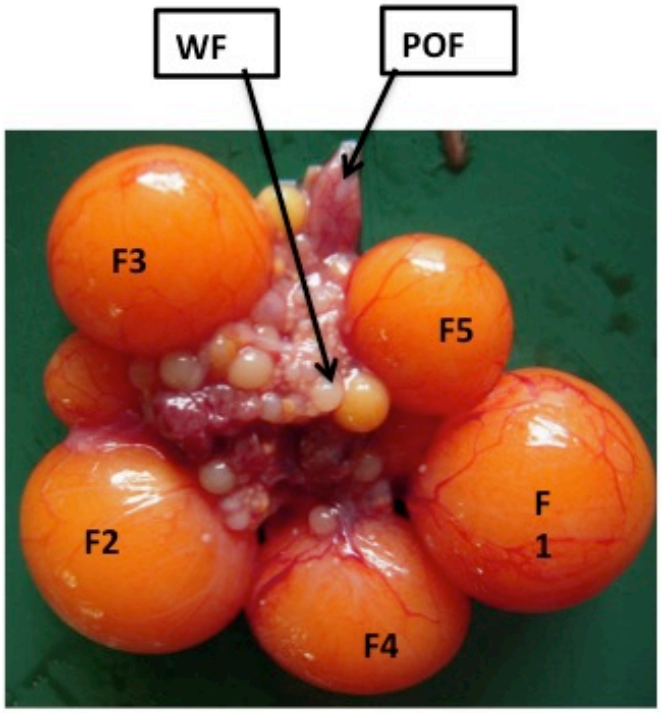

Fig. 1. Chicken ovary.
F1-F5: the largest to fifth largest preovulatory yellow follicles; WF: small white follicle; POF: postovulatory follicle.

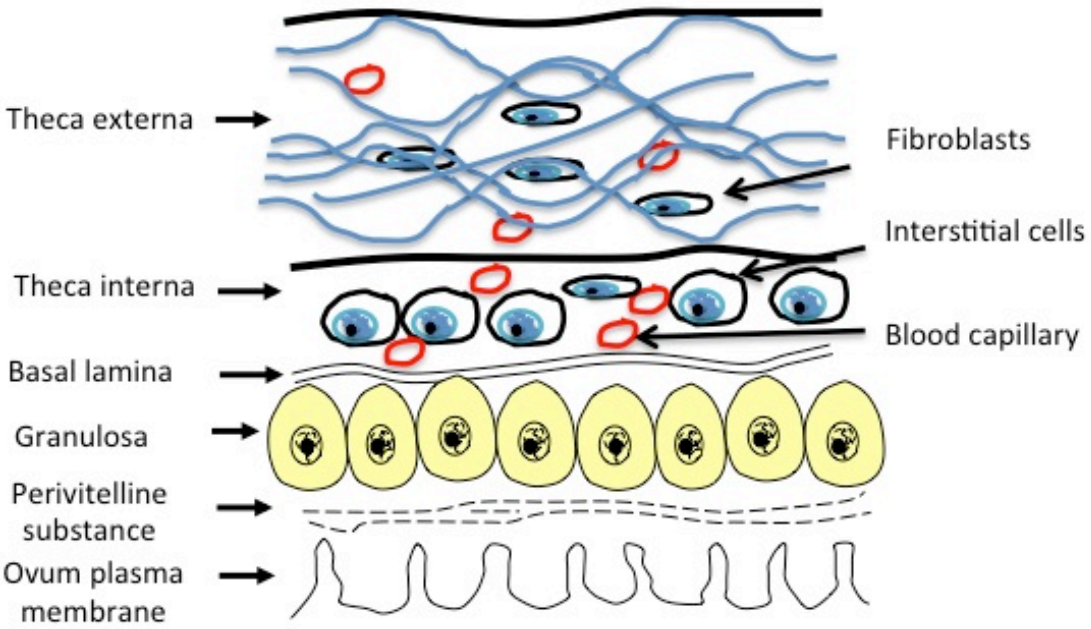

Fig. 2. Daigram of the vertical section of the preovulatory yellow follicle structure.
theca layer is differentiated into wide theca externa and narrow theca interna. Granulosa layer is a single cell layer .

Chapter 2

Changes in the Localization of Immunoreactive Avian Beta-Defensin-8, -10 and -12 in Hen Ovarian Follicles during Follicular Growth

Introduction

The hen ovary is often infected by pathogenic microorganisms such as *Salmonella* bacteria, and its infection may cause not only the ovarian functional disorder but also contamination of eggs due to bacterial transmission to the yolk (Gantois *et al.,* 2009; Neubauer *et al.,* 2009). Thus, ovarian immunity to protect the tissue from infection is essential to maintain the normal functions of the ovary and the production of hygienic eggs safe for human consumption. The ovary consists of stromal follicles embedded in the ovarian stroma, prehierarchal and hierarchal follicles. The follicles undergo changes in the tissue structures, responsiveness to gonadotropins, steroidogenic activities and yolk absorption ability during their growth (Johnson, 2000). The existence of immunocompetent cells involved in adaptive immunity such as antigen presenting cells expressing major histocompatibility complex class I and II (Subedi and Yoshimura, 2005a), $CD4^+$ and $CD8^+$ T cells (Barua and Yoshimura, 1999a; Subedi and Yoshimura, 2005b), and B cells (Barua *et al.,* 1998a) have been identified in the growing follicles. In contrast, reports on the innate immune functions in the follicles are relatively limited. Toll-like receptors (TLRs) are one of the receptors able to recognize microbial agents in the innate

immune system, identifiable by the type of conserved pathogen-associated molecular patterns in a variety of animals (Roach *et al.*, 2005). Subedi *et al.* (2007a) identified the mRNA expression of TLRs has been identified in the ovarian tissue. TLRs recognize the pattern of microorganisms and induce the synthesis of antimicrobial peptide and cytokines in the theca and granulosa layers of follicles.

Beta-defensins are antimicrobial peptides that play a significant role in the innate immune response in a variety of animals including avians (Evan *et al.*, 1994; Harwing *et al.,* 1994; Fogaça *et al.*, 2004; Lynn *et al.*, 2004; Xiao *et al.*, 2004). Some avian beta-defensisns (avβDs) that have been studied for their antimicrobial activities displayed a wide range of antimicrobial or microbistatic activities against Gram-positive and Gram-negative bacteria and fungi (Lehrer and Ganz, 1999; Higgs *et al.*, 2007; Ma *et al.*, 2008; van Dijk *et al.*, 2008; Soman *et al.*, 2009). The avβDs may kill the microorganisms by disrupting their membranes (van Dijk *et al.,* 2008). Until now, the sequences of fourteen different *avβDs* genes have been reported (Lynn *et al.*, 2007; van Dijk *et al.*, 2008). These *avβD* genes are located in relatively close positions, namely approximately 86 Kb single *avβD* cluster on chromosome *3q3.5 – q3.7* (Lynn *et al.*, 2007; van Dijk *et al.,* 2008). Moreover, Subedi *et al.* (2007b) identified the gene expression of 6 types of *avβDs* in the theca and 4 types of *avβDs* in the granulosa layer of white and yellow follicles. The *avβD-1, -8, -10* and *-12* were expressed in both layers. Intravenous injection of birds with LPS caused an increase in the expression of *avβD-1, -8* and *-12* in the theca and a decrease in *avβD-1* and *-12* expressions in the granulosa layer. Subedi *et al.* (2008) identified the immunoreactive (ir) avβD-12 in the growing follicles. The ir- avβD-12 was negligible in the white follicles, whereas it was identified in the theca and granulosa layers of yellow follicles. Although the gene

expression profiles of *avβDs* in the follicles have been shown, the localization of their proteins remains to be studied except for *avβD*-12.

Thus, the aim of this study was to determine the presence of avβD proteins in the follicles and the changes in their localization during follicular growth. The avβD-8, -10 and -12, whose gene expression had been identified in both the theca and granulosa layers, were examined. Specific questions were addressed as follows: (1) which follicular cells express these avβDs, (2) whether specific follicular cells express different types of avβDs, and (3) whether the localization of avβDs changes with follicular growth, using stromal follicles, white follicles and the three largest hierarchal follicles (yellow follicles). Although the localization of ir-avβD-12 in prehierarchal and hierarchal follicles has been reported (Subedi *et al.*, 2008), it was further examined for comparison with the localization of avβD-8 and -10.

Materials and Methods

Experimental animals

White Leghorn hens (approximately 400-d-old) laying 5 or more eggs in a sequence were kept in individual cages under a 14 h light: 10 h dark regime. They were provided with free access to feed and water. Handling of the hens was conducted in accordance with the regulations of Hiroshima University for animal experiments.

Preparation of rabbit anti- avβD-8, -10 and -12 polyclonal antibodies

Antibodies to avβD-8 and -10 were raised in rabbits using synthetic peptides conjugated with keyhole limpet haemocyanin (KLH), whereas the antibody to avβD-12 had been prepared in the previous study (Subedi *et al.*, 2008). Amino acid sequences of synthetic peptides of avβD-8, avβD-10 and avβD-12 used for immunization were as follows: avβD-8 (NNEAQCEQAGGI), avβD-10 (DTVACRTQGNF) and avβD-12 (GPDSCNHDRGLCRVGNCNPGEYLA-KYCFEPVILCCKP). The sequences of those peptides corresponded to their specific sequence (Xiao *et al.*, 2004). The anti- avβD-12 antibody in the antiserum was purified using HiTrap affinity column conjugated with synthetic avβD-12 peptide (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's directions. The antibodies of avβD-8 and -10 in the antiserum and normal IgG in the preimmune rabbit serum (for control staining) were purified using Hi Trap affinity Protein G HP column (GE Healthcare Bio-Sciences).

Immunocytochemistry

The ovarian stroma containing stromal follicles, white follicles approximately 7 mm in diameter, the third largest (F3), the second largest (F2) and the largest follicles (F1) were collected 6 h after oviposition (n=4). They were fixed in Bouin fixative, dehydrated with graded ascending series of alcohols, cleared with xylene and embedded in paraffin. Sections (4 μm in thickness) were air-dried in MAS coated pre-cleaned slides (Matsunami Glass Inc., Osaka, Japan). Then, the sections were deparafinized with xylene and rehydrated. Antigen retrieval was performed by autoclaving the sections for 1 min in 0.1M citric acid, pH 6.0. The immunohistochemistry was performed using Vecta Stain ABC kit (Vector Laboratories, Burlingame, CA, USA). The slides were incubated with blocking

solution [1.5 % (v/v) normal goat serum in PBS] for 1 h at room temperature. Sections were incubated overnight with rabbit antibodies to avβD-8, -10 or -12 diluted at a concentration of 20 μg/mL, followed by washing with PBS (3 × 5 min). The sections were then incubated with biotinylated anti-rabbit IgG and avidin-biotin-peroxidase complex for 1 h each, and were washed with PBS (3 x 5 min) after each step. Immunoprecipitates were visualized by incubating the sections with 0.02% (w/v) 3, 3,-diaminobenzidine tetrahydrochloride and 0.005% (v/v) H_2O_2 in 0.05 M Tris-HCl (pH 7.6). The sections were counterstained with hematoxylin and dehydrated with graded series of alcohols and mounted. They were examined under a light microscope (Nikon Eclipse E, Tokyo, Japan) using a Nomarsky filter. Control staining was carried out simultaneously in which the first antibody was replaced with normal rabbit IgG. Simultaneously, other sections were also stained with haematoxylin and eosin for the observation of the general tissue structure.

SDS-PAGE and Western blotting for avβDs in the follicular tissue

Sample preparation

The F1, F2 and F3 follicles were collected 6 h after oviposition. The surface connective tissue on the theca was removed. The theca was separated from the granulosa layer in PBS and the granulosa layer was washed in PBS to remove the yolk. The tissues of the three follicles were pooled and used as one sample. Each tissue was homogenized in 5 times volume of homogenization buffer consisting of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 0.1% (w/v) SDS and 1 mM phenylmethylsulfonylfluoride using a polytron

homogenizer (Kinematica AG, Switzerland). The samples were centrifuged at 12,000 X g for 20 min. The supernatant was collected and the protein concentration was measured using protein assay reagent (Bio-Rad Lab, Hercules, CA, USA) as described by the manufacturer.

Tricine-SDS-PAGE

The samples were separated by Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Tricine SDS-PAGE; 16% separating gel and 4% stacking gel) as described by Schägger (2006) with minor modifications. Samples containing 10 μg protein were mixed with sample buffer composed of 30% (v/v) glycerol, 5% (v/v) mercaptoethanol, 4% (w/v) SDS, 0.06% (w/v) bromophenol blue and 150 mM Tris-HCl, pH 7.0, and boiled for 10 min. Each 15 μl of sample mixture was loaded onto gels and run at 50 V in the stacking gel and at 150 V in the separating gel.

Western blotting

After SDS-PAGE, the proteins in the gel were electrophoretically transferred onto a nitrocellulose membrane (PALL Gelman Laboratory, Ann Arbor, MI, USA). The membrane was washed briefly with western buffer [0.02 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.5% (v/v) Tween 20, and 0.05% (w/v) BSA] and incubated with 5% (w/v) casein milk (Roche, Mannheim, Germany) solution in western buffer for 60 min. The membrane was then incubated with avβD-8, -10 or -12 antibodies diluted at a concentration of 10 μg/ml in Can Get Signal immunoreaction enhancer solution 1 for primary antibody (Toyobo Co., Ltd., Osaka, Japan) containing 1% (w/v) casein milk

for 1 h at room temperature. The membrane was then washed in western buffer for 30 min (10 min X 3) before incubation with alkaline phosphatase conjugated goat anti-rabbit IgG (Assay Designs, Inc., Ann Arbor, MI, USA) diluted at 1:5,000 in Can Get Signal immunoreaction enhancer solution 2 for second antibody (Toyobo Co., Ltd.) for 1 h at room temperature. The membrane was washed with western buffer for 30 min (10 min X 3 times) and the immunoprecipitates on the membrane were visualized by incubating in a reaction mixture composed of 0.017% (w/v) 5-bromo-4-chloro-3-indolyl phosphate disodium salt (Sigma-Aldrich, Inc., St. Louis, MO, USA) and 0.45% (w/v) nitro blue tetrazolium (Nacalai Tesque, Inc. Kyoto, Japan) in substrate buffer (0.1 M Tris-HCl, pH 9.5, 0.1M NaCl and 0.5M $MgCl_2$). The membrane was finally washed in running water to stop the reaction. For the control staining, the first antibodies were replaced with normal rabbit IgG. The results were confirmed by three different trials.

Results

Figure 3 shows the structures of stromal, small white and yellow follicles. The stromal follicles consisted of a granulosa layer that is single cell layered and a thin theca layer containing the interstitial cell islets (Fig. 3a). In the white follicles, the granulosa layer had several cell layers, and the theca layer was differentiated into the theca externa and interna where theca interna cells and blood capillaries were observed (Fig. 3b). The yellow follicles (F1-F3) showed more developed features than the white follicles; namely, the granulosa layer consisted of a single cell layer and the

thickness of theca externa and interna increased. In the theca interna, the theca interna cells were located near the outer border of this layer (Fig. 3c).

In the granulosa layer, immunolabeling of avβD-8 was identified in the cytoplasm of granulosa cells facing to the theca layer in stromal follicles, whereas their density was faint in the white follicles (Fig. 4a, b). Dense immunolabeling occurred in the whole cytoplasm of granulosa cells in the yellow follicles, namely F3 – F1 (Fig. 4c, d). The same immunolabeling pattern as avβD-8 was observed for avβD-10 (Fig. 5) and -12 (Fig. 6) in the stromal, white and yellow follicles.

In the theca layer, immunoreaction products of avβD-8 were negligible in the stromal follicles (Fig. 4a), whereas the thecal interstitial cells were faintly immunostained in white follicles (Fig. 4b). In the yellow follicles, the theca interna cells near the outer boarder of the theca interna showed immunolabeling for avβD-8 (Fig. 4c, d). The immunolabeling of avβD-10 (Fig. 5) and -12 (Fig. 6) in the theca layer showed the same profile as avβD-8, except that some immunoreaction products of avβD-12 were identified within interstitial cells in the stromal follicles.

Immunoreaction products were not observed in the control sections examined using normal rabbit IgG in place of the first antibodies (Fig. 7).

Table 3 summarizes the results of immunostaining of avβDs in the follicles. The immunostaining pattern was the same in avβD -8, -10 and -12. The density of immunoreaction products in the granulosa layer was initially reduced in white follicles compared with stromal follicles, but then increased in yellow follicles. The immunoreaction products in the thecal cells increased with follicular growth with their first appearance in the white follicles (avβD-8 and -10) and stromal follicle (avβD-12).

Western blot analysis showed a single immunoreactive band for each of avβD-8 (21 KDa), -10 (11 KDa) and -12 (28 KDa) in both the granulosa and theca layers of yellow follicles (Fig. 8).

Discussion

The results of this chapter reported that ir- avβD-8, -10 and -12 proteins were localized in the theca and granulosa layers of hen ovarian follicles. Significant findings were (1) the three types of ir-avβDs appeared in the interstitial cells or theca interna cells as well as granulosa cells, (2) hierarchal yellow follicles contained more amount of ir-avβDs than stromal and white follicles, and (3) Western blot analysis showed a single band for each avβD in both granulosa and theca layers. It is suggested that each antibody to avβD-8, -10 and -12 specifically recognized the antigens because Western blot analysis revealed single bands for each avβD but control staining with normal rabbit IgG (negative control) did not show bands. The sizes of the bands appeared in Western blot were 21, 11 and 28 KDa for avβD-8, -10 and -12, respectively. In contrast, the calculated molecular weights based on the amino acid sequences (total of signal peptide, propiece and mature peptide) reported by Xiao *et al.* (2004) are 7.431, 7.166 and 7.205 KDa. Although the exact reason why the bands of avβDs appeared at larger sizes than expected sizes is not known, it is assumed that the molecules might have formed some complexes with other residues. Yudin *et al.* (2005) reported that carbohydrates residues were added to the defensin molecules to enhance their activity. The molecular weight of chicken oviductal avβD-12 was 34 KDa in a recent study (Abdel Mageed *et al.*, 2009).

It is reported that the theca layer expressed mRNAs of *avβD-1, -2, -7, -8, -10* and *-12*, whereas granulosa layers expressed those of *avβD-1, -8, -10* and *-12* (Subedi *et al.*, 2007b). The current study using Western blot and immunohistochemical analysis showed the presence of avβD-8, -10 and -12 in both layers. The expression of ir-avβD-12 in the theca interna and granulosa layers has been also reported by Subedi *et al.* (2007a). These results suggest that the theca and granulosa layers express not only mRNA but also the protein products of *the avβD-8, -10 and -12* genes.

The ir-avβD-8, -10 and -12 were observed in the cells near the outer border of the theca interna and the granulosa cells in the hierarchal yellow follicles. They were also localized in the interstitial cells and granulosa cells in the white follicles. Thus, it is likely that the cells synthesizing them are common among the three different avβDs. Secretion of different types of avβDs in a tissue may enable to kill a wider spectrum of microorganisms if the antimicrobial activities differ among the different types of avβDs. It is reported that the innate immune system via avβDs responds differently to *Salmonella* and *Campylobacter* infection in chickens; namely avβD-3, -10 and -12 were significantly increased in response to *Salmonella*, whereas avβD-3, -4, -8, -13 and -14 were reduced by *Campylobacter* (Meade *et al.*, 2009).

Circulating microorganisms may infiltrate and colonize first in the theca interna because capillary beds are well developed there (Perry *et al.*, 1978). Then, the invasive microorganisms may migrate into the theca externa and granulosa layers (Gantois *et al.*, 2009). SE experimentally injected in birds was identified in the theca interna and granulosa layers (Takata *et al.*, 2003). *In vitro* experiments also showed *Salmonella* bacteria attached to the surface of the granulosa cells followed by cytoplasm invasion (Thiagarajan *et al.*, 1996). The avβDs in theca interna and granulosa layers may play roles in host defense against the pathogens migrating into these tissues.

The follicular tissues undergo marked changes not only in structures, cell proliferation, yolk uptake and endocrine functions (Johnson, 2000), but also in immune functions during the recruitment process from prehierarchal to hierarchal phase. The granulosa cells show a high proliferative activity before entering the hierarchal stage (Yoshimura *et al.*, 1996). Compared with the prehierarchal follicles, the hierarchal yellow follicles receive more blood flow (Scanes *et al.*, 1982), suggesting that more circulating antigens may migrate into the larger follicles. More macrophages and more developed phagocytotic activity of the theca interna cells were observed in the hierarchal yellow follicles (Barua *et al.*, 1998b; Yoshimura and Okamoto, 1998). The current study showed that the density of ir-avβDs in the interstitial cells of the stromal follicles and white follicles was negligible or faint, whereas that in the theca interna cells of the yellow follicles were high. Their density in the granulosa cells was higher in the yellow follicles than the stromal and white follicles. These results suggest that host defense system mediated by avβDs may develop with the follicular growth in association with immunocompetent cell members including macrophages. Such development of the host immunity in larger follicles may be necessary to protect the tissues from infection because opportunities of influx of the circulating pathogens may be increased with follicular growth. It is reported that the smaller follicles are more susceptible to *Salmonella* invasion than large yellow follicles (Howard *et al.*, 2005).

In conclusion, the current results suggest that avβD-8, -10 and -12 proteins are expressed in specific cells in the follicles, namely interstitial or theca interna cells and granulosa cells. Their amounts in the cells are likely to increase during follicular growth from prehierarchal to hierarchal phase. These avβDs may play significant roles in the host innate immune system in the follicles.

Abstract

Avian beta-defensins (avβDs) play significant roles in the innate immune system. The aim of this study was to identify immunoreactive (ir) avβDs proteins in the hen ovarian follicles and the changes in their localization with follicular growth. The ovarian follicles at different growth stages, namely the largest (F1), second and third largest (F2 and F3), prehierarchal (white) and stromal follicles, were collected. The presence of ir-avβD-8, -10, and -12 were examined by immunohistochemistry and Western blot. The three ir-avβDs showed a similar pattern of immunostainings in the follicular tissues at different growth stages. In the granulosa cells, the ir-avβDs were identified in the stromal follicles, whereas their density was reduced in white follicles. The granulosa cells of yellow follicles (F3-F1) showed dense immunolabelings. The interstitial cells showed a faint immunolabeling for avβD-12 but not for avβD-8 and -10 in the stromal follicles, whereas they were weakly stained in the white follicles. Dense immunoreaction products were noticed in the theca interna cells of F3 - F1 follicles. Western blot analysis showed a single band for each defensin. These results suggest that avβD-8, -10 and -12 proteins are expressed in the specific cells in the follicles, namely interstitial or theca interna cells and granulosa cells, where their amounts are likely increased with follicular growth. These avβDs may play significant roles in the host innate immune system in the follicles.

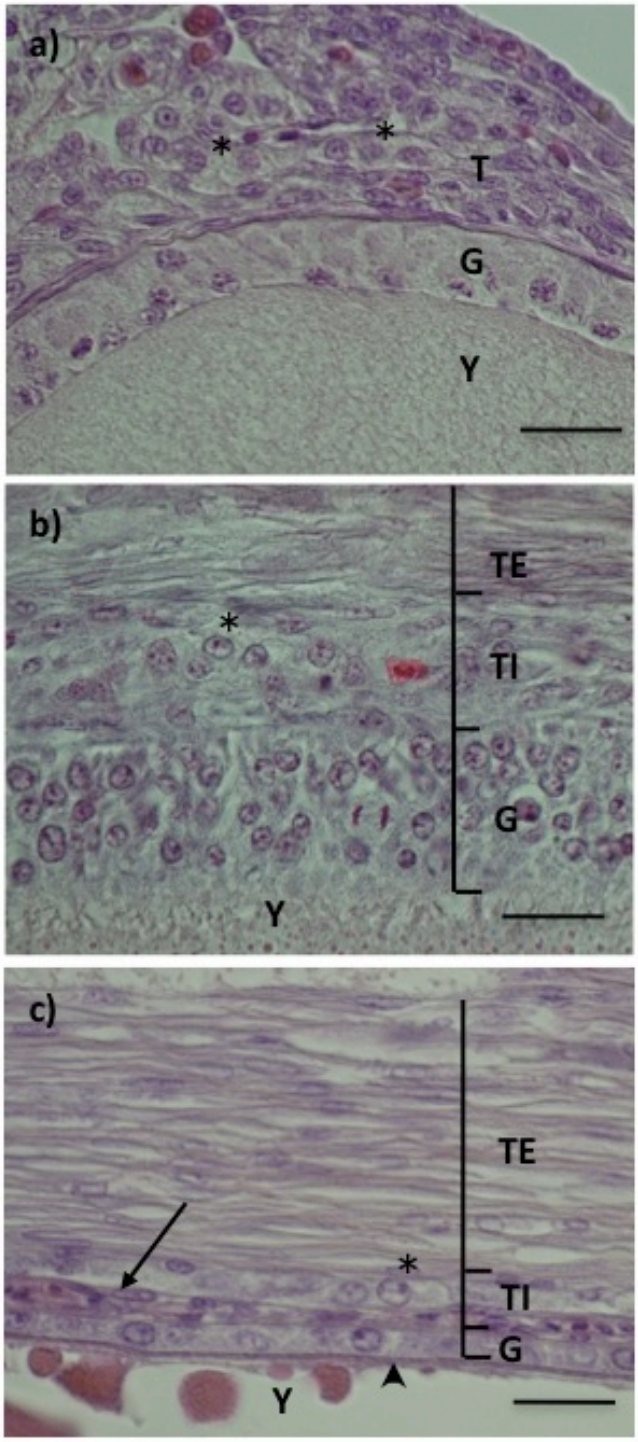

Fig. 3. Sections of hen ovarian follicles at different growth stage.
a) stromal follicle, b) white follicle, c) the largest yellow follicle. Note that theca layer is differentiated into externa and interna in the white and the largest yellow follicles unlike the stromal follicle. Granulosa layer of the small yellow follicle shows a multi-cell layer structure. G = granulosa layer; T = theca layer, TI = theca interna, TE = theca externa, Y= yolk, * = interstitial cells and theca interna cells, arrow= capillary, arrow head = perivitelline membrane. HE staining. Scale bars represent 20 μm.

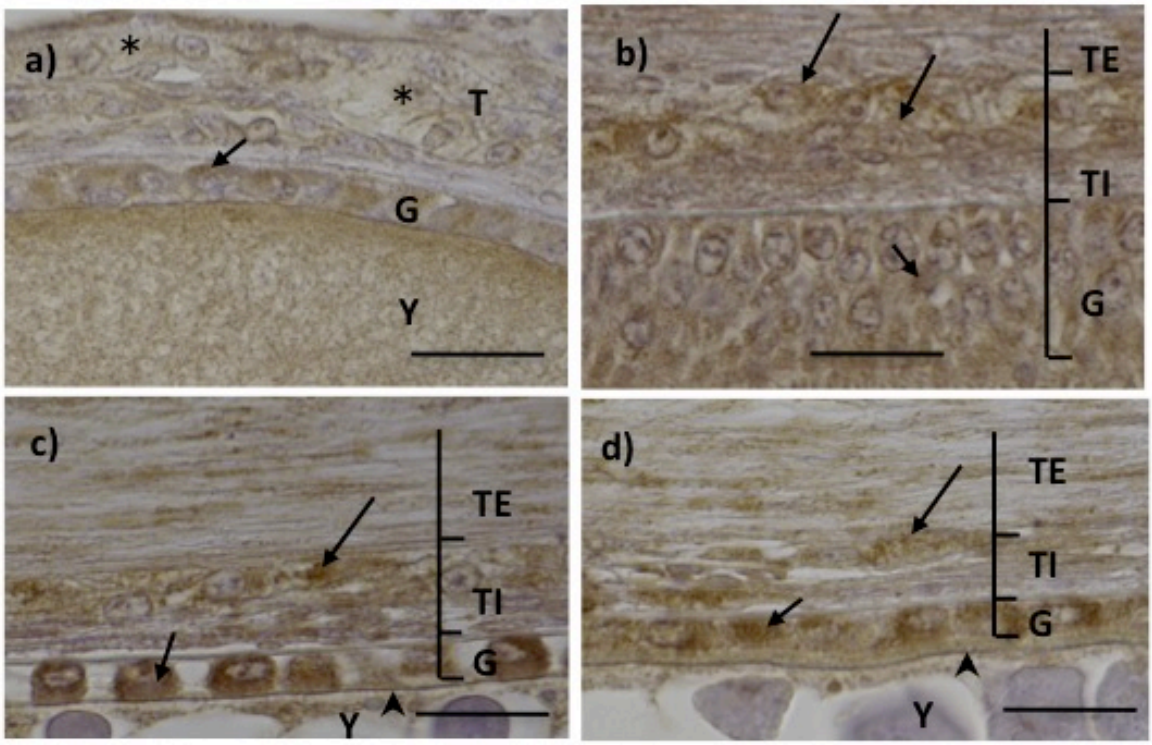

Fig. 4. Sections of growing ovarian follicles immunostained for avβD-8.
a) stromal follicle, b) whhite follicle, c) the third largest yellow follicle, d) the largest yellow follicle. Note that the granulosa cells are immunolabeled in all the follicles (short arrows), whereas the density is faint in the white follicle. Interstitial cells of white follicle show a faint immunostaining (b), and theca interna cells in the largest and third largest follicles contain dense immunoreaction products (c and d) (long arrows). * = interstitial cells. See Fig. 3 for other abbreviations. Scale bars represent 20 μm.

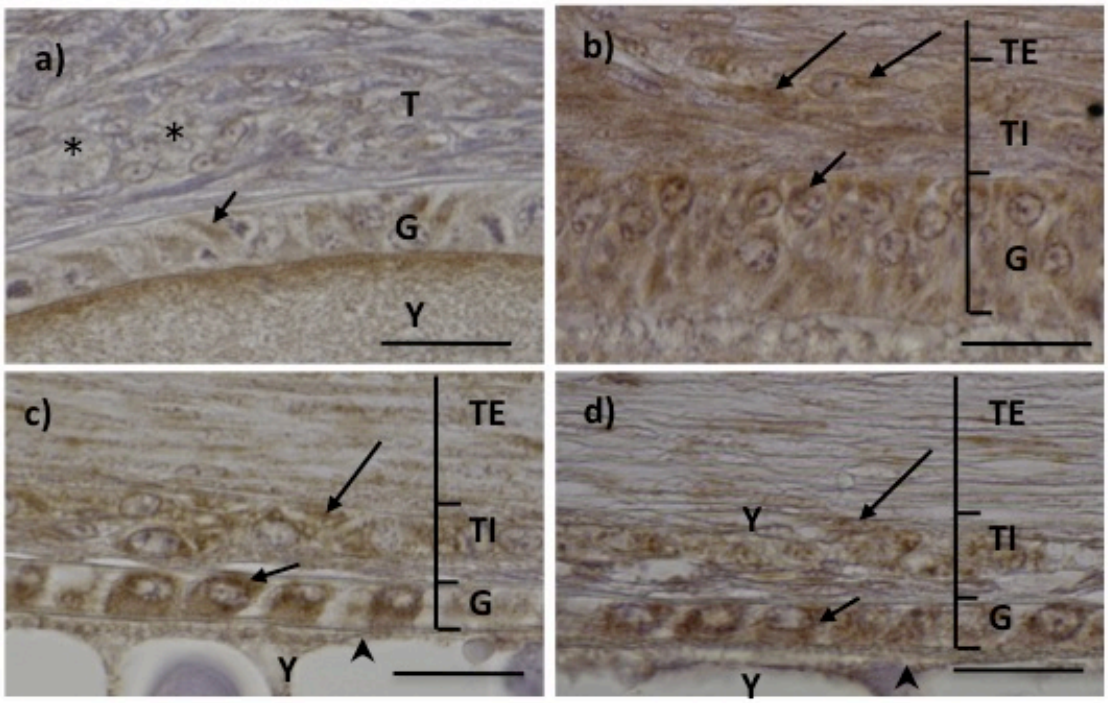

Fig. 5. Sections of growing ovarian follicles immunostained for avβD-10.
a) stromal follicle, b) white follicle, c) the third largest yellow follicle, d) the largest yellow follicle. Granulosa cells contain small amount of immunoreaction products in stromal and white follicles, whereas they are densely immunolabeled in the largest and third largest yellow follicles (short arrows). Interstitial cells of white follicle contain small amount of immunoreaction products and theca interna cells in the largest and third largest yellow follicles contain dense immunoreaction products (c and d) (long arrows). See Figs. 3 for abbreviations. Scale bars represent 20 μm.

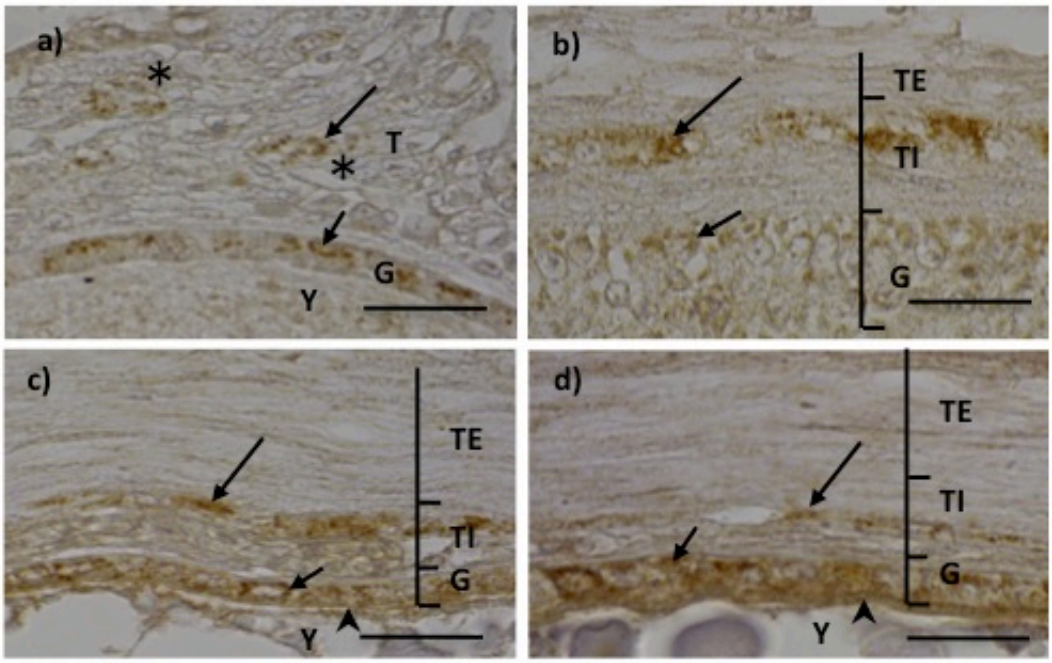

Fig. 6. Sections of growing ovarian follicles immunostained for avβD-12.
a) stromal follicle, b) white follicle, c) the third largest yellow follicle, d) the largest yellow follicle. Granulosa cells contain small amount of immunoreaction products in cortical and white follicles, whereas they are densely immunolabeled in the largest and third largest yellow follicles (short arrows). Interstitial cells of white follicle contain moderate amount of immunoreaction products and theca interna cells in the largest and third largest yellow follicles contain dense immunoreaction products (c and d) (long arrows). See Figs. 3 for abbreviations. Scale bars represent 20 μm.

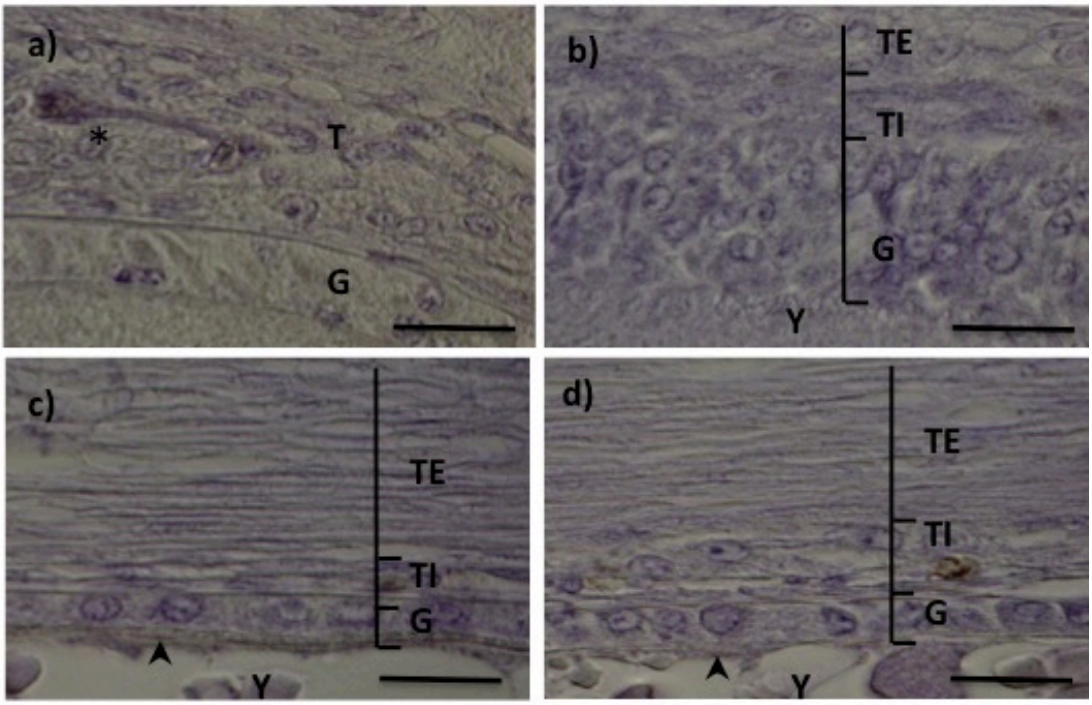

Fig. 7. Sections of growing ovarian follicles immunostained using normal rabbit IgG for control.

a) stromal follicle, b) white follicle, c) the largest yellow follicle. No staining is observed in all sections. See Figs. 3 for abbreviations. Scale bars represent 20 μm.

Table 3. Summary of the immunolabeling profiles in the ovarian follicles at different growth stages

Tissues	Follicles	avβD-8	avβD-10	avβD-12
Granulosa layer	Stromal follicle	+	+	+
	WF	+/-	+/-	+/-
	F3	++	++	++
	F2	++	++	++
	F1	++	++	++
Theca layer	Stromal follicle	-	-	+/-
	WF	+/-	+/-	+
	F3	+	+	+
	F2	+	+	+
	F1	+	+	+

-= no immunoreaction products, +/-= faintly immunolabeled, += moderately immunolabeled, ++= densely immunolabled. WF= small white follicle, F1 – F3= the largest to third largest follicles.

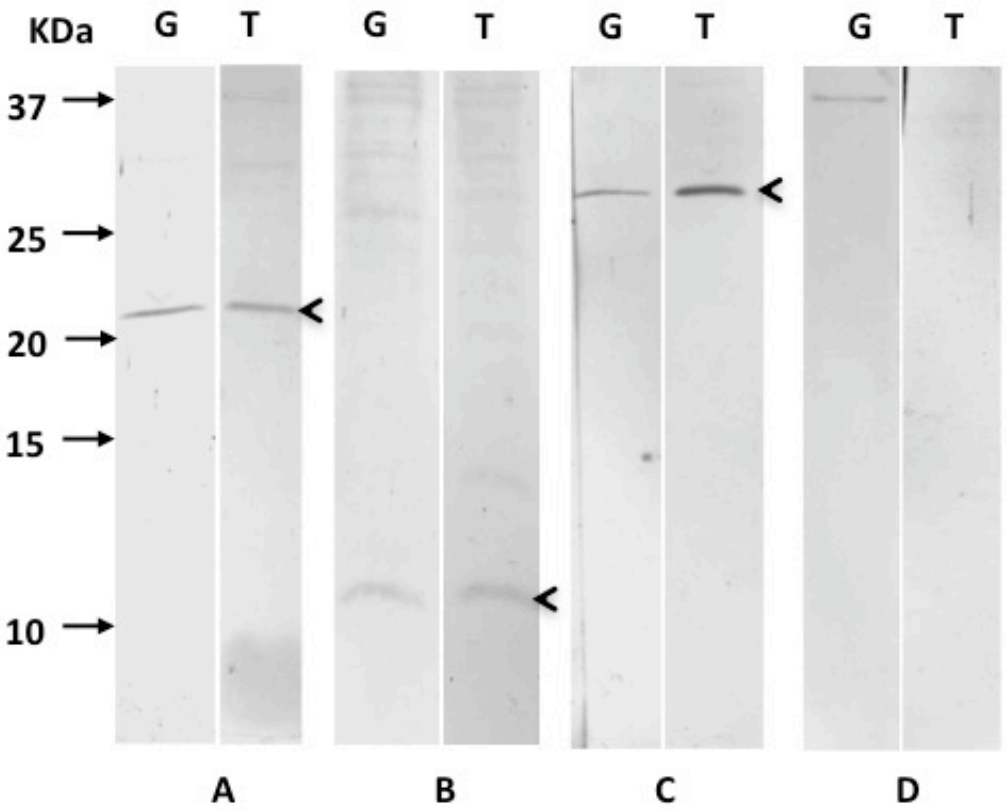

Fig. 8. Western blot of avβD-8, -10 and -12 in the ovarian follicular tissues.

Lanes A = avβD-8, Lanes B = avβD-10, Lanes C = avβD-12, Lanes D = control staining using normal IgG antibody. The bands of avβD-8, -10 and -12 exist at 21, 11 and 28 KDa (arrow heads). G= granulosa layer, T= theca tissue.

Chapter 3

Effects of lipopolysaccharide on the expression of proinflammatory cytokines and chemokines and influx of leukocytes in hen ovary

Introduction

Hen ovaries can be infected by pathogenic bacterial and viral agents that may be transmit to the egg yolk. Infection may cause ovarian disorders and the production of contaminated eggs. One of the microorganisms infecting the ovary is *Salmonella*, a Gram-negative bacterium (review by Gantois *et al.*, 2009). The follicular wall surrounding the yolk consists of granulosa and theca layers, and SE could invade these layers (Takata *et al.*, 2003). The local immune system plays essential roles in host defense against infection. Previous reports identified antigen-presenting cells, T and B cells in the follicles that showed unique changes in density affected by age and estrogen (Barua *et al.*, 1998b, 2001; Barua and Yoshimura, 1999b). The expression of Toll-like receptors (TLRs), a class of pattern recognition receptor responsible for initiation of the innate immune response, has also been identified in the theca and granulosa layers (Subedi *et al.*, 2007a; Woods *et al.*, 2009). Furthermore, the synthesis of avian beta-defensins, antimicrobial peptides, in the follicular wall has also been reported (Chapter 2; Subedi *et al.*, 2007b, 2008; Michailidis *et al.*, 2010).

Proinflammatory cytokines and chemokines play a key role in initiating innate and adaptive immune responses and assist in generating a local inflammatory response (Staeheli *et al.*, 2001; Ferro *et al.*, 2004; Hughes *et al.*, 2007). The primary

structures of cytokines are markedly different between avian and mammals, although they may perform similar tasks (Staeheli *et al.*, 2001). The pleiotropic activities of interleukin (IL)-1 include its T cell proliferation, induction of fever, glucocorticoid secretion, triggering of the acute-phase response, and the activation of vascular endothelium, whereas many of the activities of IL-1 may be mediated through its ability to induce other proinflammatory cytokines chemokines (Staeheli *et al.*, 2001). IL-6 is a multifunctional cytokine that regulates immunoglobulin production (Kishimoto and Hirano, 1988) and T cell activation (Lotz *et al.*, 1988; Rincon *et al.*, 1997; Diehl *et al.*, 2000), the differentiation of dendritic cells (Chomarat *et al.*, 2000). CXCLi2 is one of the chicken chemokines, although its precious functions remain to be established. In general, CXC chemokines are chemoattractant for polymorphonuclear cells, whereas CC and C chemokines are chemoattractant for macrophages and lymphocytes, respectively (Zlotnic and Yoshie, 2000). Thus, proinflammatory cytokines and chemokines synthesized in the ovary may play roles in the regulation of the local immune system of the organ. Sundaresan *et al.* (2007, 2008a, b) reported the upregulation of the expression of proinflammatory cytokines and chemokines in ovarian follicles during the follicular regression process. However, it remains to be studied whether the expression of those cytokines and chemokines is modulated by bacterial components. Local immunity in the ovary may have specific regulatory functions compared to other organs as suggested by Barua *et al.* (1998b, 1999b) who showed that the localization of immunocompetent cells is significantly affected by gonadal steroids.

The goal of this study was to determine whether the expression of proinflammatory cytokines and chemokines in ovarian cells changed to recruit heterophils and T cells in response to bacterial components. Changes in *IL-1β, IL-6*

and *CXCLi2* expressions in association with the frequency of heterophil-like cells and T cells in response to LPS, a cell wall component of Gram-negative bacteria were examined.

Materials and Methods

Birds and treatments

White Leghorn hens approximately 300-d-old and laying 5 or more eggs in a sequence were used. They were kept in individual cages under a lighting schedule of 14 L: 10 D and provided with feed and water *ad libitum*. The hens were i.v. injected with saline or 0.5 mg LPS (E. coli 0111:B4, extracted by phenol; Wako Pure Chemical Industries, Osaka, Japan) dissolved in 100 μl saline, and then the ovary was collected 0, 3, 6 and 12 hrs after injection. The time of tissue collection was adjusted to 6 hrs after oviposition in each hen (18 hrs before the predicted time of the next ovulation). When ovary samples were collected, hens were euthanized under anesthesia with Somnopentyl (0.4 ml/kg BW; Kyoritsu Pharmaceutical Co., Ltd., Tokyo, Japan). Birds were handled in accordance with the regulations of the Animal Experiment Committee of Hiroshima University.

Experiment 1. Analysis of changes in the RNA expression of IL-1β, IL6 and CXCLi2 in the theca and granulosa layers

The F2 follicles and ovarian stroma containing stromal follicles were collected from birds treated with LPS or saline before 0 (non-treated), 3, 6, or 12 hrs of

examination (n = 4 birds each at each examination time point). The outer superficial connective tissue surrounding the theca layer of each follicle was removed, and theca and granulosa layers were isolated separately from the follicles in sterile phosphate-buffered saline (PBS) as described previously (Porter *et al.*, 1989). RNA samples were isolated from the granulosa, theca and stroma cells using Sepazaol 1 super (Nacalai Tesque, Kyoto, Japan) as described previously (Das *et al.*, 2010). They were purified by incubating at 42 °C for 45 min with DNase I (TaKaRa BIO Inc., Japan) at a concentration of 0.5 U/μg RNA. The purified RNA samples were reverse-transcribed using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan) following the manufacturer's instructions. Briefly, 10 μl reaction mixture that contained 1 μg total RNA, 1 × RT buffer, 1 mM each deoxyribonucleotide triphosphate mixture, 20 U RNase inhibitor, 0.5 mM oligo (dT) and 50 U ReverTra Ace was placed in a Programmable Thermal Controller PTC-100 (MJ Research Inc., Waltham, MA, USA) and incubated at 42 °C for 30 min followed by heat inactivation at 99 °C for 10 min. Quantitative PCR analysis was performed for *IL-1β, IL6* and *CXCLi2* expression using a Roche Light Cycler (Roche Applied Science, IN, USA) as described previously (Das *et al.*, 2010). Expression of *RPS17* was examined as an internal control to normalize the values of each sample. A total of 20 μl reaction mixture containing l of 1 X SYBR Premix Ex Taq II (TaKaRa Co., Tokyo, Japan), 0.2 μM of each forward and reverse primer (Table 4), 100 ng of cDNA was prepared. The reaction mixture was placed in 20 μl capillaries (Roche Diagnostics GmbH, Mannheim, Germany). Following denaturation at 95 °C for 10 sec, PCR was carried out with a thermal protocol of 95 °C for 5 sec and 60 °C for 20 sec. Specificity of the amplified products was verified by melting curve analysis and by sequencing the PCR products. Data analysis was performed as described previously by Das *et al.* (2010). Briefly, the ΔCT was calculated for each sample by subtracting the threshold cycle (C_T) value of *RPS17* (internal control) from

the C_T of the respective target gene. For relative quantification, the ΔCT value of *RPS17* was then subtracted from the ΔCT of each experimental sample to generate the ΔΔCT. The ΔΔCT value was therefore fit to the formula $2^{-\Delta\Delta CT}$ to calculate the approximate fold difference.

Experiment 2. Western blot analysis of changes in IL1β and IL6 protein expression in ovarian tissues

The F2 and F3 follicles and ovarian stroma were collected from another 9 birds injected with or without LPS or saline (3 birds each) before 6 hrs of examination as described in Experiment 1. The collected F2 and F3 follicles of each bird were pooled as one follicular sample. The superficial connective tissue on the theca was removed. The theca was separated from the granulosa layer in PBS, and the granulosa layer was washed in PBS to remove the yolk. Each theca and granulosa tissue was homogenized separately in a 5 times volume of homogenization buffer consisting of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 0.1% (w/v) SDS and 1 mM phenylmethylsulfonylfluoride using a polytron homogenizer (Polytron PT1200c, Kinematica AG, Switzerland) as described in Chapter 2. The samples were centrifuged at 12,000 X g for 20 min at 4 °C. The supernatant was collected and the protein concentration was measured using protein assay reagent (Bio-Rad Lab, Hercules, CA, USA) using bovine serum albumen as the standard protein.

The samples were separated by Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Tricine SDS-PAGE; 16% separating gel and 4% stacking gel) as described in Chapter 2. Samples were mixed with sample buffer

composed of 30% (v/v) glycerol, 5% (v/v) mercaptoethanol, 4% (w/v) SDS, 0.06% (w/v) bromophenol blue and 150 mM Tris-HCl, pH 7.0, at a sample protein concentration of 1 μg/μl and boiled for 5 min. A 15 μl sample was loaded onto gels and run at 50 V in the stacking gel and at 150 V in the separating gel.

After SDS-PAGE, the proteins in the gel were electrophoretically transferred onto a nitrocellulose membrane (PALL Gelman Laboratory, Ann Arbor, MI, USA). The membrane was washed briefly with buffer (0.02 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.5% (v/v) Tween 20, and 0.05% (w/v) BSA) and incubated with 5% (w/v) casein milk (Roche, Mannheim, Germany) solution in Tris-buffered saline containing 1 % Tween 20 (TBS-T) for 60 min. The membrane was then incubated with antibodies to rabbit anti-chicken IL-1β (Abcam Co., Tokyo, Japan) or rabbit anti-chicken IL-6 (Abcam Co.) diluted at a concentration of 10 μg/ml in TBS-T overnight at 4 °C. The membrane was then washed in TBS-T for 30 min (10 min X 3) before incubation with peroxidase labeled anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK) diluted at 1:5,000 in TBS-T for 1 h at room temperature. The membrane was washed with Western buffer for 30 min (10 min X 3 times) and the immunoprecipitates on the membrane were treated by Amersham ECL™ Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK) for 1 min. Images were taken using an ATTO cooled CCD camera system Ez-Capture II (ATTO Corporation, Japan). The results were confirmed by three repeated trials.

Experiment 3. Localization of heterophil-like cells, CD4$^+$ and CD8$^+$ T cells

The F3 and F4 follicles and ovarian stroma were collected from the birds used for Experiment 1 (n = 4 birds at each examination time point). For general histology,

the F4 follicles and stroma were fixed in Bouin solution and processed for paraffin sections (4 μm). They were stained with hematoxylin and eosin. F3 follicles and stroma were embedded in Tissue Tek OCT compound (Sakura Finetek, Tokyo, Japan) and snap-frozen in a mixture of isopentane and solid carbon dioxide. Cryostat sections (15 μm in thickness) were air-dried on slides treated with 3-aminopropyl-triethoxysilane. The sections were fixed with cold acetone and methanol on ice for 10 min each. They were washed with PBS for 15 min (5 min x 3 times) and incubated with 1 % (v/v) goat serum for 15 min to block nonspecific binding of antibodies. Sections were then incubated overnight with mouse anti-chicken $CD4^+$ or $CD8^+$ antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) diluted with PBS at a concentration of 1:100, followed by washing with PBS for 15 min (5 min x three times). The immunoreactions of the first antibody on the sections were detected using a Histofine SAB-PO (M) kit (Nichirei Co., Tokyo, Japan) according to the manufacturer's instructions. Briefly, the sections were incubated with the biotinylated anti-mouse IgG + IgA + IgM second antibody and with avidin-biotin-peroxidase complex for 1 h each. Finally, immunoprecipitates were visualized by incubating with a mixture of 0.02% (w/v) diaminobenzidine (Sigma, St. Louis, MO) and 0.001 % H_2O_2 (v/v) in 0.05 M Tris-HCl (pH 7.6). The sections were dehydrated and covered.

The immunostained sections were examined under a light microscope with a computer-based image analyzer software (Image-pro plus; Media Cybemetics, Silver Spring, MD, USA). The numbers of immunopositive cells were determined by observing three different regions in each theca layer and stroma. The cell number in $1x10^5$ μm^2 was calculated. The average of the three counts was expressed as the cell frequency in one tissue of one hen.

Statistical analysis

The significance of differences of cytokine and chemokine expressions and T cell frequencies among different times of treatment within the LPS or saline group was determined by one-way ANOVA, followed by Tukey's test. The differences between LPS-treated and saline-treated groups were examined by Student's t test. Differences were considered significant at $P < 0.05$.

Results

Experiment 1. Analysis of changes in the RNA expression of IL-1β, IL6 and CXCLi2 in ovarian tissues

Figure 9 shows the effects of LPS injection on *IL-1β* expression in ovarian tissues. The expression of *IL-1β* was significantly increased in the granulosa layer, theca layer and stroma tissues with a peak at 3, 3-6 and 3 hrs after injection, respectively (Fig. 9 a-c). The expression in each tissue showed a tendency to decline after the peak. Saline injection showed no significant effects on *IL-1β* expression in the three tissues. The expression was significantly different between LPS-injected and saline-injected groups at 3, 6 and 12 hrs after injection in all tissues.

The expression of *IL-6* was significantly increased at 3 hrs after LPS injection, followed by a decline thereafter in the granulosa, theca and stromal tissues (Fig. 10 a-c). No significant changes in the expression were identified in the saline-injected group. Significant differences in the expression between the LPS-injected and saline-injected

groups were found at 3, 3-12 and 3-6 hrs after injection in the granulosa, theca and stromal tissues, respectively.

The *CXCLi2* expression in the granulosa, theca and stromal tissues was also significantly increased at 3 hrs after LPS injection and declined after that peak (Fig. 11 a-c). Saline injection did not affect the *CXCLi2* expression, and significant differences in the expression compared to the LPS-injected group were identified at 3, 3-12, and 3 hrs after injection in the granulosa, theca and stromal tissues, respectively.

Experiment 2. Western blot analysis of changes in IL1β and IL6 protein expression in ovarian tissues

Western blot analysis showed specific bands for IL-1β and IL-6 at the expected sizes of 17 KDa and 25 KDa, respectively, in the granulosa, theca and stromal tissues at 6 hrs after LPS injection (Fig. 12). The bands of both IL-1β and Il-6 were faint or negligible in the three tissues obtained before injection (0 hr) and at 6 hrs after saline injection.

Experiment 3. Localization of heterophil-like cells, $CD4^+$ and $CD8^+$ T cells

Only a small number of heterophil-like cells were identified in the capillaries in the theca interna and veins in the superficial connective tissues before LPS injection; however, many heterophil-like cells were found in the capillaries in the theca interna 12 hrs after LPS injection, whereas this population was not affected by saline injection. In the stromal tissue, a small number of heterophil-like cells were observed in the

connective tissues, whereas noticeable differences in their localization were not identified in the LPS- and saline-injected groups (Fig. 13).

Both $CD4^+$ and $CD8^+$ T cells were localized in the theca interna and externa but not in the granulosa layer in all birds. These T cells were also localized in the stromal connective tissues (Figs. 14 and 15). The frequency of $CD4^+$ T cells in the theca layer showed a significant increase at 6 hrs after LPS injection, and that in the LPS-injected group was greater than the saline-injected group at 6 and 12 hrs (Fig. 16a). In the stromal tissues, the frequency of $CD4^+$ T cells increased at 3 and 6 hrs after LPS injection and was significantly greater in the LPS-injected groups than in the saline-injected groups at 6 and 12 hrs (Fig. 16b). The frequency of $CD8^+$ T cells was not affected significantly by injection with LPS or saline in both theca and stroma tissues (Fig. 16c and d). Saline injection did not affect the frequency of $CD4^+$ and $CD8^+$ T cells in the theca and stroma (Fig. 16).

Discussion

This Chapter describes that hen ovarian tissues expressed cytokines as well as chemokines, and their expression and influx of leukocytes in the ovary were enhanced by LPS stimulation. The significant findings were (1) *IL-1β, IL-6* and *CXCLi2* were expressed in the theca and granulosa layers, (2) their expression was significantly upregulated by injection of birds with LPS, and (3) influx of heterophil-like cells and $CD4^+$ T cells was also enhanced by LPS stimulation. The identification of *IL-1β, IL-6* and *CXCLi2* expression in the follicles supports the previous report of Sundaresan *et al.* (2008a) who identified the expression of those cytokines and chemokines in the

regressing preovulatory follicles; however, they used mixed samples of the granulosa and theca layers. The current study further suggests that both of these layers express proinflammatory cytokines and chemokines.

Many workers have shown that *salmonella* organisms colonize the hen ovary, including the attachment of *Salmonella* organisms to granulosa cells and vitelline membranes (review by Gantois *et al.,* 2009). Experimental inoculation of Japanese quail hens with SE resulted in the invasion of these microorganisms to the ovarian stroma, follicular granulosa and theca layers (Takata *et al.*, 2003). Intravenous LPS injection may mimic the effects of such bacterial invasion into ovarian tissues. It has been reported that TLR4, which plays role to recognize LPS of Gram-negative bacteria, is expressed in the granulosa and theca layers (Subedi *et al.*, 2007a, Woods *et al.*, 2009). The upregulation of *IL-1β, IL6* and *CXCLi2* expression by LPS in the current study could probably be initiated by the interaction of LPS and TLR4 in ovarian tissues. The upregulation of IL-1*β*, IL-6 and CXC chemokines by *Salmonella* organisms, Gram-negative bacteria, has been shown in various tissues, including gut and visceral organs (Withanage *et al.*, 2004; Cheeseman *et al.*, 2008) and heterophil and monocytes/macrophages (Kaiser *et al.*, 2006; Kogut *et al.*, 2006; Wigley *et al.*, 2006). In mammals, it has been shown that IL-1β has pleiotropic activities, such as T cell proliferation, triggering the acute phase response and activation of the vascular endothelium, and also the upregulation of *IL-6* expression (Staeheli *et al.*, 2001). IL-6 is a multifunctional cytokine, and one of its roles is to induce the proliferation and maturation of T cells during infection (Lotz *et al.*, 1988). Avian and mammalian cytokines may perform similar tasks, although their primary structures in most cases are remarkably different (Staeheli *et al.*, 2001). The current results showed the upregulation of gene and protein expressions of IL-1β and IL-6 by LPS in the ovarian

stroma and follicles. These cytokines may lead to the inflammatory process as a local immune response in the ovary.

The frequencies of heterophil-like cells in the capillaries of theca interna and $CD4^+$ T cells in the theca and stromal tissues were increased by injection with LPS. Also, the expression of *CXCLi2* was significantly upregulated by LPS in the granulosa and theca layers of follicles and stroma. Although the exact function of chicken chemokines remains to be determined, it is reasonable to postulate that CXC chemokine members recruit heterophils, as CXCL8 preferentially acts on mammalian neutrophils (Gangur *et al.*, 2002). Kogut (2002) reported that CXCLi2-like chemokines were involved in heterophil recruitment to the site of infection following challenge with SE in chickens. Thus, one of the factors responsible for the recruitment of heterophil-like cells into ovarian follicles may be CXCLi2 synthesized in the theca and granulosa cells by LPS. It remains unknown why marked changes in the frequency of heterophil-like cells were not observed in the stroma. Chicken CXCLi2 may also be chemotactic for monocytes/macrophages and lymphocytes (Kaiser *et al.*, 1999). Although it is not clear whether CXCLi2 recruits T cells, it induces the degranulation of neutrophils and releases potent chemoattractants for human T cells (Taub *et al.*, 1996). Thus, it is assumed that CXCLi2 expressed in the granulosa and theca layers of follicles and stroma in response to LPS might directly or indirectly recruit $CD4^+$ T cells in the ovarian follicles and stroma. In a mouse model study, IL-1β stimulated the clonal expression of $CD4^+$ T cells and elevated CD4/CD8 ratio due to the increased $CD4^+$ T cells (Mansilla-Roselló *et al.*, 1996). In the current study, $CD4^+$ T cells were increased in frequency in both theca and stroma, but the changes in the frequency of $CD8^+$ T cells were not significant. Thus increased expression of IL-1β in the ovarian tissues by LPS may be also responsible in the direct or indirect regulation of T cell

recruitment in hen ovary as suggested in mouse model. Heterophil-like cells and T cells that migrated into the ovary may play roles in the elimination of Gram-negative bacteria by their phagocytic functions and enhancement of the T cell-mediated immune response.

Sundaresan *et al.* (2007) reported that the expression of *IL-6* and *IL-8* in the ovary was elevated in association with a decline of the gonadal steroid level and tissue regression in molting hens. The current histological study that examined the follicles at 12h after LPS injection could not identify marked cell death or atretic signs of follicular tissues; however, it is possible that examination of follicles later than 12 hrs after LPS injection shows atretic changes. If steroidogenesis and tissue integrity are affected by the elevated expression of proinflammatory cytokines and chemokines in response to Gram-negative cell infection, it may lead to ovarian disorders.

In conclusion, we suggest that ovarian follicular cells and stromal cells have the ability to express proinflammatory cytokines and chemokines, and their expression is upregulated by LPS, a Gram-negative bacterial component, in association with the recruitment of heterophil-like cells and T cells. The response may play roles in local host defense in ovarian follicles.

Abstract

The aim of this study was to determine whether the expression of proinflammatory cytokines and chemokines in ovarian cells was changed to recruit heterophils and T cells in response to LPS, a Gram-negative bacterial component. White Leghorn laying hens were intravenously injected with LPS or saline, and their

ovarian follicles and stroma were collected. Changes in the mRNA expression of *IL-1β*, *IL6* and *CXCLi2* in the theca and granulosa layers and ovarian stroma were analyzed by quantative reverse transcriptase (RT) -PCR, whereas proteins of IL-1β and IL6 were also identified by Western blot analysis. Localization of heterophil-like cells, $CD4^+$ and $CD8^+$ T cells was examined by general histology and immunohistochemistry. The expressions of *IL-1β*, *IL6* and *CXCLi2* were significantly increased in the granulosa layer, theca layer and stroma tissues by 3 to 6 h after LPS injection. Increase of IL-1β and IL6 proteins in those tissues was also identified 6 hrs after LPS injection. The LPS stimulation resulted in the increased influx of heterophil-like cells and $CD4^+$ T cells, but not $CD8^+$ cells, in the theca layers of follicles. Saline injection affected neither expression of examined proinflammatory cytokines and chemokines nor frequencies of immunocompetent cells. These results suggest that ovarian follicular cells and stromal cells have the ability to express proinflammatory cytokines and chemokines, and their expression is upregulated by LPS in association with the recruitment of heterophil-like cells and T cells. These responses may play roles in local host defense in ovarian follicles.

Table 4. Real-time quantitative RT-PCR primer

Gene	Primer Sequences	Accession No.
IL-1β	F: ACTGGGCATCAAGGGCTA R: GGTAGAAGATGAAGCGGGTC	NM_204524
IL-6	F: AGAAATCCCTCCTCGCCAAT R: AAATAGCGAACGGCCCTCA	NM_204628
CXCLi2	F: GGCTTGCTAGGGGAAATGA R: AGCTGACTCTGACTAGGAAACTGT	AJ009800
RPS17	F: AAGCTGCAGGAGGAGGAGAGG R: GGTTGGACAGGCTGCCGAAGT	NM_204217

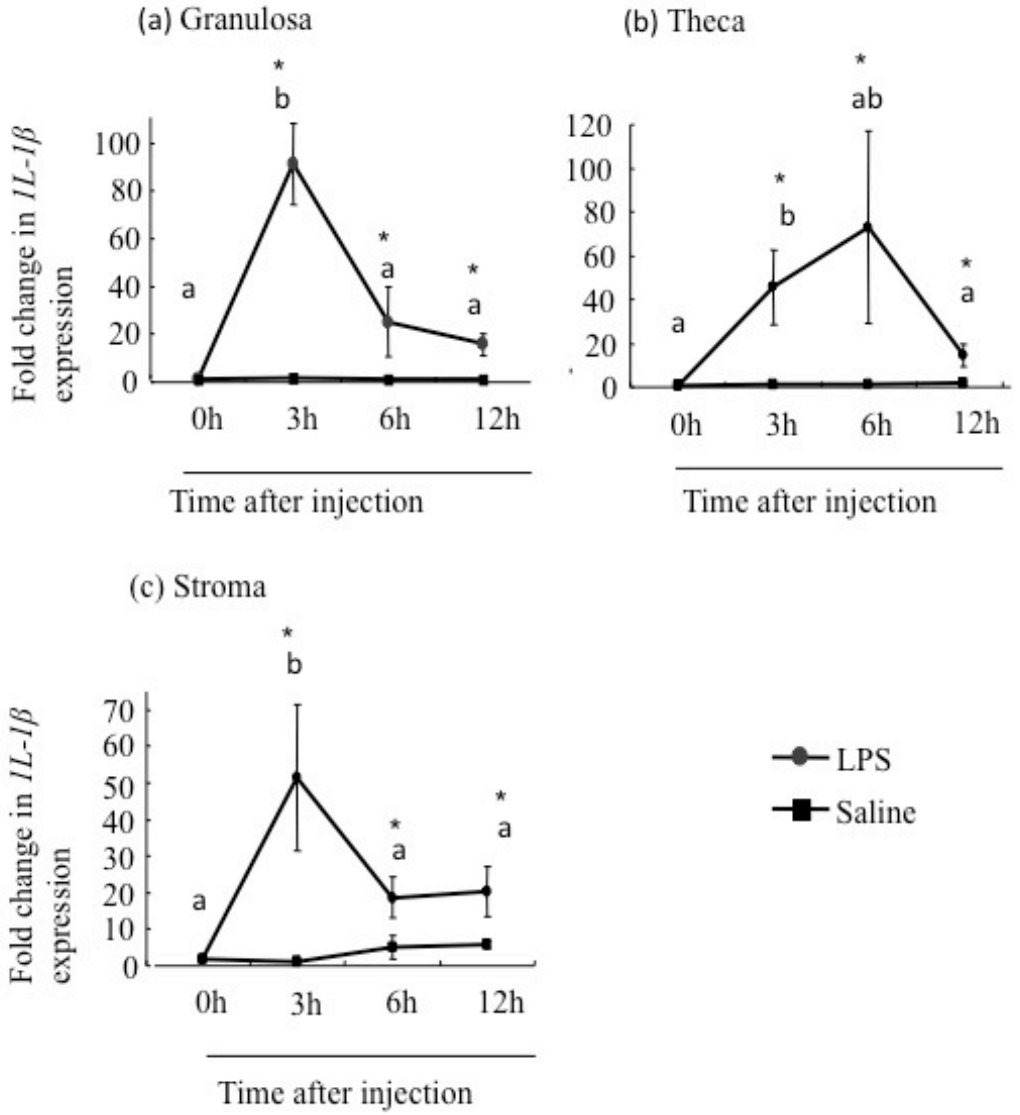

Fig. 9. Effects of lipopolysaccharide injection on the mRNA expression of interleukin 1-β (IL-1β) in hen ovarian tissues. (a): Granulosa layer. (b): Theca layer. (c): Stroma. Ovarian tissues were collected 0, 3, 6 and 12 hrs after injection with LPS or saline. Each value shows the mean ± SEM of fold changes in expression. [a-b]Bars with different letters are significantly different within LPS-injected groups (P<0.05). Asterisks (*) represent a significant difference between LPS-injected and saline-injected groups at corresponding treatment times (P<0.05).

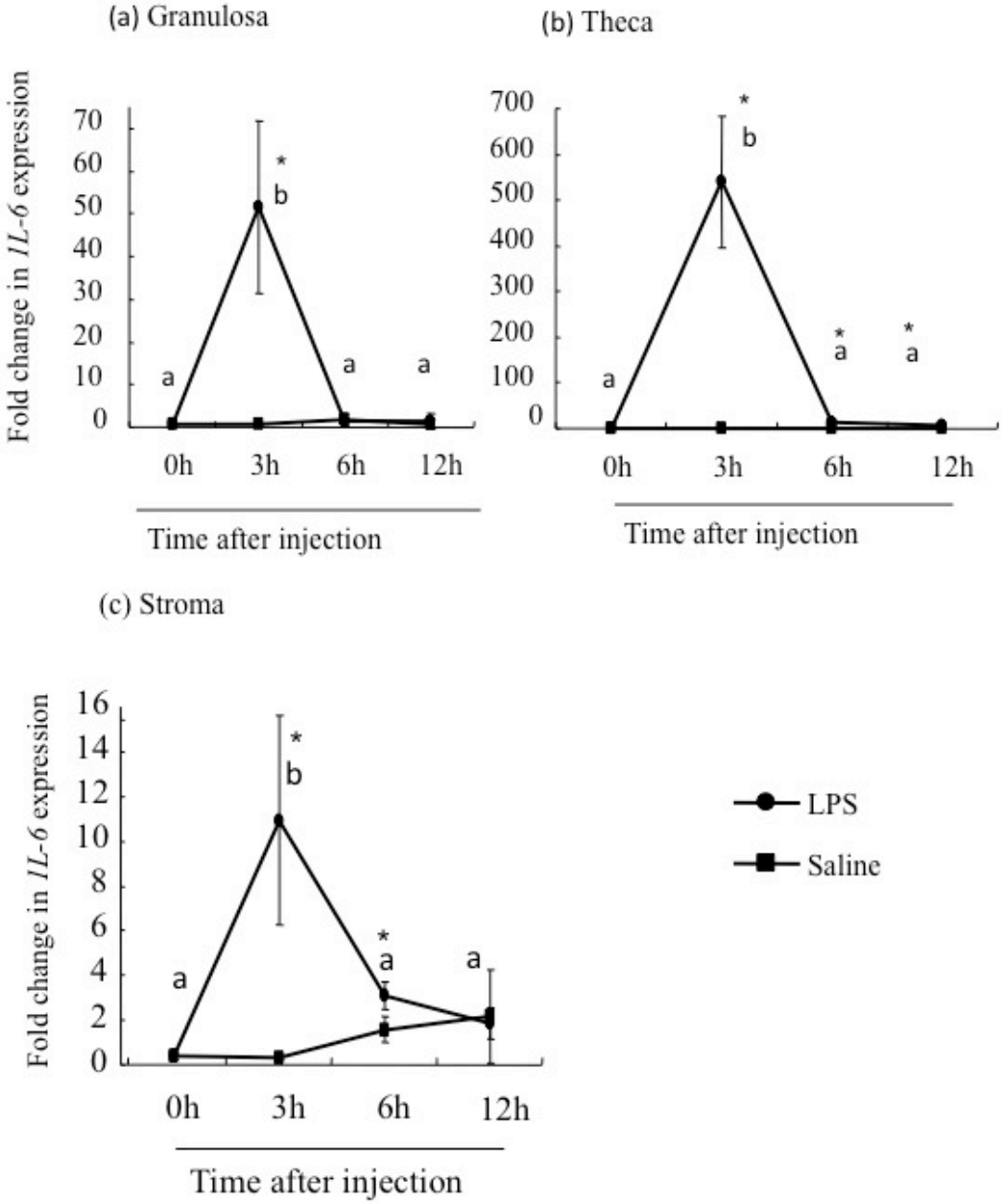

Fig. 10. Effects of lipopolysaccharide injection on the mRNA expression of interleukin 6 (IL-6) in hen ovarian tissues. (a): Granulosa layer. (b): Theca layer. (c): Stroma. Ovarian tissues were collected 0, 3, 6 and 12 hrs after injection with LPS or saline. Each value shows the mean ± SEM of mRNA expression fold changes. See Fig. 9 for explanations of letters.

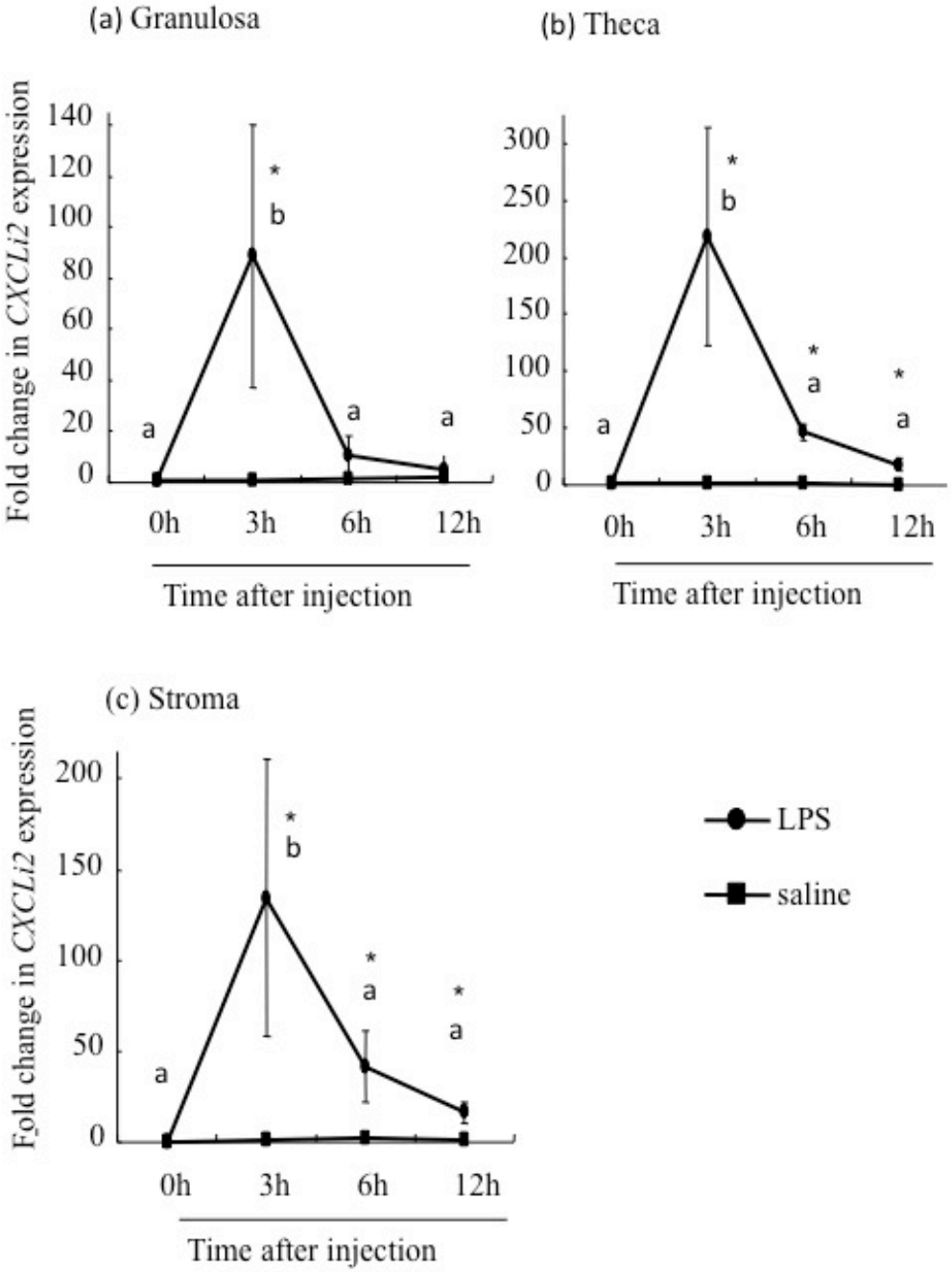

Fig. 11. Effects of lipopolysaccharide injection on the mRNA expression of CXCLi2 chemokines in hen ovarian tissues. (a): Granulosa layer. (b): Theca layer. (c): Stroma. Ovarian tissues were collected 0, 3, 6 and 12 hrs after injection with LPS or saline. Each value shows the mean ± SEM of mRNA expression fold changes. See Figure 9 for explanations of letters.

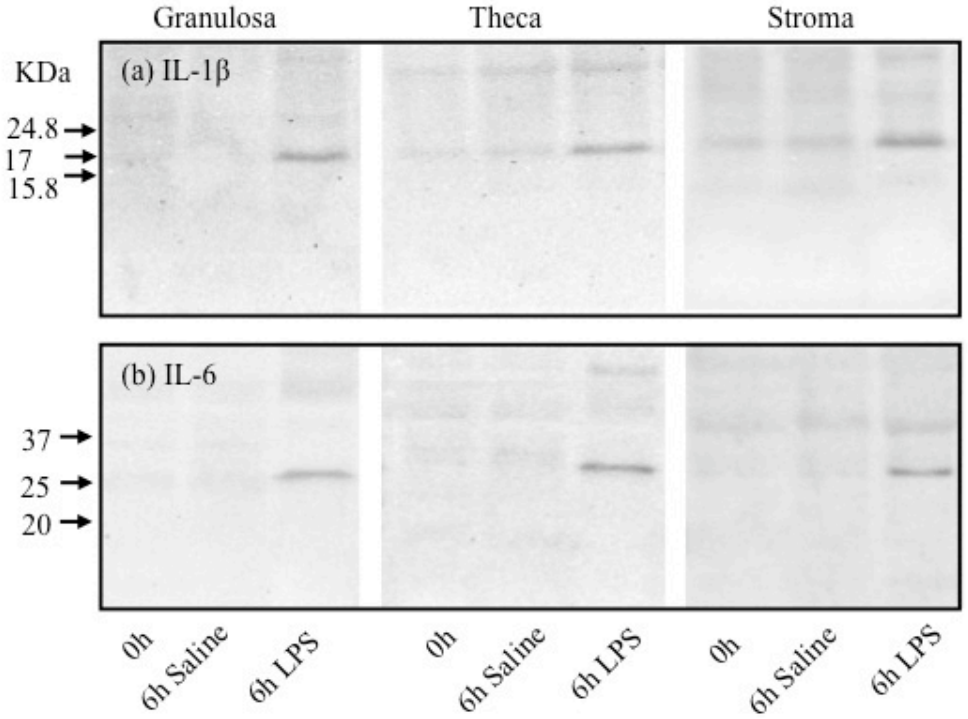

Fig. 12. Western blot analysis of interleukin-1β (IL-1β) and IL-6 in hen ovarian tissues treated with or without lipopolysaccharide and saline. (a): Analysis of IL-1β. (b): Analysis of IL-6. Tissues were collected form hens before (0 hr) or 6 hrs after injection with lipopolysaccharide or saline. Note that clear bands of IL-1β and IL-6 appear only 6 hrs after lipopolysaccharide injection.

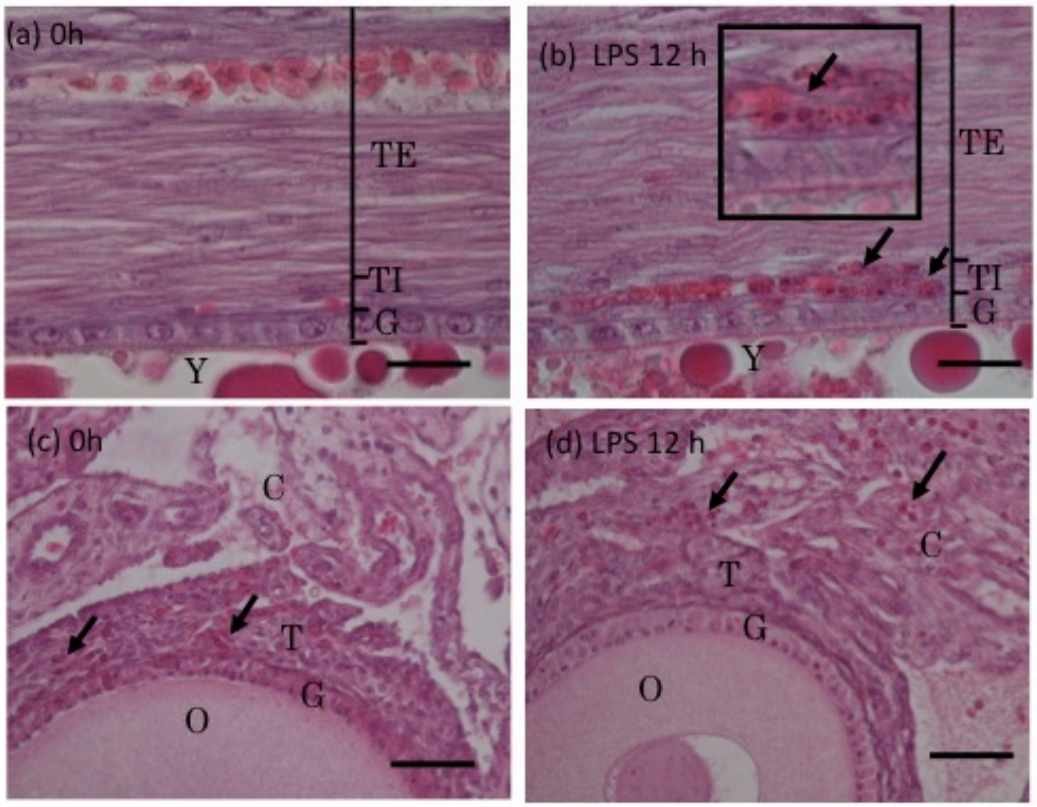

Fig. 13. Sections of hen ovarian follicles of non-treated birds (a) and after treatment with LPS for 12 hours. Stroma of non-treated birds (c) and after treatment with LPS for 12 hours. Note the increase of heterophile-like cells in the theca interna of LPS treated birds (arrows).

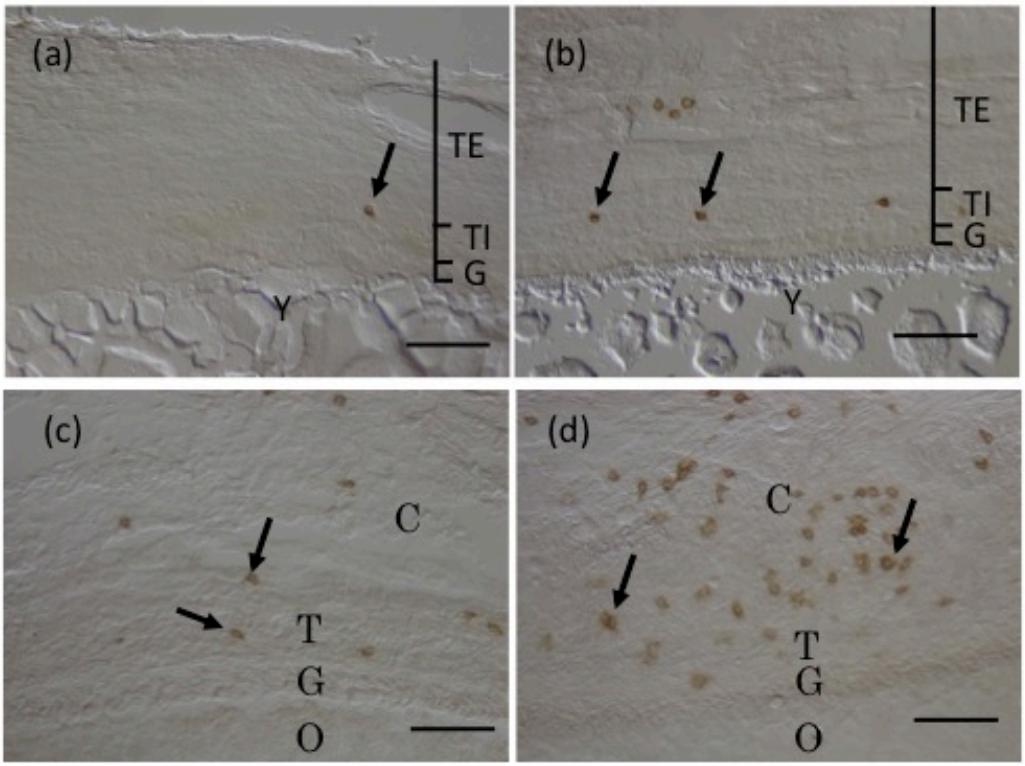

Fig. 14. Sections of ovarian follicles and stroma immunostained for $CD4^+$ T cells treated with or without lipopolysaccharide. (a and b): Follicular walls of the third largest yellow follicle before (a) and 6 hrs after lipopolysaccharide injection (b), respectively. (c and d): Ovarian stroma of before (c) and 12 hrs after lipopolysaccharide injection (d), respectively. Arrows show positive cells in the theca layer (a and b) and stromal connective tissues (c and d). C = stromal connective tissue, G = granulosa layer, O = oocyte, T = theca layer, TE = theca externa, TI = theca interna, Y = yolk. Scale bars represent 50 μm.

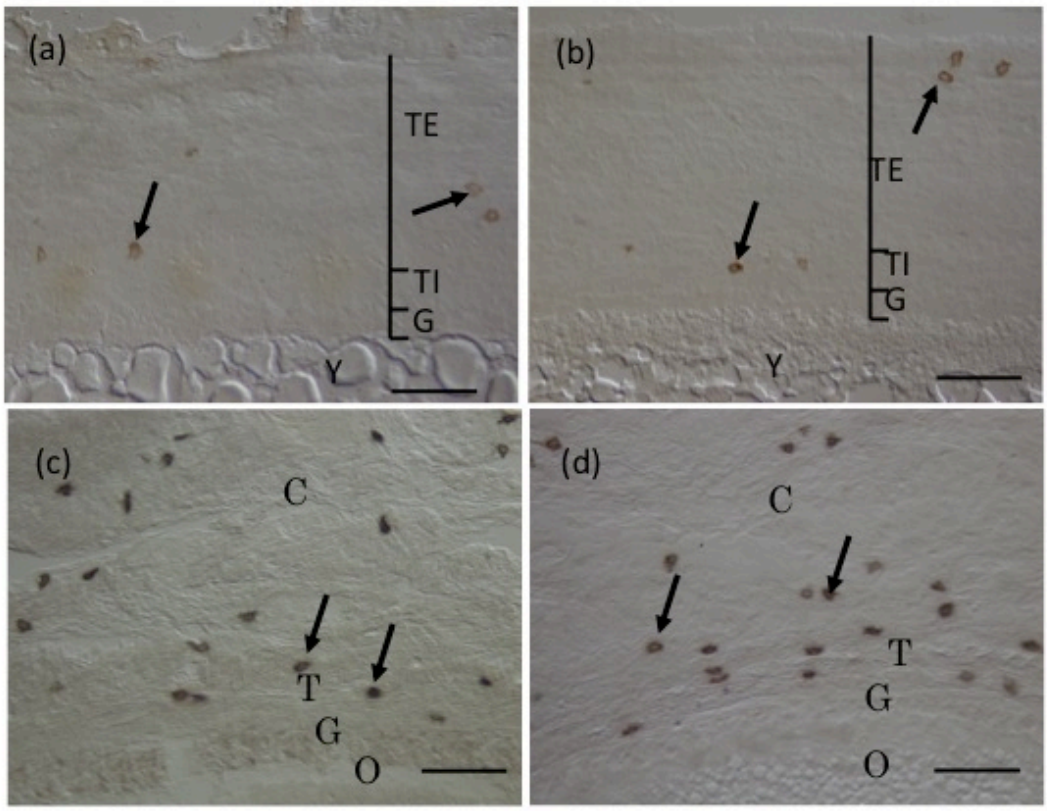

Fig. 15. Sections of ovarian follicles and stroma immunostained for $CD8^+$ T cells treated with or without lipopolysaccharide. (a and b): Follicular walls of the third largest yellow follicle before (a) and 6 hrs after lipopolysaccharide injection (b), respectively. (c and d): Ovarian stroma of before (c) and 12 hrs after lipopolysaccharide injection (d), respectively. Arrows show positive cells. See Fig. 14 for explanation of letters. Scale bars represent 50 μm.

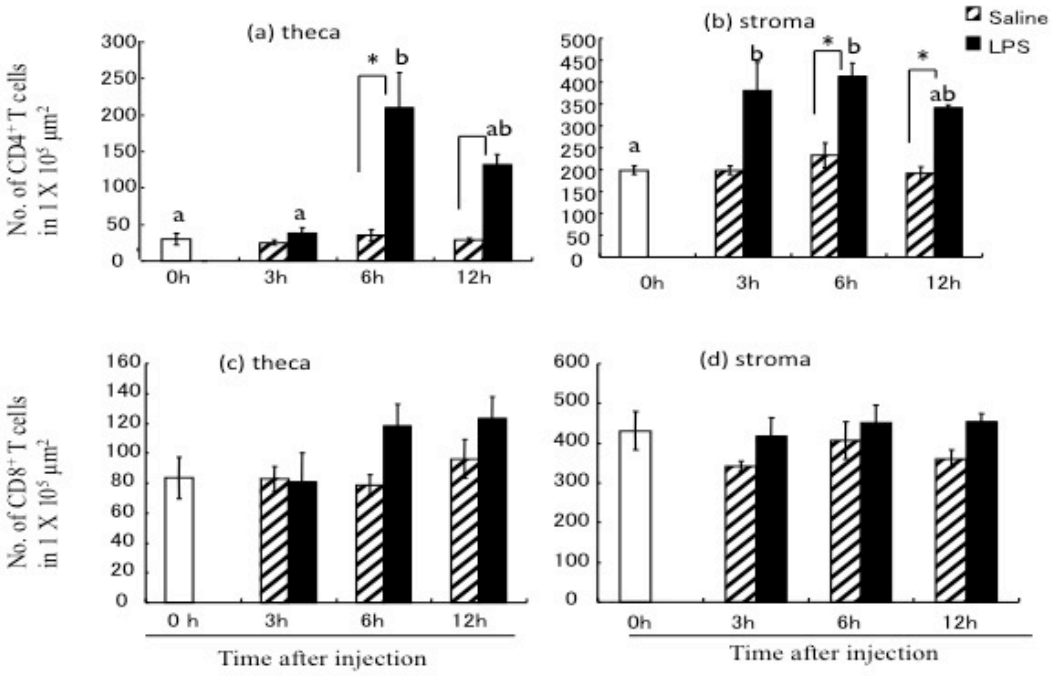

Fig. 16. Effects of lipopolysaccharide injection on the frequencies of CD4⁺ and CD8⁺ T cells in ovarian tissues. (a and b): CD4⁺ T cells in the theca and stroma, respectively. (c and d): CD8⁺ T cells in the theca and stroma, respectively. Birds were injected with lipopolysaccharide or saline 0–12 hrs before tissue collection. Values are the mean ± SEM of the number of positive cells in 1 X 10^5 µm^2 area. $^{a-b}$Bars with different letters are significantly different within LPS-injected or saline-injected groups (P<0.05). Asterisks (*) represent a significant difference between LPS-injected and saline-injected groups (P<0.05).

Chapter 4

Effects of lipopolysaccharide and interleukins on the expression of avian β-defensisns in hen ovarian follicular tissue

Introduction

Salmonellosis is reported to be one of the most widely spread food borne diseases worldwide. Human salmonellosis frequently results from the consumption of contaminated chicken eggs (Guard-Petter, 2001). Systemic SE infection in laying hens can lead to colonization of the ovary or oviduct (Keller *et al.,* 1995; Okamura *et al.*, 2001; De Buck *et al.,* 2004). Invasion of the ovarian follicles by SE during egg formation is one of the major mechanisms of egg contamination (Takata *et al.*, 2003; Gontois *et al.*, 2009).

Cytokines are key factors for triggering the immune response and inflammation. They are a group of mediators regulating cellular functions that are secreted by specific cells to affect the functions and behavior of other cells, playing a role in the regulation of immune and inflammatory processes (Giansanti *et al.,* 2006). Cytokines are well-established factors of the host immune response to *Salmonella* infection. Chicken proinflammatory cytokines such as IL-1β and IL-6 appear to function, as do their mammalian counterparts, in mediating an inflammatory response (Staeheli *et al.,* 2001). The gene expression levels of IL-1 and IL-6 were increased upon administration of LPS or *Salmonella* (Chapter 3; Withanage *et al.*, 2004; Tsai *et al.,* 2010). IL-1β is produced by phagocytes and other cell types in response to viral, bacterial and protozoal infections (Okamura *et al.,* 2004; Withanage *et al.,* 2004;

Dalloul *et al.,* 2006). IL-6 is a multi-functional cytokine that plays a role in the proinflammatory response (Giansanti *et al.*, 2006). In previous studies, IL-6 responded to *Salmonella* infection, whereas the response varied according to the chicken line studied and the period of exposure to the bacteria (Chiang *et al.*, 2008).

Defensins are small cationic antimicrobial peptides that may kill various microorganisms, such as Gram-negative and -positive bacteria, viruses and fungi (Lehrer and Ganz, 1999; Higgs *et al.*, 2007; Ma *et al.*, 2008; van Dijk *et al.*, 2008; Soman *et al.*, 2009). In chickens, the sequences of fourteen types of avian beta-defensins (*avβDs*), previously known as gallinacins, have been described to date (Michailidis *et al.*, 2010). In the chicken ovary, the expressions of several types of avβD genes and immunoreactive avβD-8, -10, and -12 have been identified (Chapter 2; Subedi *et al.*, 2007b; Michailidis *et al.,* 2010). Intravenous injection of chickens with LPS caused an increase in the expressions of some avβDs including avβD-12 as well as IL-1β and IL-6 in the theca layer of ovarian follicles (Chapter 3; Subedi *et al.*, 2007b). Also, the expressions of several avβDs in the chicken oviduct were significantly increased by LPS injection (Abdel Mageed *et al.*, 2008). In humans, it is reported that beta defensisn-2 (HBD-2) expression was induced in different cells by IL-1β (McDermott *et al.*, 2003; Liu *et al.,* 2003). Although avβD expression was modulated by LPS in ovarian tissue, it remains unknown whether the expression was induced by the direct effect of LPS stimulation or mediated by cytokines synthesized in response to LPS. The aim of this study was to determine whether the induction of avβDs in theca tissue by LPS was mediated by IL-1β and IL-6. We choose avβD-10 and -12 among fourteen types of avβDs because they responded differently to LPS in previous *in vivo* study; namely expression of avβD-12 was increased by LPS, but changes in the expression of avβD-10 was not significant (Subedi *et al.*, 2007b).

Materials and methods

Birds and tissue sampling

White Leghorn hens approximately 300-d-old and laying 5 or more eggs in a sequence were used. They were kept in individual cages under a lighting regimen of 14L: 10D and provided with feed and water *ad libitum*. The hens were euthanized under anesthesia with Somnopentyl (Kyoritsu Pharmaceutical Co., Ltd., Tokyo, Japan). Six hours after oviposition, the second (F2) and third (F3) largest preovulatory follicles were collected. Birds were handled in accordance with the Animal Experiment Committee regulation of Hiroshima University. Superficial connective tissue of the theca was removed. The theca was separated from the granulosa layer and washed in phosphate-buffered saline (PBS). Theca tissue was cut into small pieces (approximately 5 x 5 mm) and placed in a sterile tube for culture (Greiner Bio-one Co., Ltd, Tokyo, Japan) containing 4 ml culture medium.

Experimental design

The theca tissue was cultured as described below, and the effects of LPS on cytokines and avβDs expression (Experiment 1) and that of cytokines on avβD gene and protein expression (Experiment 2) were examined.

Experiment. 1. Effects of LPS on the expression of cytokines and avβDs

The dose dependency and time course of the effects of LPS on the cytokines (IL-1β and IL-6) and avβDs (avβD-10 and-12) were examined. The theca layer isolated from F2 was used for dose-dependency examination and F3 for time-course analysis (n= 5 birds). They were cultured as described below and stimulated by LPS at concentrations of 0, 10^2, 10^3, or 10^4 ng/ml for 3 h (dose dependency) or 10^3 ng/ml

LPS for 0, 0.5, 1, or 3 h (time course).

Experiment. 2.Effects of IL-1β and IL-6 on avβDs expression

The dose dependency and time course of the effects of IL-1β and IL-6 on avβD-10 and 12 gene and protein expression in the cultured theca were examined. The theca of F2 and F3 were isolated for dose and time dependency analysis of avβD gene expression, respectively (n= 5 and 4 birds for IL-1β and IL-6 stimulation, respectively). They were cultured as described below and stimulated with IL-1β or IL-6 (0, 10^2, or 10^3 ng/ml for 3 h (dose dependency); 10^3 ng/ml for 0, 1, or 3 h (time course). The theca tissue of those follicles were also incubated with 0 to 10^3 ng/ml IL-1β for 5 h to examine the effects on the avβD-12 protein expression, whose gene expression was upregulated (n= 5).

Tissue culture

Incubation was performed in a CO_2 incubator at 37 °C under a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was TCM-199 medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% (v/v) bovine serum (Biological industries, Kibbutz Beit Haemek, Israel), 100 U/ml penicillin and 100 μ g/ml streptomycin (Cosmo Bio, Tokyo, Japan).

The following chemicals were used in this study: LPS from *Sallmonella minnesota* (Invivogen, San Diego, CA, USA) dissolved in sterile/endotoxin free water (Invivogen), recombinant chicken interleukin-1β (IL-1β) (Abdserotec, Ltd., Morphosys UK, Oxford, UK).

Real-time PCR analysis

Total RNA was extracted from cultured tissues using Sepazol 1 super (Nacalai

Tesque Inc., Kyoto, Japan) as described previously (Chapter 3). It was purified by incubating at 42 °C for 45 min with DNase I (TaKaRa Bio Inc., Japan) at a concentration of 1 U/μg RNA. Purified RNA samples were reverse transcribed using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan) following the manufacturer' s instructions. Briefly, 10 μl reaction mixture containing 1 μg total RNA, 1 × RT buffer, 1 mM each deoxyribonucleotide triphosphate mixture, 20 U RNase inhibitor, 0.5 mM oligo (dT) and 50 U ReverTra Ace was placed on a Programmable Thermal Controller PTC-100 (MJ Research Inc., Waltham, MA, USA) and incubated at 42 °C for 30 min followed by heat inactivation at 99 °C for 10 min. Quantitative PCR analysis was performed for IL-1β, IL-6, avβD-10 and avβD-12 expression using the Roche Light Cycler (Roche Applied Science, IN, USA) as described previously (Chapter 3). Expression of RPS17 was examined as an internal control to normalize the values of each sample. A total of 20 μl reaction mixture containing 1 X SYBR Premix Ex Taq II (TaKaRa Co., Tokyo, Japan), 0.2 μM of each forward and reverse primers (Table 5), 1 μl cDNA and DNase free water was prepared. The reaction mixture was placed into 20 μl capillaries (Roche Diagnostics GmbH, Mannheim, Germany). Following denaturation at 95 °C for 30 s, PCR was carried out with a thermal protocol of 95 °C for 5 s and 60 °C for 20 s. Specificity of the amplified products was verified by melting curve analysis and by running the products on 2 % (w/v) agarose gel. Data analysis was performed as described previously in Chapter 3. Briefly, ΔCT was calculated for each sample by subtracting the cycle threshold (CT) value from the RPS17 (internal control) CT value of the respective target gene. For relative quantification, the ΔCT value of RPS17 was then subtracted from the ΔCT of each experimental sample to generate ΔΔCT. The ΔΔCT value was then fit in the formulae 2−ΔΔCT to calculate the approximate fold difference.

SDS-PAGE and Western blot

Theca tissue was homogenized separately in a 5 times volume of homogenization buffer consisting of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 0.1% (w/v) SDS and 1 mM phenylmethylsulfonylfluoride using a polytron homogenizer (Polytron PT1200c; Kinematica AG, Switzerland). The samples were centrifuged at 12,000 X g for 20 min at 4 °C. The supernatant was collected and the protein concentration was measured using a protein assay reagent (Bio-Rad Lab, Hercules, CA, USA) using bovine serum albumen as the standard protein.

The samples were separated by Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Tricine SDS-PAGE; 16% separating gel and 4% stacking gel) as described in Chapter 3. Samples were mixed with sample buffer composed of 30% (v/v) glycerol, 5% (v/v) mercaptoethanol, 4% (w/v) SDS, 0.06% (w/v) bromophenol blue and 150 mM Tris-HCl, pH 7.0, at a sample protein concentration of 1 μg/μl and boiled for 5 min. Each 10 μl sample mixture was loaded onto gels and run at 30 V in stacking gel and at 150 V in separating gel.

After SDS-PAGE, the proteins in the gel were electrophoretically transferred onto a PVDF membrane (Bio-Rad Lab.) at 270 mA for 1 h. The membrane was soaked in methanol for 10 min and then washed briefly with Tris-buffered saline containing 0.1 % Tween20 (TBS-T) (20 mM Tris HCl, pH 7.6, 0.8 % (w/v) sodium chloride and 0.1 % (v/v) Tween 20). It was incubated with 5% (w/v) casein milk (Roche, Mannheim, Germany) solution in TBS-T for 60 min and then incubated with antibodies to rabbit anti-chicken avβD-12 (Subedi *et al.*, 2008) diluted at a concentration of 10 μg/ml in TBS-T or mouse monoclonal anti-chicken β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:5,000 in TBS-T overnight at 4 °C. The anti- avβD-12 antibody in the antiserum had been purified using a HiTrap

affinity column conjugated with synthetic avβD-12 peptide (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and was same antibody used for previous study (Abdel Mageed *et al.,* 2009). The membrane was then washed in TBS-T for 30 min (10 min X 3) before incubation with peroxidase labeled anti-rabbit IgG for avβD-12 or anti-mouse IgG for β-actin (GE Healthcare, Buckinghamshire, UK) diluted at 1:5,000 in TBS-T for 1 h at room temperature. The membrane was washed with TBS-T for 30 min (10 min X 3 times) and the immunoprecipitates on the membrane were treated by Amersham ECL Western blotting detection reagents (GE Healthcare) for 1 min. Images were taken using an ATTO cooled CCD camera system EZ-Capture II (ATTO Co., Tokyo, Japan). Band intensity was measured using computer software CS analyzer version 3 (ATTO Co.).

Statistical analysis

The significance of differences in the expressions of avβD-12 among different treatments with LPS or IL-1β groups was determined by one-way ANOVA, followed by Tukey`s test. Differences were considered significant at $P < 0.05$.

Results

Experiment. 1. Effect of LPS on the expression of cytokines and avβDs

Figure 17 shows the effect of LPS stimulation on IL-1β and IL-6 expression in the theca of the ovarian follicle. The expressions of IL-1β and IL-6 were significantly upregulated with the increase of LPS doses from 0 to 10^4 ng/ml with a peak at 10^3 ng/ml. Expressions of both cytokines also increased with time after stimulation with 10^3 ng/ml.

Figure 18 shows the effect of LPS stimulation on the expression of avβD-10 and -12 in the theca. There was no significant change in the expression of both avβDs in response to different doses of LPS from 0 to 10^4 ng/ml, and to different time of incubation (0-3h) with 10^3 ng/ml LPS.

Experiment.2. Effects of IL-1β and IL-6 on avβD expression

Figure 19 shows the effect of recombinant IL-1β stimulation on the expression of avβD-10 and avβD-12 in theca tissue. IL-1β stimulation showed no significant effect on the expression of avβD-10. Meanwhile, stimulation of theca tissue for 3 h resulted in the increased expression of avβD-12 at doses 10^2 and 10^3 ng/ml. Also, avβD-12 expression significantly increased with the incubation time from 0 to 3 h with a peak at 3 h.

In contrast, IL-6 did not affect the expression of avβD-10 and -12 by incubating at different doses (at 0 to 10^3 ng/ml, for 3 h) or with different time (for 0 to 3 h, at 10^3 ng/ml) (Fig. 20).

The results of Western blot analysis for avβD-12 in the theca after stimulation for 5 h with different doses of IL-1β (0 to 10^3 ng/ml) are shown in Fig. 21. The density of an immunoreactive band for avβD-12 was increased significantly by 10^2 and 10^3 ng/ml IL-1β compared with tissue incubated without IL-1β.

Discussion

The expression of avβD-12 in the theca was upregulated by IL-1β that may be synthesized in response to LPS. Significant findings of this study were 1) LPS was able to induce the mRNA expression of proinflammatory cytokines, IL-1β and IL-6, while it did not induce avβD-10 or -12; 2) IL-1β, but not IL-6, induced avβD-12 mRNA

and protein expression, although it did not affect the expression of avβD-10.

Proinflammatory cytokines play a key role in initiating an innate immune response and assist in generating a local inflammatory response (Staeheli *et al.,* 2001). In the current *in vitro* study, it was found that LPS increased the expression of proinflammatory cytokines IL-1β and IL-6 in a dose- and time-dependent manner. These results support our previous *in vivo* study showing that injection of chickens with LPS resulted in a marked increase of proinflammatory cytokine genes and protein expression in the theca (Chapter 3). In broilers, SE infection resulted in the increased expression of proinflammatory cytokines (IL-1β and IL-6) in spleen and cecum (Cheeseman *et al.*, 2007). In newly hatched chicks, oral infection with *Salmonella* resulted in the upregulation of mRNA expression of proinflammatory cytokines of the intestinal and liver tissues in correlation with inflammatory signs (Withanage *et al.*, 2004). The current study used LPS of *Salmonella*, and thus the expressions of IL-1β and IL-6 could be probably upregulated in the theca in response to SE, as observed in other organs. In theca tissue the expression of TLR4 increased significantly after LPS injection (Subedi *et al.*, 2007a). It is likely that TLR4 in the theca could recognize LPS to induce IL-1β and IL-6 under current experimental conditions.

The current *in vitro* study showed that LPS did not directly affect the expression of avβD-10 and 12 in theca tissue. In a previous study, intravenous injection of chickens with LPS increased the expression of avβD-12 but not avβD-10 in the theca and granulosa layers (Subedi *et al.*, 2007b). The expression of avβD-12 was also upregulated in hen ovarian tissues by oral *Salmonella* inoculation (Michailidis *et al.*, 2010); thus, it is likely that LPS or *Salmonella* stimulated the expression of avβD-12 *in vivo* but not *in vitro*, whereas it might not affect avβD-10 expression significantly both *in vivo* and *in vitro*. These results suggest that LPS may not exert significant effects to induce avβD-10. It is necessary to examine why the effect of LPS to induce the

avβD-12 was different between *in vitro* and *in vivo* studies. One possibility is that the induction of avβD-12 by LPS in the theca layer might occur indirectly through the production of cytokines, which in turn might induce avβD-12.

In experiment 1, LPS stimulation upregulated expression of IL-1β and IL-6, but not avβD-10 and -12. The results of experiment 2 showed that stimulation of theca tissue with IL-1β increased the gene and protein expression of avβD-12, although avβD-10 gene expression was not affected. Thus, it is suggested that, in theca tissue, IL-1β is synthesized in response to LPS, and then IL-1β stimulates the expression of avβD-12. Since the expression of avβD-10 and 12 was not changed significantly by IL-6, IL-6 may not play roles in the regulation of expression of those avβDs. In humans also, human beta defensin2 (HBD2) expression in gingival keratinocyte cultures was increased by IL-1β up to ~16-fold, whereas it was increased by LPS only up to ~5-fold (Mathews *et al.*, 1999). It remains unknown why the expressed IL-1β did not affect avβD-12 within the culture. It is assumed that (1) the amount or biological activity of IL-1β synthesized *in vitro* was not enough to stimulate avβD-12 expression; (2) the incubation time was short because time for IL-1β synthesis is necessary before IL-1β stimulates avβD-12 expression. Significant increase of avβD-12 expression appeared 3 h after IL-1β stimulation (Fig. 3).

In conclusion, the results of this study suggest that theca tissue expresses IL-1β and IL-6 in response to LPS, and then IL-1β stimulates avβD-12 expression. This process of avβD-12 synthesis may occur against infection by *Salmonella* and other Gram-negative bacteria in ovarian follicles.

Abstract

The aim of this study was to determine whether the expression of avβDs in the follicular theca tissue was stimulated directly by LPS or indirectly through IL-1β induced by LPS. Theca tissues of ovarian follicles were collected from White Leghorn hens. Tissue specimens of those theca tissues were cultured in TCM-199 culture medium and stimulated by LPS from *Sallmonella minnesota*, IL-1β or IL-6. In the first experiment, changes in the expression of IL-1β, IL-6, avβD-10, and avβD-12 in response to LPS stimulation was examined by qRT-PCR. The expression of avβD-10 and 12 had been known to be expressed in the theca. In the second experiment, changes in the expression of avβD-10 and -12 in response to recombinant chicken IL-1β or IL-6 stimulation were examined by qRT-PCR. Expression of avβD-12 protein after IL-1β stimulation that showed changes in the gene expression was analyzed by Western blotting. In the first experiment, LPS was able to induce IL-1β and IL-6, but not avβD-10 or -12. In the second experiment, IL-1β was able to upregulate significantly the avβD-12 gene expression and protein. However, IL-6 did not exert significant effects on the expression of avβD-10 and -12. It is suggested that LPS may stimulate theca cells to produce proinflammatory cytokines while, in turn, IL-1β stimulates those cells to synthesize avβD-12, which may be able to attack infectious *Salmonella* and other Gram-negative bacteria.

Table 5. Rrimers used for RT-PCR analysis

Gene	Primer sequences	Accession No.
avβD-10	F: CTGTTCTCCTCTTCCTCTTCCAG	NM_001001609
	R: AATCTTGGCACAGCAGTTTAACA	
avβD-12	F: GGAACCTTTGTTTCGTGTTCA	AY534898
	R: GAGAATGACGGGTTCAAAGC	
β-actin	F: TTCCAGCCATCTTTCTTG	X00182
	R: TCCTTCTGCATCCTGTCA	
IL-1β	F: ACTGGGCATCAAGGGCTA	NM_204524
	R: GGTAGAAGATGAAGCGGGTC	
IL-6	F: AGAAATCCCTCCTCGCCAAT	NM_204628
	R: AAATAGCGAACGGCCCTCA	
RPS17	F: AAGCTGCAGGAGGAGGAGAGG	NM_204217
	R: GGTTGGACAGGCTGCCGAAGT	

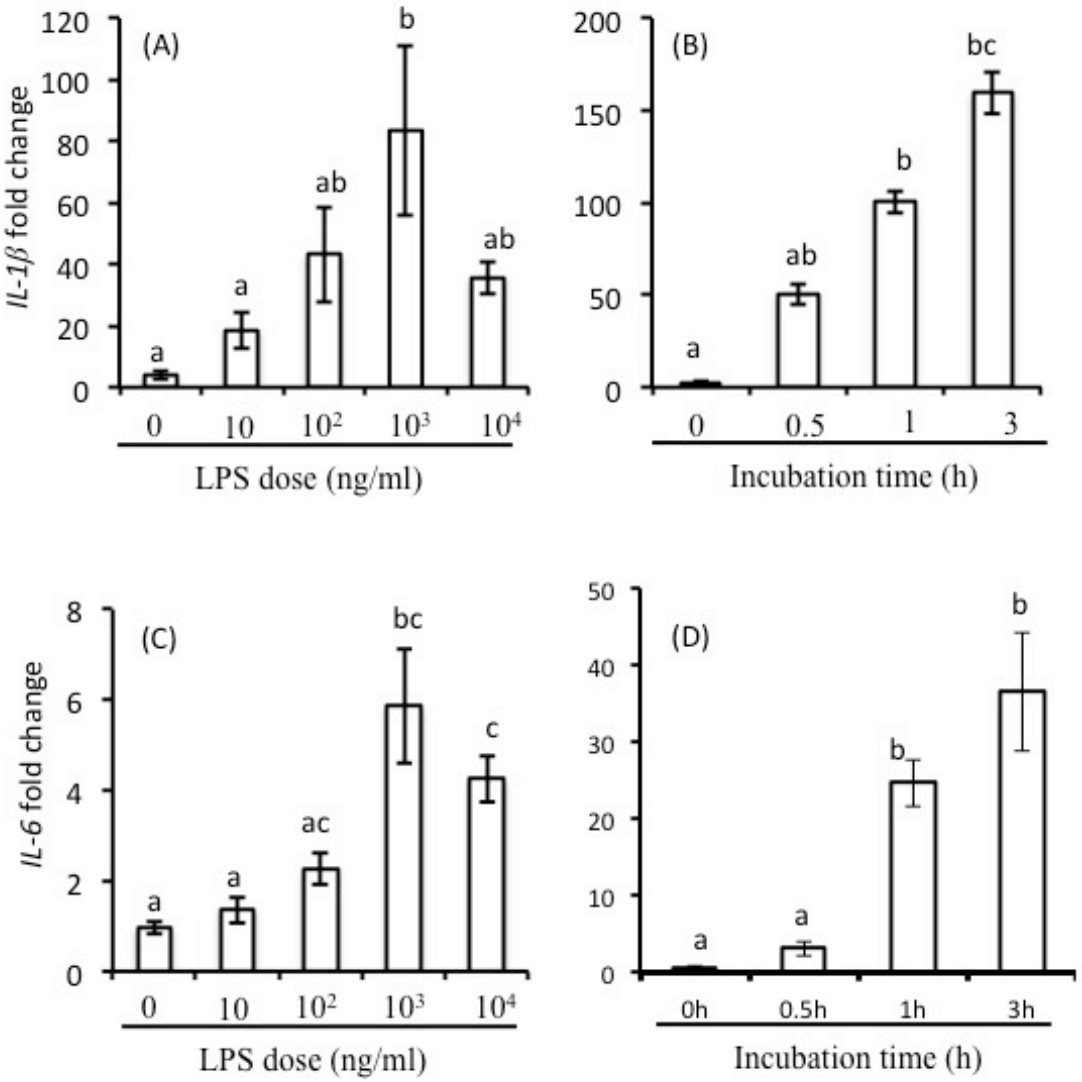

Fig. 17. Effects of lipopolysaccharide (LPS) stimulation on the mRNA expression of IL-1β and IL-6 in hen ovarian theca layer. A and C. The expression level of IL-1β and IL-6 mRNA in the theca tissue stimulated for 3 h with 0, 10, 10^2, 10^3, 10^4 ng LPS/ml. B and D. The expression level of IL-1β and IL-6 mRNA in the theca tissue stimulated with 10^3 ng LPS/ml for 0, 0.5, 1, and 3 h. Each value shows the mean ± SEM of fold changes in expression. Bars with different letters are significantly different within LPS-stimulated treatments ($P < 0.05$).

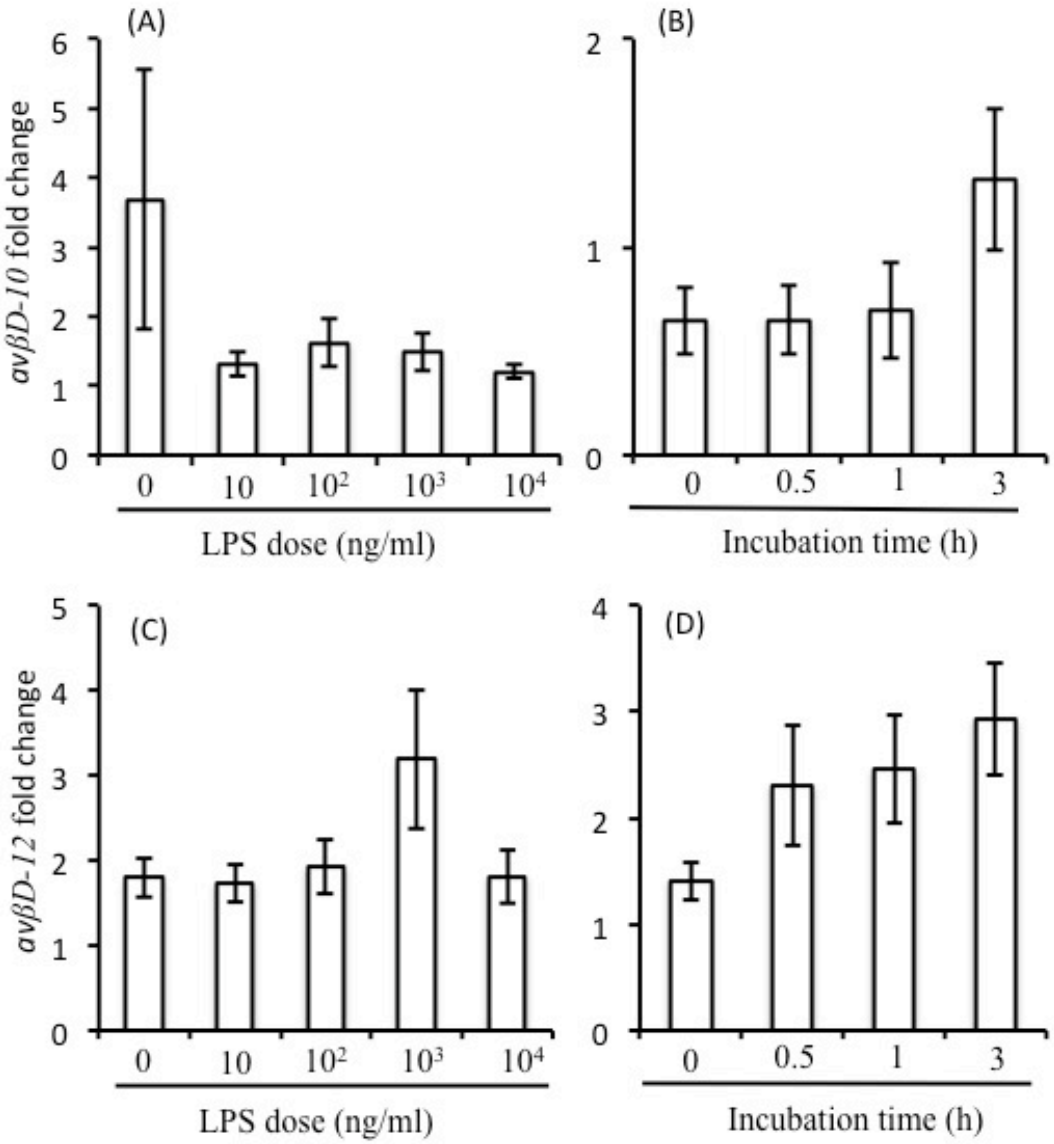

Fig. 18. Effects of lipopolysaccharide (LPS) stimulation on the mRNA expression of avβD-10 and avβD-12 in hen ovarian theca layer. A and C. The expression level of avβD-10 and avβD-12 mRNA in the theca tissue stimulated for 3 h with 0, 10, 10^2, 10^3, 10^4 ng LPS/ml. B and D. The expression level of avβD-10 and avβD-12 in the theca tissue stimulated with 10^3 ng IL-1β /ml for 0, 0.5, 1, and 3 h. Each value shows the mean ± SEM of fold changes in expression. There was no significant difference within LPS-stimulated treatments ($P < 0.05$).

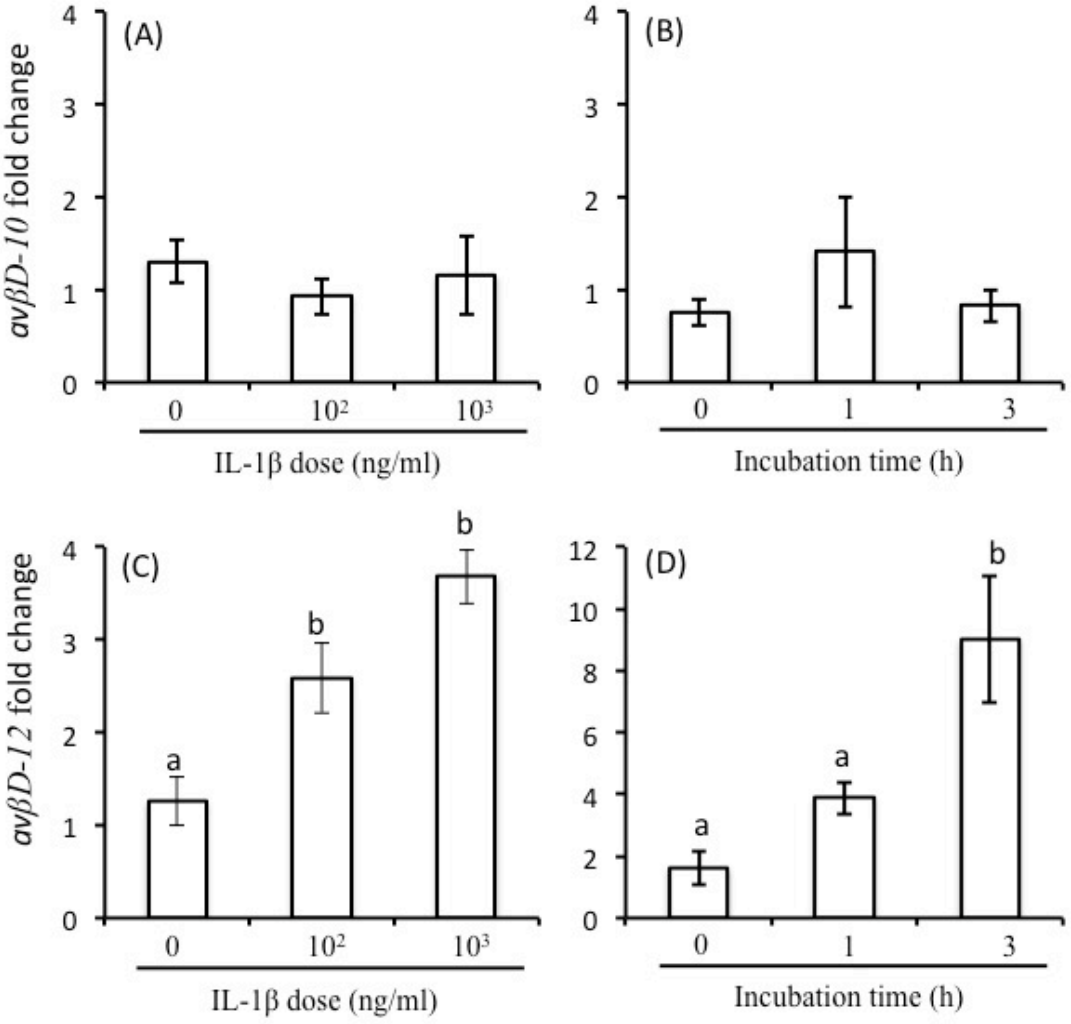

Fig. 19. Effects of interleukin-1β (IL-1β) stimulation on the mRNA expression of avβD-10 and avβD-12 in hen ovarian theca layer. A and C. The expression level of avβD-10 and avβD-12 mRNA in the theca tissue stimulated for 3 h with 0, 10^2, and 10^3 ng IL-1β /ml. B and D. The expression level of avβD-10 and avβD-12 in the theca tissue stimulated with 10^3 ng IL-1β /ml for 0, 1, and 3 h. Each value shows the mean ± SEM of fold changes in expression. Bars with different letters are significantly different within IL-1β -stimulated treatments ($P < 0.05$).

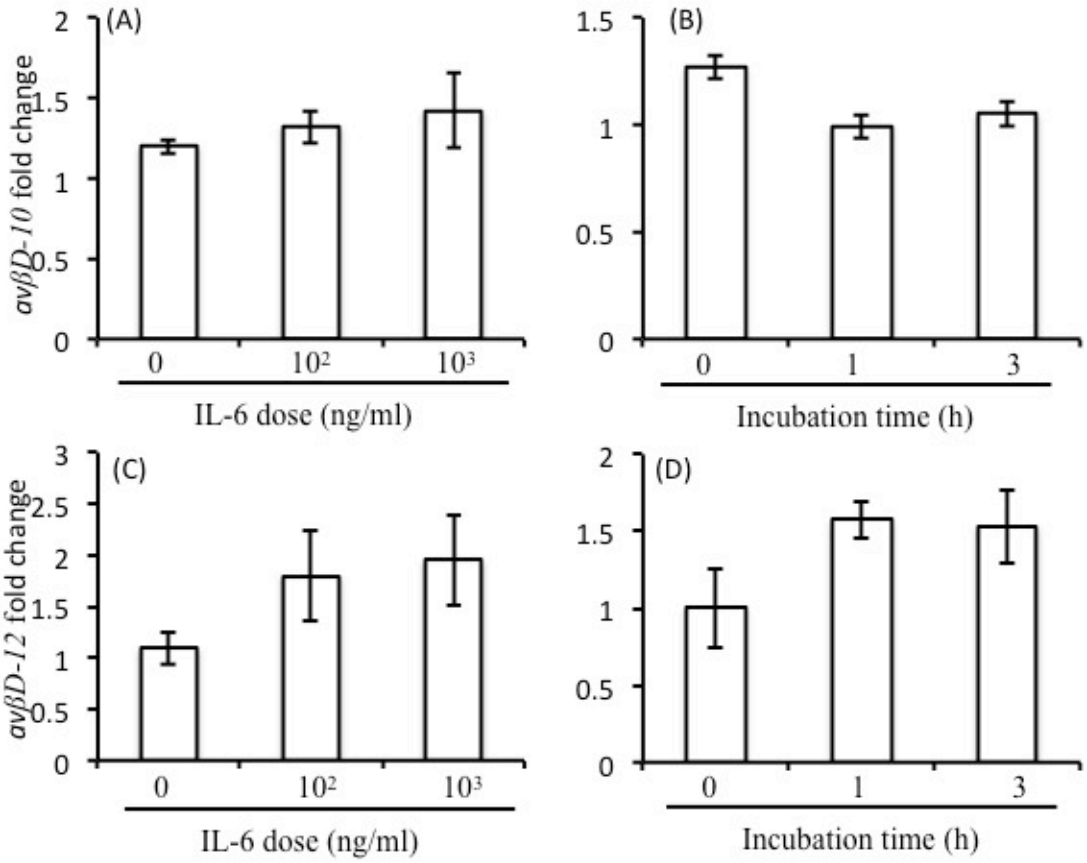

Fig. 20. Effects of interleukin-6 (IL-6) stimulation on the mRNA expression of avβD-10 and avβD-12 in hen ovarian theca layer. A and C. The expression level of avβD-10 and avβD-12 mRNA in the theca tissue stimulated for 3 h with 0, 10^2, and 10^3 ng IL-6/ml. B and D. The expression level of avβD-10 and avβD-12 in the theca tissue stimulated with 10^3 ng IL-6 /ml for 0, 1, and 3 h. Each value shows the mean ± SEM of fold changes in expression. there was no significant difference within IL-6 - stimulated treatments ($P < 0.05$).

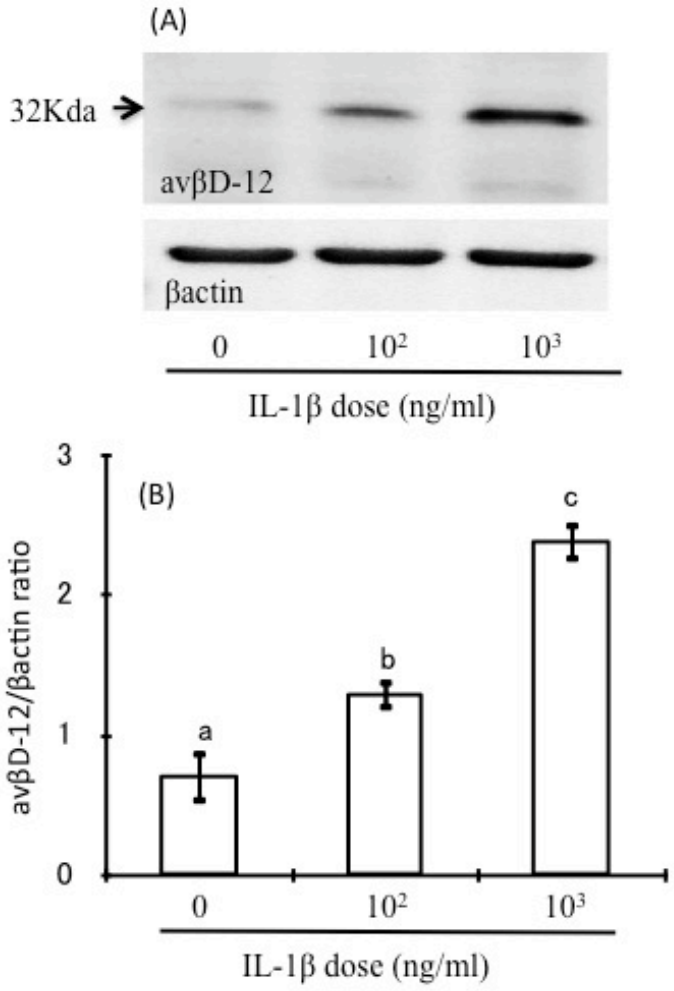

Fig. 21. Western blot analysis of avBD-12 and β-actin in the theca layer of the ovarian follicle. A. Immunoblot of the theca tissue treated with 0, 10^2, and 10^3 ng IL-1β/ml for 5 h. Bands immunostained with avBD-12 or β-actin antibody. B. Value of avβD-12 band intensity change relative to that of β-actin band density value. Data presented are means ± SEM of 5 independent experiment . Bars with different letters are significantly different within IL-1β -stimulated treatments ($P < 0.05$).

Chapter 5

General Discussion

Infection of hen ovary by pathogenic agents may cause contamination of egg yolk by pathogens. Contaminated eggs may induce human food poisoning. Also, ovarian infection may cause ovarian disorders that may result in decreased egg production and vertical transmission of infection to embryo and chicks originating from the contaminated eggs. In chickens, one of the most noticeable pathogen that infect ovary is *Salmonella* organisms. Infection of the ovary and the reproductive tract and subsequent transmission to eggs are important pathological features of Salmonellosis (Keller *et al.*, 1995; Gantois *et al.*, 2008). In the past decade, several studies have been conducted to elucidate the chicken host cell immune response to SE or to other serotypes' infections (Kaiser *et al.,* 2000; Swaggerty *et al.*, 2006; van Hemert *et al.,* 2006 ; Berndt *et al.*, 2007). After inoculation of laying hens with SE, the organism was isolated from tissues of the ovarian folliclular wall and yolk (Gast and Beard, 1990; thiagarajan *et al.*, 1994). SE was immunohistochemically identified in the ovarian stroma and preovulatory follicles of Japanese quail after intraperitonial inoculation with SE (Takata *et al.*, 2003). Interaction between ovarian follicular wall of preovulatory follicles and SE may be involved in the colonization of *Salmonella* in the preovulatory follicles. It has been reported that SE can attach to granulosa cells *in vitro,* suggesting that granulosa cells of the preovulatory follicles is a preferred site for colonization of SE (Thiagarajan *et al.*, 1996). It is believed that SE organisms are incorporated into macrophages in the intestinal mucosa, which may migrate to other

organs via blood circulation. The high vascularization of the preovulatory follicles and increased permeability of the blood vessels in large follicles (Griffin *et al.*, 1984) may facilitate the transport of such SE from the blood stream to the developing follicle (Barrow and Lovell, 1991). The organisms may penetrate the basement membrane and reach the granulosa cells and yolk (Thiagarajan *et al.*, 1994). A lot of scientific groups worked to study the local immunity of the chicken ovary and the mechanism of egg infection. Understanding the local immune system of the avian ovary will enable us to develop tools to produce eggs free of microbial contamination. The current study investigated the local immune functions mediated by antimicrobial peptides and cytokines in hen ovary.

Antimicrobial peptides provide the first line of defense against attacking pathogens. Recently antimicrobial peptides were recognized as key mediators of the innate immune responses (Zasloff, 2002; Sugiarto and Yu, 2004; Townes *et al.*, 2004, 2009). Defensins are one of the key antimicrobial components of vertebrates and invertebrates innate immunity (Lehrer and Ganz, 2002; Selsted and Ouellette, 2005). avβDs, which are members of antimicrobial molecules, attack a wide range of microorganisms including and Gram positive and Gram negative bacteria, fungi and yeast (Evans *et al.,* 1995; Harmon, 1998; Sugiarto and Yu, 2004; van Dijk *et al.*, 2008).

In Chapter 2, immunoreaction products of avβD-8, -10 and -12 proteins were localized in hen ovarian follicles. We found out that avβDs proteins located in the theca interna and granulosa, and their amount was increased with follicular growth from white follicles to preovulatory yellow follicles. During follicular growth the theca and granulosa layers undergo structural and functional changes. The theca layer becomes distinguishable into theca interna and theca externa. Also, the granulosa cells of stromal and white follicles show a high proliferative activity and

undergo differentiation before developing into preovulatoy follicle (Yoshimura *et al.*, 1996). Estrogen production in the theca was greater in white follicles than yellow follicles (Yoshimura *et al.,* 1995). The changes in the avβDs synthesis may occur in association with differentiation in the structure and endocrine activity of the ovarian follicles cells during growth from white to preovulatory yellow follicles. The blood flow was greater in the yellow follicles compared to the white follicles (Scanes *et al.*, 1982; Griffin *et al.,* 1984), which may increase the chance of pathogens to reach the yellow follicles. The development of host immunity may be necessary to protect the preovulatory yellow follicles from circulating pathogens.

Expression of *avβD-1, -2, -4* to *-12,* and *-14* was identified in the theca layer of the ovarian follicle, while that of *avβD-3* and *-13* was not identified using real time PCR (Chapter 4). In a previous study, only *avβD-1, -2, -7, -8, -10,* and *-12* were identified in the theca layer of the third largest follicle (Subedi *et al.*, 2007b). Lynn *et al.* (2004) studied the differential expression of *avβDs* among 21 different chicken tissues, including reproductive, digestive, respiratory, nervous, and lymphoid organs. They showed that every tissue exerts a pattern of *avβDs* expression different from other body tissues, and suggested that there may be the tissue specificity in β-defensin expression. Expression of avβD genes may be different according types of hosts, tissues, or even cells (Ebers *et al.*, 2009). However, it was likely that *avβD-10* and *-12* were usually expressed in the follicular theca layer.

Only *avβD-10* and *-12* were expressed in the ovary of 2 month old chickens (Xiao *et al.*, 2004). These results indicate the importance of these two defensins in providing a defense line against pathogens in the chicken ovarian follicles. The expression of *avβD-1, -7* and *-12* in the theca layer of the third largest follicles was increased in response to LPS (Subedi *et al.,* 2007b). A significant up-regulation of

avβD-4, -5, -7, -11 and *-12* was observed in the ovary of SE infected sexually mature birds (Michailidis *et al.*, 2010). From these *in vivo* study reports, the avβD-12, but not avβD-10 was upregulated by LPS inoculation or SE infection. In the current *in vitro* study, LPS was not able to upregulate the expression of both of *avβD-10* and *-12* suggesting that LPS could not induce both *avβDs* in the theca cells directly, and other molecules may be involved in the upregulation of these *avβDs* expression (Chapter 4).

In Chapters 3 and 4, the induction of proinflammatory cytokines IL-1β and IL-6 by LPS was shown both *in vivo* and *in vitro*. In contrast, LPS could not induce *avβD-10* and *12*, meanwhile IL-1β induced *avβD-12* (Chapter 4). It was suggested that increase in cytokine levels might trigger protective mechanisms indirectly resulting in the induction of antimicrobial genes (Biswas and Yenugu, 2011). In human, proinflammatory cytokines, such as IL-1β and TNF-α, are potent stimulators of HBD-2 expression in corneal epithelial cells (McDermott *et al.,* 2003), A549 cells (Jang *et al.*, 2007), astrocytes (Hao *et al.*, 2001), and human dental pulp cells (Kim *et al.*, 2010). Because IL-1β was able to induce avβD-12 *in vitro* (Chapter 4), it is possible that LPS induces IL-1β synthesis. In turn IL-1β induces the avβDs.

In human, IL-1β induces HBD-2 mRNA expression in A549 cells (Jang *et al.*, 2004). Also, in human dental pulp cells, IL-α increased HBD-2 mRNA expression through the induction of IL-1 receptor, in which PKC, P38, MAPK, JNK, ERK, AMPK, and NFκB pathways were responsible (Kim *et al.,* 2010). We hypothesize that proinflammatory cytokines induce avβDs synthesis through the activation of NFκB pathway. Further studies are necessary to investigate the proinflammatory cytokines receptors in the ovarian tissue and intra cellular molecules involved in the induction of avβDs in the ovarian tissue.

Cytokines regulate not only the innate immune response but also the adaptive immune response. In Chapter 3, the inoculation with LPS recruited avian heterophils and T cells to the ovarian tissue. Chemokines show chemotactic activity that acts primarily by attracting leukocytes to the sites of inflammation and facilitating their migration from the circulation into infected tissue to mediate host defense mechanisms (Ebnet and Vestweber, 1999). In a human study on the epithelial rests of Malassez *in vitro*, the mRNA expressions of IL-1α, IL-6, IL-8 (CXCL8), and GM-CSF were upregulated by stimulation with LPS in a dose- and time dependent manner (Liu *et al.,* 2001). Also in human, oral bacteria, such as *Porphyromonas gingivalis*, stimulated the *IL-1, IL-6,* and *IL-8* expression in oral epithelial cells (Sandros *et al.*, 2000). Elevated expressions of proinflammatory cytokine mRNAs have been also found after viral, bacterial and protozoal infections in chickens (Laurent *et al.,* 2001; Okamura *et al.*, 2004; Khatri *et al.*, 2005). Increased levels of CXCLi2 and IL-1β mRNA have been described in the intestinal tissues and in the livers of birds infected with SE (Withanage *et al.*, 2004). *In vitro* stimulation of macrophages isolated from Salmonella-resistant chickens by *Salmonella* upregulated pro-inflammatory cytokine and chemokine mRNA expression levels, including *IL-1β, IL-6*, and *CCLi2* (Wigley *et al.,* 2006). *IL-1β* and *IL-6* mRNA expression was increased in broiler after SE infection (Cheeseman *et al.*, 2007). Increased expression of the proinflammatory cytokines and chemokines (up to several hundred-fold) correlated with the presence of inflammatory signs in the liver, spleen, jejunum, ileum, and cecal tonsils in newly hatched chickens (Withanage *et al.,* 2004). An avian *in vitro* epithelial model has indicated that production of the proinflammatory cytokine *IL-6* was induced by invasion with both *Salmonella* serovar Typhimurium and *Salmonella* serovar Enteritidis (Kaiser *et al.,* 2000). Expression of both *IL-6* and *CXCLi2* mRNA were upregulated in heterophils after *S. Enteritidis* exposure (Kogut *et al.*, 2003). Tsai *et al.* (2010) suggested that during the SE

infection, chicken granulosa cells recruit cells of the innate immune responses through the production of proinflammatory cytokines and chemokines. From these studies, it would be accepted that proinflammatory cytokines IL-1β and IL-6 and CXCLi2 (chemokine) were upregulated in most species and tissues after infection by pathogens including *Salmonella* organisms.

In mammals, as a part of the infection process, proinflammatory cytokines and chemokines, in particular the CXC chemokines (IL-8) are elicited (Zhang *et al.*, 2003), and play roles in the recruitment of neutrophils to the site of infection, leading to inflammation and damage. In human, CXC chemokines recruit neutrophils (Laurent *et al.*, 2001). In chicken, Cheeseman *et al.* (2008) postulated that CXCLi2 was able to recruit avian heterophils as CXCL8 preferentially attracts mammalian neutrophils (Gangur *et al.,* 2002). IL-6, a pro-inflammatory cytokine, is involved in the transition of immune response from innate to acquired immunity and plays a key role in the recruitment of immune cells to sites of infection (Kaiser *et al.,* 2000). Heterophils are the avian equivalent of mammalian neutrophils and play a key role in protecting chickens from the development of systemic disease following infection with SE and other microbes (Kogut *et al.,* 1994). Heterophils migrated to the liver and intestinal villi of newly hatched chickens infected with *Salmonella* typhimurium, accompanied by elevated levels of pro-inflammatory cytokines in the tissues (Withanage *et al.*, 2004), indicating a potential role of pro-inflammatory cytokines in the acute inflammatory response. Infection with *Salmonella* serovar Typhimurium leads to some diarrhea and intestinal lesions in young chickens (Barrow *et al.*, 1987) and to an influx of heterophils into the gut accompanied by inflammation and damage to villi (Henderson *et al.*, 1999). The early expression of CXC chemokines correlated with inflammation and pathology seen in the intestines and ceca, and was consistent with an influx of

polymorphonuclear heterophils to these sites (Withanage *et al.*, 2004). The results of this study demonstrated the upregulation of cytokines and chemokines by LPS, which was accompanied by increased influx of heterophils and T cells to the theca layer of the preovulatory follicles (Chapter 3). Thus, it is suggested that infection of Gram negative bacteria induces proinflammatory cytokines and chemokines, followed by heterophils and T cells that are responsible for cellular immune response in the follicles like other organs.

SE infection resulted in a significant induction of TLR4 and TLR15 in the ovary of mature birds (Michailidis *et al.*, 2010). TLR4 that recognizes LPS was increased with follicular growth and in response to LPS in the granulosa and theca layers of hen preovulatory follicles (Subedi *et al.*, 2007a). Induction of proinflammatory cytokines by LPS possibly may be mediated by TLR4 in the theca (Chapter 4). Previous research has shown that functional TLR15 is unique to chickens and may be essential to the response to *Salmonella* infection (Higgs *et al.*, 2006; Nerren *et al.*, 2009). Thus, it is assumed that the expression of proinflammatory cytokines is upregulated in the theca in response to *Salmonella* infection though interaction with TLR4 and 15.

In conclusion, the results of this study suggest that avβDs, proinflammatory cytokines and ckemokines could by synthesized in hen ovary. Their expression are upregulated in response to LPS. The synthesized proinflammatory cytokines in response to LPS may upregulate avβDs production to kill bacteria. The proinflammatory cytokines and chemokines may also recruit the cellular immune system elements eliciting the inflammatory process for encountering the pathogens. These innate immune functions mediated by avβDs and cellular immune response may play essential role in the local host defense in the ovary against Gram-negative

bacteria including *Salmonella* organisms. It may be also possible that this immune system responds to other pathogenic microbe species.

Chapter 6

Summary

Immune function in the ovary plays essential roles in the defense to pathogens in the ovarian tissue and suppression of transmission of the microorganisms to the eggs. The goal of this study was to determine the innate immune function mediated by avian β-defensins (avβDs), a group of antimicrobial peptides, in chicken ovary. Specifically, it was examined whether avβDs proteins were expressed in the ovary, and then whether lipopolysaccharide (LPS), or cytokines induced by LPS enhanced the expression of avβDs. It was also examined whether LPS enhanced T cell influx in association with the changes in cytokine expression to know the presence of linkage between innate and adaptive immunity in the ovary.

1- Changes in the localization of immunoreactive avian beta-defensin-8, -10 and -12 in hen ovarian follicles during follicular growth

Defensins are small cationic antimicrobial peptides that may kill various microorganisms, such as Gram-negative and -positive bacteria, viruses and fungi. The aim of this study was to identify immunoreactive (ir) avβDs proteins in the hen ovarian follicles and the changes in their localization with follicular growth. The ovarian follicles of White Leghorn hens at different growth stages, namely the largest (F1), second and third largest (F2 and F3), prehierarchal small yellow and cortical follicles in the stroma, were collected. The presence of ir-avβD-8, -10, and -12 was examined by immunohistochemistry and western blot. The three ir-avβDs showed a similar pattern of immunostainings in the follicular tissues at different growth stages. In the granulosa cells, the immunoreactions' products of the three avβDs were identified in the cortical follicles, whereas their density was reduced in small yellow follicles. The

granulosa cells of yellow follicles (F3-F1) showed dense immunolabelings for three avβDs. The interstitial cells showed a faint immunolabeling for avβD-12 but were negative for avβD-8 and -10 in the cortical follicles, whereas they were weakly stained in the small yellow follicles. Dense immunoreactions products were noticed in the theca interna cells of F3 - F1 follicles. Western blot analysis showed a single band for each defensin in the theca and granulosa layers of F2 Follicle. These results suggest that av D-8, -10 and -12 proteins are expressed in the specific cells in the follicles, namely interstitial or theca interna cells and granulosa cells, where their amounts are likely increased with follicular growth. These avβDs with follicular growth may protect the ovarian tissues from infection by pathogens.

2- Effects of lipopolysaccharide on the expression of proinflammatory cytokines and chemokines and influx of leukocytes in hen ovary

Cytokines are key factors for triggering the immune response and inflammation. Cytokines are a group of mediators regulating cellular functions that are secreted by specific cells to affect the behavior of other cells, playing a role in the regulation of immune and inflammatory processes. The aim of this study was to determine whether the expression of proinflammatory cytokines and chemokines in ovarian cells was changed in response to lipopolysaccharide (LPS), a gram-negative bacterial component, to recruit heterophils and T cells. White Leghorn laying hens were intravenously injected with LPS or saline, and their ovarian follicles and stroma were collected. Changes in the mRNA expression of interleukin (IL)-1β, IL6 and CXCLi2 chemokine in the theca and granulosa layers and ovarian stroma were analyzed by quantative reverse transcriptase PCR (qRT-PCR), whereas proteins of IL-1β and IL6 were also identified by Western blot analysis. Localization of heterophil-like cells, CD4+ and CD8+ T cells was examined by general histology and

immunohistochemistry. The expressions of IL-1β, IL6 and CXCLi2 were significantly increased in the granulosa layer, theca layer and stroma tissues by 3 to 6 h after LPS injection. Increase of IL-1β and IL6 proteins in those tissues was also identified 6 hrs after LPS injection. The LPS stimulation resulted in the increased influx of heterophil-like cells and CD4+ T cells, but not of CD8+ cells, in the theca layers of yellow follicles. Saline injection affected neither expression of examined proinflammatory cytokines and chemokines nor frequencies of immunocompetent cells. These results suggest that ovarian follicular cells and stromal cells have the ability to express proinflammatory cytokines and chemokines, and their expression is upregulated by LPS in association with the recruitment of heterophil-like cells and T cells. Those proinflammatory cytokines and chemokines may play key roles in the induction of immune response for protection of the chicken ovary from infection with gram negative bacteria.

3- Effects of lipopolysaccharide and interleukins on the expression of avian β-defensisns in hen ovarian follicular tissue

Chicken proinflammatory cytokines such as IL-1β and IL-6 appear to function, as do their mammalian counterparts, in mediating an inflammatory response. In humans, it is reported that beta defensisn-2 (HBD-2) expression was induced in different cells by IL-1β . The aim of this study was to determine whether the expression of avβDs in the follicular theca tissue was stimulated directly by LPS or indirectly through IL-1β induced by LPS. Theca tissues of ovarian follicles were collected from White Leghorn hens. Tissue specimens of those theca tissues were cultured in TCM-199 culture medium and stimulated by lipopolysaccharide from Sallmonella minnesota (LPS), IL-1β or IL-6. In the first experiment, changes in the

expression of IL-1β, IL-6, avβD-10, and avβD-12 in response to LPS stimulation were examined by qRT-PCR. The expression of avβD-10 and 12 has known to be expressed in the theca. In the second experiment, changes in the expression of avβD-10 and -12 in response to recombinant chicken IL-1β or IL-6 stimulation were examined by qRT-PCR. Expression of avβD-12 protein after IL-1β stimulation that showed changes in the gene expression was analyzed by Western blotting. In the first experiment, LPS was able to induce IL-1β and IL-6, but not avβD-10 or -12. In the second experiment, IL-1β was able to upregulate significantly the avβD-12 gene expression and protein. However, IL-6 did not exert significant effects on the expression of avβD-10 and -12. It is suggested that LPS may stimulate theca cells to produce proinflammatory cytokines while, in turn, IL-1β stimulates those cells to synthesize avβD-12, which may be able to attack infectious Salmonella and other Gram-negative bacteria.

4. Conclusion

The results of the study revealed that avβDs, proinflammatory cytokines and ckemokines could be synthesized in hen ovary, and their expressions were upregulated in response to LPS. The synthesized proinflammatory cytokines in response to LPS may upregulate avβDs to kill bacteria. The proinflammatory cytokines and chemokines may also recruit the cellular immune system elements eliciting the inflammatory process for encountering the pathogens. These innate immune functions mediated by avβDs and cellular immune response may play essential role in the local host defense in the ovary against gram-negative bacteria including Salmonella organisms.

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