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Regulation of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells by gut microbiota in chicken

닭 장내 미생물에 의한 $CD4^+CD8^-CD25^+$ 및 $CD4^+CD8^+CD25^+$ T 세포 조절 기전 연구

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By

In Kyu Lee

School of Agricultural Biotechnology

Graduate School

Seoul National University

농 학 박 사 학 위 논 문

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지도교수 윤 철 희

이 논문을 농학 박사학위논문으로 제출함

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이 인 규

이인규의 박사학위논문을 인준함

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위 원 장	한 승 현	(인)
부위원장	윤 철 희	(인)
위 원	박 병 철	(인)
위 원	강 석 성	(인)
위 원	박 태 섭	(인)

Summary

Gut microbiota in chicken has long been studied and considered for mostly growth performance point of view. And therefore, immunological studies regarding gut homeostasis in chicken have been insufficiently achieved. Regulatory T cells (Tregs) are a notable subtype of $CD4^+$ T cells playing an important role to maintain gut homeostasis in humans and animals. Intestinal Tregs are induced by gut microbiota, such as, *Clostridium* spp. cluster IV and XIVa strains, altered Schaedler flora (ASF), or *Bacteroides fragilis* in mice. Although it has been suggested that $CD4^+CD25^+$ T cells act as Tregs, there are no such studies showing the relationship between gut microbiota and Tregs in chickens.

The first, I established the model for ABX-treated chickens by the administration of various concentrations of antibiotic cocktail consisting of ampicillin, gentamycin, neomycin, metronidazole, and vancomycin in water. Cecal contents from chickens treated with antibiotic cocktail consisting of 100 μg/ml of ampicillin, gentamycin, neomycin and metronidazole, and 50 μg/ml of vancomycin for 7 days eliminated colony forming unit (CFU) over 99%. These chickens treated by certain concentration of antibiotics cocktail (ABX) were referred as ‘ABX-treated chickens’. There were no changes on physiological traits, for example, weight of body and immune organs (spleen, bursa and liver), length of intestine (duodenum, jejunum, ileum and large intestine) and the concentration of glucocorticoid in the serum. Furthermore,

the population and MHC class II expression on B cells and macrophages in the cecal tonsils and spleen were not changed. I concluded that physiological traits, B cells and macrophages were not changed in ABX-treated chickens.

The second, I examined whether subtype of $CD4^+$ T cells was changed in ABX-treated chickens. In cecal tonsil, $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells were significantly decreased in ABX-treated chickens, however these cells in the spleen were not changed. The expression of IL-10 and IFN-γ was significantly decreased in $CD4^+CD8^-CD25^+$ T cells from cecal tonsils of ABX-treated chickens. It was noting that $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells from ABX-treated chickens did not suppress the proliferation of $CD4^+CD25^-$ T cells. The reduction of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in cecal tonsils from ABX-treated chickens expressed high level of $CD5^{hi}$. Interestingly, the percentage of thymic $CD4^+CD8^+CD25^+$ T cells was not changed in ABX-treated chickens. Conclusively, the population and suppressive function of peripheral $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells decreased in ABX-treated chickens.

The third, I examined what factors affected the population of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells. ABX-treated chickens co-housed with wild type chickens recovered the number of gut microbiota, and the proportion of $CD4^+CD8^-CD25^+$ or $CD4^+CD8^+CD25^+$ T cells in cecal tonsils to similar levels as those of wild type chickens. The results further showed that Gram-positive bacteria appeared to be responsible for the changes of $CD4^+CD8^-CD25^+$ or $CD4^+CD8^+CD25^+$ T cells in cecal tonsils. Feeding acetate, one of the

short chain fatty acids, in ABX-treated chickens recovered $CD4^+CD8^-CD25^+$ T cells and $CD4^+CD8^+CD25^+$ T cells in cecal tonsils. Both butyrate and propionate did not show the effect to recover these cells. Interestingly, GPR43 mRNA level was highly expressed in $CD4^+CD8^-CD25^+$ T cells.

Conclusively, my study demonstrated that gut microbiota can regulate the population and suppressive function of $CD4^+CD8^-CD25^+$ or $CD4^+CD8^+CD25^+$ T cells, and acetate can induce $CD4^+CD8^-CD25^+$ T cells in cecal tonsils via GPR43.

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List of Abbreviations

ABX	Antibiotics cocktail
Ahr	Aryl hydrocarbon receptor
APCs	Antigen presenting cells
APRIL	A proliferation-inducing ligand
ASF	Altered Schaedler flora
BAFF	B cell activating factor
CFU	Colony forming unit
c-Maf	Cellular homolog of the avian virus oncogene musculoaponeurotic fibrosarcoma
CTs	Cecal tonsils
CTV	CellTrace™ Violet
DCs	Dendritic cells
EHEC	Enterohemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
Foxp3	Forkhead box P3
GALT	Gut-associated lymphoid tissue
GC	Germinal center
GPR	G protein coupled receptor
H3K27	H3 lysine 27
HDAC	Histone deacetylase
IBD	Inflammatory bowel disease
IELs	Intraepithelial lymphocytes
IgA	Immunoglobulin A
IL	Interleukin
ILCs	Innate lymphoid cells

iNOS	Inducible nitric oxide synthase
LP	Lamina propria
Maf	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog
PPs	Peyer's patches
pTregs	Peripheral Tregs
RA	Retinoic acid
RegIIIγ	Regenerating islet-derived protein 3 γ
SAA	Serum amyloid A protein
SCFAs	Short chain fatty acids
SFB	Segmented filamentous bacteria
SIgA	Secreted IgA
TCRs	T cell receptors
TGF	Transforming growth factor
Th17	T helper 17
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tr1	Type 1 regulatory T
Tregs	Regulatory T cells

I. Review of Literature

1. Gut homeostasis

Mouse and human studies suggested that the microbiota continuously interact with the intestinal immune system for the balance between pro-inflammatory and tolerogenic immune responses, called gut homeostasis [1-3]. Various immune cells and their products are associated with gut homeostasis including forkhead box P3 $(Foxp3)^+$ regulatory T cells (Tregs), T helper 17 (Th17) cells, IgA^+ B cells, innate lymphoid cells (ILCs), transforming growth factor (TGF)-β and interleukin (IL)-10. Gut homeostasis of chicken has not been fully understood and it is assumed to be similar to that of mammals [4].

1.1. Regulatory T cells

Tregs are a subset of $CD4^+$ T cells that exist in peripheral organs and intestine, where they help to maintain gut homeostasis. The absence of Tregs results in the abnormal expansion of $CD4^+$ T cells expressing commensal bacteria-specific T cell receptors (TCRs) resulting in intestinal inflammation [5]. The development of peripheral Tregs is known to partly depend on the gut

microbiota [2, 6]. As shown in Fig. 1, Tregs are induced by specific populations of commensal bacteria which comprise *Clostridium spp*. cluster IV and XIVa strains [2], altered Schaedler flora (ASF) [6], or *Bacteroides fragilis* [3] and/or short chain fatty acids (SCFAs) [7] produced as gut microbial product through IL-10, TGF-β or retinoic acid (RA) expressing antigen presenting cells (APCs), such as, $CD103^+$ dendritic cells (DCs) and $CD11b^+CD11c^-$ macrophages.

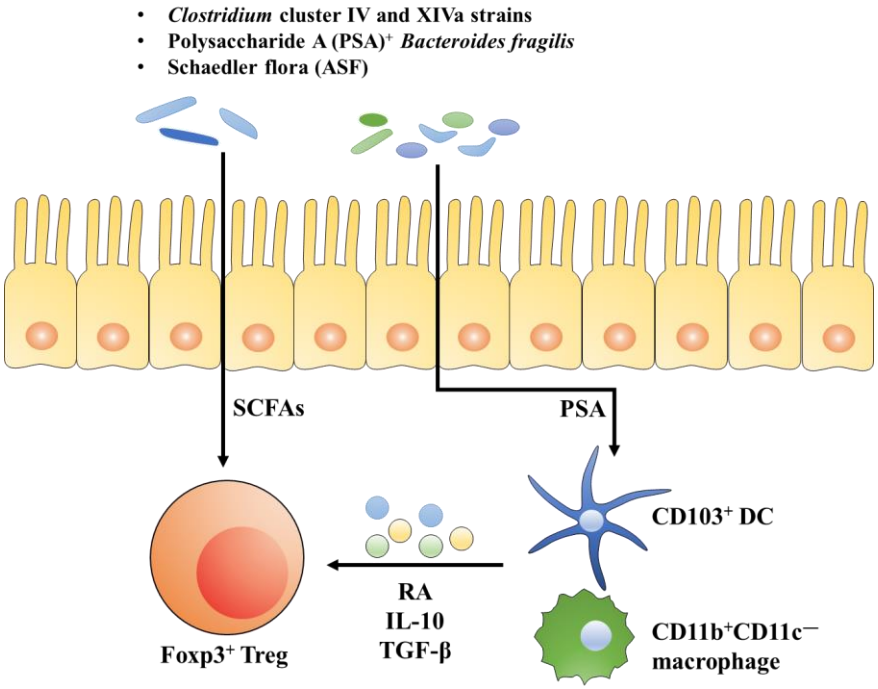


Figure 1. Induction of Tregs by host cells, gut microbiota and their products. Tregs are known to be induced by specific populations of commensal bacteria together with their product, SCFAs and PSA, through IL-10, TGF-β or RA produced by $CD103^+$ DC and $CD11b^+CD11c^-$ macrophages.

Besides Foxp3$^+$ Tregs, CD4$^+$Foxp3$^-$ type 1 regulatory T (Tr1) cells, one of Treg subsets, could contribute to gut homeostasis by suppressing inflammatory condition. In SCID mice with inflammatory bowel disease (IBD), antigen specific Tr1 cells (pre-induced *in vitro* with IL-10) prevented to the progression of colitis [8]. It has been suggested that *Bifidobacterium breve* and *B. longum*, as probiotics, induced Tr1 cells in mouse and alleviated the development of intestinal inflammation [9]. CD4$^+$CD25$^+$ T cells of chicken are known as Tregs [10], which are absent of Foxp3 gene [11, 12], unlike their mammalian counterpart. Furthermore, chicken CD4$^+$CD25$^+$ T cells migrated preferentially cecal tonsils rather than spleen and lung [13]. Chicken Tregs are not fully investigated, for example, intrinsic and extrinsic factors conditions to induce Tregs.

1.2. T helper 17 cells

Th17 cells are one of CD4$^+$ T cell lineages, producing IL-17A, IL-17F and IL-22, which have a role for host defense and development of autoimmune disease in human and mouse [14]. Intestinal Th17 cells are significantly reduced in antibiotic-treated or germ-free mice [1, 15-18], suggesting that the microbiota play a crucial role to develop Th17 cells in gut. Segmented filamentous bacteria (SFB), one of *Clostridia*-related bacteria, induces the

generation of Th17 cells [1, 16, 17]. SFB stimulates the host epithelium to upregulate serum amyloid A protein (SAA) production, which is known to promote IL-6 and IL-23 from $CD11c^{+}$ lamina propria (LP) DCs [1]. ATP produced by gut microbiota, but not much of pathogens, for instance, *Salmonella typhimurium* which secreted ATP lesser than gut microbiota resulting in induction of Th1 cells, promotes LP $CD70^{hi}CD11c^{low}$ cells to develop Th17 cells [15]. Regenerating islet-derived protein 3 γ (RegIIIγ), as a C-type lectin antimicrobial peptide, from Th17 cells prevents the intestinal infection by pathogens including *Citrobacter rodentium* and *Listeria monocytogenes* [19-21]. In chicken studies, IL-17 is assumed as Th17 response, simply because anti-chicken IL-17 antibody to measure the cells secreting IL-17 directly is not available. *Salmonella enterica* serovar Enteritidis infection in chicken induced IL-17 in ceca [22]. It has been suggested that IL-17 was increased in chicken infected with *Eimeria tenella*, a protozoan parasite [23, 24]. The role of Th17 cells in the gut of chicken has yet to be fully understood.

1.3. Immunoglobulin A

The relationship between gut microbiota and gut-specific B cell responses, for instance, immunoglobulin A (IgA) secretion, is closely associated. IgA is an active component involving host protection and a major class of

immunoglobulin in the intestine. IgA exists as a polymeric IgA in the intestinal lumen [25]. Secreted IgA (SIgA) can recognize commensal bacteria and soluble antigens to inhibit penetration into the lamina propria [25]. IgA regulates the composition of the gut microbiota [26, 27]. Activation-induced cytidine deaminase, which is known to be essential enzyme for class switching, deficiency mice showed increase anaerobic bacteria, including *Peptostreptococcus*, *Bacteroidacease*, *Eubacterium* and *Bifidobacterium* in small intestine, whereas cecum microbiota was not changed [28]. Furthermore, gut microbiota regulates IgA production, as the number of IgA-producing cells in the intestine, for example, is decreased significantly in germ-free mouse [25]. Commensal bacteria induces various effector molecules, such as tumor necrosis factor (TNF), inducible nitric oxide synthase (iNOS), B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) that are involved in the induction of IgA^+ B cells in lamina propria [29, 30]. It is probable that gut microbiota stimulates DCs in lamina propria to induce IgA^+ B cells, and in return, SIgA regulates the function and composition of the gut microbiota to maintain gut homeostasis. In chicken, IgA expression of ileum, ceca and cecal tonsils was burst at 7 days post hatching [31]. Probiotics mixture, consisting of *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Streptococcus faecalis*, induced natural IgA from intestinal contents reacting tetanus toxoid and *Clostridium perfringens* alpha-toxin [32]. However, there is no molecular mechanism study on IgA for gut homeostasis in chicken.

1.4. Innate lymphoid cells

ILCs are known as immune cells involved in innate immune responses [33, 34] in human and mouse studies. It has been suggested that gut microbiota is required for the differentiation of ILCs and the production of IL-22 [35, 36]. In other study, gut microbiota suppressed IL-22 production by RORγt$^+$ ILCs [37]. A role of IL-22 in the gut is to promote antimicrobial peptide production by intestinal epithelial cells. IL-22 induces the expression of the C-type lectin antimicrobial peptides, for example, RegIIIγ, which protect the host from the infection of pathogens, for example, enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) and *C. rodentium* [19]. RegIIIγ limited the number of surface-associated Gram-positive bacteria, Firmicutes phylum (*Eubacterium rectale*, and SFB), and activation of adaptive immunity, for instance, IgA and IFN-γ$^+$ cells [21]. Conclusively, ILCs regulate not only both commensal and harmful bacteria but also host immunity in the gut. There is no report about chicken ILCs.

2. Gut microbiota in chicken

2.1. Intestine

The intestine is important in converting the feed into the nutrients for animals' maintenance and growth. Digestive tract of chicken is composed of beak/mouth, esophagus, crop, proventriculus, gizzard, small intestine, ceca, large intestine and cloaca. During the digestion, morphology and chemical composition of feed change as they passing through several organs. Since chicken does not have teeth, they pick up feed with beak and it enters the mouth without chewing. Crop, an out-pocketing of the esophagus, is located in the neck region and stores feed and water [38]. When crop is empty, or near empty, hunger signal transmit to the brain [39]. Very little digestion occurs in crop by amylase secreted in mouth [40].

Proventriculus plays as the true stomach and begins to digest feed with hydrochloric acid and pepsin [41]. However, feed is not yet ground at this point. Gizzard is a unique digestive organ in chicken. It is referred to as 'mechanical stomach' since strong muscles of gizzard as acts like the bird's teeth [42]. Furthermore, feed is grinding, mixing, and mashing with digestive enzymes in gizzard [41].

Small intestine in chicken is consisted of the duodenum, jejunum and ileum similar to that of a mammal. Duodenum secrets digestive enzymes and bicarbonate to counter the hydrochloric acid [43]. Digestive enzymes produced by the pancreas are primarily involved in protein digestion. Digestion of lipids and absorption of fat-soluble vitamins, such as, vitamins A, D, E and K in here occurs with bile [44]. Nutrients are absorbed mainly in the jejunum and ileum.

Ceca are two blind pouches located and a joint point of the small and large intestine. Water in fecal material is reabsorbed and fermentation of indigestible materials at here. It is known that the fermentation produces short fatty acids and vitamin B [45, 46]. Large intestine in chicken is much shorter than the small intestine in chicken. The last of water re-absorption occurs in here. In cloaca, feces are mixed with urine from urates.

2.2. Establishment of gut microbiota

Microorganisms in animal gut has evolved with host [47]. Microorganisms are abundant in the colon and ceca of chicken [48]. Domestic birds, including chicken, duck, and turkey, have about 1×10^{11} cells/g in ceca [49, 50].

The chicks are initially exposed to microbes from the surrounding environment. Therefore, the early stage of the post hatching period would be critical for the formation of gut microbial community. The density of gut

microbiota in chicken increased rapidly within 24 h post hatching [51, 52]. Aerobes such as *Enterobacteriaceae*, *Lactobacillus*, and *Streptococcus* colonized initially in small intestine show a positive oxidation at hatching [53, 54]. Then, oxygen consumption by aerobes causes more anaerobic conditions in lower gut environment, which facilitates growth and colonization of the obligate anaerobes [49, 55, 56].

Ceca contain a more diverse community of gut microbiota, including *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Escherichia*, *Fusobacterium*, *Lactobacillus*, *Streptococcus* and *Campylobacter* in chicken [56-58]. Density of gut microbiota increases throughout the digestive tract, for example, duodenum, ileum and ceca contain 10^3–10^5, 10^8–10^9, $<10^{12}$ colony forming unit (CFU) $gram^{-1}$ of digesta by microscope-counts, respectively [56, 58].

2.3. Gut microbiota on growth performance

The role of gut microbiota in chicken has long been interested for research scientists, industry and the field, because of its impact on growth performance. Probiotics have several positive effects in chicken, (1) improvement of weight gain and feed utilization, (2) decrease mortality through preventing enteric pathogens (3) to attach and colonize in intestine [59, 60]. Non-degradable

complex carbohydrates from diet in the small intestine, such as non-starch polysaccharides and resistant starch, are the main sources of carbon and energy for the commensal bacteria [61]. The metabolites are derived from fermentation by intestinal bacteria, which are consumed as the energy source for host [62]. For example, SCFAs, which are the metabolites by anaerobic microbes utilizing carbohydrates, are considerable energy source in animal [63]. Several pathways associated with production of SCFAs were detected in a meta-genomic analysis of cecal microbiota in chicken [64]. SCFAs from fermentation of non-hydrolysable oligo- and polysaccharides feeding may provide extra energy and a better feed conversion ratio in chicken.

2.4. Effects of gut microbiota on immunological aspect

Gut immune homeostasis in chicken, although seemingly similar to that of mammals, has not yet been fully understood. However, there are some studies about the relationship between gut microbiota and immune system in chicken. The complexity of gut microbiota impacts the repertoire of TCR in the gut [65] and the kinetics on the expression of immune-associated genes during the maturation of gut immune system [66]. It has been suggested that gut microbiota also indirectly affects the development of B cells in the bursa. When the bursal duct is ligated during embryonic development to preclude the normal

traffic of gut-derived molecules into the bursa, cortico-medullary structure in bursal follicle fails to develop normally after hatching [67, 68]. The mechanism of the phenomenon is explained that these gut-derived molecules, probably and mostly bacterial mitogens, could directly induce maturation and proliferation of bursal B cells [69, 70], or indirectly stromal cells to produce cytokines, perhaps via Toll-like receptor (TLR) signaling, for B cell development [71].

2.5. Cecal tonsils

The gut-associated lymphoid tissue (GALT) consists of multiple lymphoid follicles and these are made up of cecal tonsils (CTs), Peyer's patches (PPs), the bursa of Fabricius, Meckel's diverticulum located along the digestive tract in chicken [72]. Especially, CTs are located on the entrance of each cecum, which consist of a pair of fingerlike pockets located in end of small intestine. CTs are histologically [73, 74] and immunologically [75] as secondary lymphoid tissue, similar to the spleen. CTs consist of a cryptosporidians, diffuse lymphoid follicles and germinal centers [76]. Considering cellular and morphological features, a role in antigen sampling of CTs could be similar to mammalian PPs [72, 77]. Within organized lymphoid structures, such as CTs, $CD4^+$ αβ T cells and B cells exist [78, 79], whereas γδ T cells predominate in dispersed areas, such as the epithelium and LP [80].

The development of CTs during embryogenesis has not been described in detail. CTs are appeared at near hatching [76, 81], unlike lymphoid cells infiltrate at presumptive sites of PPs [72]. During embryogenesis, clusters of MHC class II^+ cells, a few scattered Bu-1^+ cells and IgM^+ cells were observed at E13. At E17, MHC class II^+ cells were widely and densely expended, and Bu-1^+ cells and IgM^+ cells are increased more than those at E13 [82]. It suggests that MHC class II^+ cells, presumably antigen presenting cells including dendritic cells [83], provide a microenvironment for lymphocytes.

II. Introduction

Tregs are a subtype of $CD4^+$ T cells, known to play an important role in maintaining gut immune homeostasis since the gastrointestinal tract is constantly exposed to inflammatory condition by a huge microbial components [84]. In mouse and human, Foxp3 is the master transcription factor for Tregs [8, 85]. Common surface molecule and cytokines as makers for Tregs are high expression of CD25 (IL-2 receptor α), and IL-10 and TGF-β, respectively [86]. Non-Foxp3 Tregs, also called Tr1 cells [87], induced by chronic activation of $CD4^+$ T cells with antigen and IL-10 [8] are also reported. Although the master transcription factor is unknown for Tr1 cells yet, unique features of cytokines are suggested as IL-10^{++}, TGF-β^+, interferon (IFN)-γ^+, IL-5^+, IL-4^- and IL-$2^{low/-}$ [8, 88]. $CD4^+CD25^+$ T cells in chicken has been reported as Tregs [10], although no Foxp3 orthologue gene exists [11].

Certain gut microbiota, including *Clostridium spp*. cluster IV and XIVa strains, ASF, or *Bacteroides fragilis*, are known to induce $Foxp3^+$ Tregs in mice and human [2, 3]. These bacteria alleviate the symptom of IBD by inducing Tregs [2, 3, 6]. However, no such studies on the relationship between gut microbiota and Tregs are reported in chicken.

Gut immune homeostasis is largely regulated by microbiota in not only a direct [3] but also indirect manner. Induction [2] and function [3] of Tregs are

affected by gut microbiota related factors, such as SCFAs [89-91] including acetate (C2), propionate (C3), and butyrate (C4), which are generated especially by Firmicutes and Bacteroidetes, after fermenting undigested carbohydrates [84]. It has been shown that acetate, propionate and butyrate exist in 3:1:1 ratio, respectively at 50-150 mM in colon of mouse [92], whereas 50-70 mM of acetate, 5-30 mM of butyrate and 5-10 mM of propionate are contained in chicken ceca [93-95].

It has been shown in mouse experiments that several G protein coupled receptors (GPRs) on immune and non-immune cells recognize SCFAs [96, 97]. Activation of GPR43 using SCFAs promotes the number and function of IL-10^{+}Foxp3^{+} Tregs, and propionate directly increases Foxp3 expression and IL-10 production [91]. GPR109a, expressed on colonic epithelial cells, DCs and macrophages [98], is known to be activated by butyrate.

IL-10 and RA produced by mostly antigen presenting cells treated with SCFAs [99] could induce the differentiation of naïve T cells into Foxp3^{+} Tregs and Tr1 cells [99]. SCFAs are also known to act as a histone deacetylase (HDAC) inhibitor. For instance, butyrate enhances acetylation at histone H3 lysine 27 (H3K27) of the Foxp3 promoter causing the differentiation of naïve T cells into Tregs [89]. Acetate, on the other hand, induces the acetylation of p70 S6 kinase and phosphorylation rS6, resulted in Tr1 cell induction [100]. There are not only a very few studies on immunological effects of SCFAs in chicken but also no reports about the factors regulating gut homeostasis.

Germ-free mouse model has been a critical tool to carry out the research on immune homeostasis in the mucosal tissues as well as peripheral organs for decades [101-103]. Gut immune balance is the result of interaction among various immune cells including Tregs, Th17 cells, IgA secreting B cells, and innate immune cells [103]. In indigenous germ-free mice, peripheral Tregs (pTregs) are scarce in the lamina propria of the intestine [2, 104]. Antibiotics cocktail (ABX) treatment is an alternative way to make an intestinal germ-free animal. ABX-treated mice showed closely resembling indigenous germ-free mice in terms of immunological changes in not only the gut but also peripheral organs [105-107]. The presence of intestinal Th17 cells is dramatically reduced in ABX-treated mice [16]. Although $Foxp3^+$ Tregs are still detectable, they are significantly decreased in colonic lamina propria [2]. Unfortunately, there is no report, at the best of my knowledge, on immunological researches in germ-free or gut microbiota-free chicken model.

In the present study, the model for studying gut immune homeostasis in chicken treated with ABX was established. The main goal of the study was to (1) examine the changes in population and function of immune cells in ABX-treated chickens and (2) find the factors regulate gut homeostasis.

III. Materials and Methods

1) Experimental animal and ABX treatment

Fertile eggs of White Leghorn were provided by Animal Farm, Seoul National University, Pyeong-Chang, Korea. Fertile eggs were incubated at 37.5-38°C incubator (Rcom, Korea) for 21d. The condition of cage sustained 28-30°C and filte

red air. Care room maintained 23-25°C, 20-40% of humidity and positive pressure. The experiment was approved by Institutional Animal Care and Use Committee of Seoul National University (IACUC No., SNU-150327-2). Crumble feed was supported by SeoulFeed company and sterilized by γ-radiation by GREENPIA TECHNOLOGY company. For antibiotics treated group, chickens at hatching were treated with various concentrations of antibiotics in drinking water *ad libitum* for 7 days. I defined dilution factor (DF) 1 as an antibiotics containing ampicillin, gentamycin, neomycin (all from Sigma-Aldrich, St. Louis, MO) and metronidazole (Abcam, Cambridge, MA) for 1 mg/ml each, and vancomycin (Sigma-Aldrich) for 0.5 mg/ml. DFs were tested 1:1, 1:2, 1:10, and 1:20. As control (Con) group. ABX-treated chickens

were referred by treatment of 1:10 diluted antibiotics for 1-3 weeks.

2) Measurement of colony forming unit

Cecal contents from chickens treated with ABX were dissolved in PBS to adjust at 1 mg/ml. Dissolved cecal contents from Con group were diluted in 100-1000 times with PBS while those from ABX-treated group were used without dilution. All dissolved cecal contents were spread on Brain Heart Infusion (BHI) agar media (BD Biosciences, San Jose, CA) and then incubated at 37°C for 12 hr. CFU was determined by counting the number of colony.

3) Examination of physiological changes in ABX-treated chickens

Body weight changes were monitored in chickens every day for 7 days. At the end of the experiment, major immune organs (liver, spleen and bursa) were taken and briefly semi-dried by tapping on paper towel, and the weight was examined. Length of intestine was segmented to jejunum (J), duodenum and ileum (D+I), Ceca (C) and large intestine (L), and measured with millimeter scale. Blood samples from a wing vein were taken at 7 days after the ABX treatment. Amount of glucocorticoid in serum, which was obtained by

centrifugation at 1,000 × g at 4°C for 20 min, was measured by chicken glucocorticoid ELISA kit (MyBioSource, San Diego, CA). In brief, 50 μl of serum per well, diluted to 1:50 (pre-determined, data not shown), along with standard samples, was added to a 96-well microplate pre-coated with glucocorticoid specific antibodies. After the wash with PBS, 50 μl of secondary antibody, conjugated with HRP, was added into each well, and color was developed by the addition of 90 μl of tetramethylbenzidine. The reaction was stopped by the addition of 50 μl of stop solution. Absorbance was measured at 450 nm using an ELISA microplate reader (Molecular Device, Sunnyvale, CA) and the amount of glucocorticoid was calculated from the standard curve.

4) Flow cytometric analysis for immune cells

Chunked spleen or longitudinally cut cecal tonsils after wash were minced with the flat end of a 3 ml syringe plunger through a 40 μm cell strainer (BD Biosciences, San Jose, CA) into a 50 ml conical tube (SPL, Pocheon, Korea). In order to purify immune cells, red blood cells were lysed using ACK buffer (BD Biosciences) for 3 min at room temperature, and then washed.

For examination of B cells and macrophages, anti-chicken MHC class II-FITC (clone 2G11), Monocyte/Macrophage-PE (clone KUL01), and Bu-1-Alexa Flour® 647 (AV20) (all from Southern Biotec, Birmingham, AL) were

used. In order to examine $CD4^+$ subtype T cells, anti-chicken CD4-FITC (clone CT-4), CD8α -PE (clone CT-8), CD5-biotin (clone 2-191) (all from Southern Biotec) and CD25-Alexa Fluor® 647 (clone 13504; AbD Serotec, Puchheim, Germany), and Brilliant Violet 605 streptavidin (BioLegend, San Diego, CA) were used.

Flow cytometric data, acquired by flow cytometry (FACS Canto II, BD Biosciences), were analyzed with FlowJo software (Tree Star, San Carlos, CA). Total cell number was determined by automatic cell counter TC10 (Bio-Rad, Hercules, CA). Each number of immune cells was calculated from total cell number and the proportion of immune cells.

5) Measurement of mRNA level using RT-qPCR

$CD4^+$ subtype T cells ($CD4^+CD8^-CD25^-$, $CD4^+CD8^-CD25^+$, $CD4^+CD8^+CD25^-$ and $CD4^+CD8^+CD25^+$) B cells ($Bu\text{-}1^+$) and APCs ($KUL01^+$, MHC class $II^+Bu\text{-}1^-KUL01^-$) were sorted by using ARIAII FACS sorter (BD Biosciences). Total RNA of each $CD4^+$ subtype T cells was extracted by miRNeasy Micro Kit (QIAGEN, Hilden, Germany). The concentration of total RNA was quantified with NanoDrop (Amersham Biosciences, Piscataway, NJ) at A260. Subsequently, 100 ng of purified RNA was reverse transcribed to cDNA using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA)

according to the manufacturer's instruction.

The real-time quantitative PCR was performed on cDNA using a StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA). SYBR green PCR master Mix was used according to manufacturer's specification (Applied Biosystems). The PCR reaction was carried out in a 96-well reaction plate with 10 μl SYBR PCR master mix, 0.5 μl per primer (2 pM), 1-2 μl cDNA template and 7-8 μl nuclease-free water. Each reaction involved a pre-incubation at 95 ℃ for 10 min, followed by 45 thermal cycles at 95 ℃ for 15 s, 55 ℃ for 30 s, and elongation at 72 ℃ for 30 s. Relative quantification of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method.

Target gene expression was normalized to β-actin mRNA level. Primers for IL-10 (forward: 3'-AGCTGACGGTGGACCTATTATT-5', reverse: 3'-GGCTTTGCGCTGGATTC-5'), IFN-γ (F: 3'-CGGGAGCTGAGGGTGAA-5', R: 3'-GTGAAGAAGCGGTGACAGC-5'), Ahr (F: 3'-CAGGTCCCTGAAAACCTTGACT-5', R: 3'-ACGGCACCTGCATAACATGTT-5'), Maf (F: 3'-CCCCGTTACCTGAGGTCAGA-5', R: 3'-GTCTTCGTGCCAGAACGTTGT-5'), G-coupled protein receptor 43 (F: 3'-CTCTTTATGGCTGCCCTCAG-5', R: 3'- GTAGCCCAGGCTTGGTTGG-5') and β-actin (F: 3'-CAACACAGTGCTGTCTGGTGGTA-5', R: 3'-ATCGTACTCCTGCTTGCTGATCC-5') were synthesized from Bioneer Inc. (Daejeon, Korea).

6) Changes on the subtype of $CD4^+$ T cells treated with antibiotics *in vitro*

Spleens from 2-3 weeks old-chickens were taken and single cells were produced. Splenocytes (1×10^5 cells/well) in a 96-well culture plate (Nunc, Roskilde, Denmark) were treated with 100 μg/ml of ampicillin (A), gentamycin (G), metronidazole (M), neomycin (N) and 50 μg/ml of vancomycin (V) for 24 h. Change of $CD4^+$ subtype T cells was analyzed by flow cytometry with anti-chicken CD4-FITC, CD8α-PE and CD25-Alexa Fluor® 647. Total cell numbers were determined by automatic cell counter TC10. Cell number of each $CD4^+$ subtype T cells was calculated from total cell number and the proportion of $CD4^+$ subtype T cells was analyzed by using FlowJo software.

7) T cell suppression assay

Splenocytes from Con and ABX group were stained with anti-chicken CD4 antibody followed by the incubation with anti-mouse IgG bead (Miltenyi Biotec, Auburn, CA) for 30 min. $CD4^+$ T cells were sorted by MACS magnetic bead system (Miltenyi Biotec). $CD4^+$ T cells were stained with 1 μM

CellTrace™ Violet (CTV) dye (Invitrogen, Carlsbad, CA) for 20 min at 37°C, and then washed 3 times with pre-warm complete RPMI. $CD4^+$ T cells stained with CTV were cultured with anti-chicken CD3 and CD28 antibodies for 3 d. The cells were stained with anti-chicken CD4-FITC, CD8α-PE and CD25-Alexa Fluor® 647 and the proliferation of $CD4^+CD25^-$ T cells was determined by flow cytometry and FlowJo software.

8) Co-housing experiment

Co-housing experiment was performed for 7 days at the end of ABX treatment. Cecal contents and cecal tonsils were taken at 6 h, 1, 3, 5 and 7 days after co-housing. Cecal contents were dissolved at 1 mg/ml. Dissolved cecal contents from Con and ABX group were diluted by 10-1000 times to adjust into proper range of colony numbers (data not shown). Then, the contents were spread on BHI agar media and incubated at 37°C for 12 hr. CFU was determined by counting the number of colony. For analysis for $CD4^+$ subtype T cells, longitudinally cut cecal tonsils were processed into single cells. Anti-chicken CD4-FITC, CD8α-PE and CD25-Alexa Flour® 647 were used for examination of $CD4^+$ subtype T cells. All flow cytometric data, acquired by flow cytometry, were analyzed with FlowJo software. A total cell number was determined by automatic cell counter TC10. Each cell number of $CD4^+$ subtype T cells was

calculated with total cell number and the percentage of $CD4^+$ subtype T cells.

9) The elimination of Gram positive and negative bacteria

Chickens at hatching were treated with vancomycin (100 μg/ml; Van) for the removal of Gram positive bacteria or polymyxin B (10 μg/ml; PolyB) for Gram negative bacteria for 7 days. CFU of cecal contents (1 mg/ml) was measured. Gram staining was performed by using the kit (BD Biosciences).

10) Administration of short chain fatty acids

Chickens at hatching were fed with a diet containing SCFAs, acetate (50 mM), butyrate (30 mM), and propionate (10 mM) (concentration pre-determined, data not shown), and ABX as a positive control for 7 days. Cecal tonsils were taken and subtype of $CD4^+$ T cells was analyzed with anti-chicken CD4-FITC, CD8α-PE and CD25-Alexa® 647. All flow cytometric data were analyzed with FlowJo software.

11) Statistical Analysis

Using SAS 9.3, statistical differences were determined using T-test and one-way ANOVA with Turkey's test. Differences were considered significant at $P \leq 0.05$.

IV. Results

1) Elimination of gut microbiota in chicken

Elimination of gut microbiota in chickens administered with a various concentration of ABX in drinking water [108] containing ampicillin, gentamycin, metronidazole, neomycin, and vancomycin (Table 1) *ad libitum* for 7 days was examined. Colony from cecal tonsils of chicken treated with ABX (1:10) was not observed (Fig. 2). The result demonstrated that gut microbiota of chickens treated with ABX at 1:10 were eliminated. Therefore, ABX-treated chickens, hereafter, will be referred as ABX (1:10) treatment.

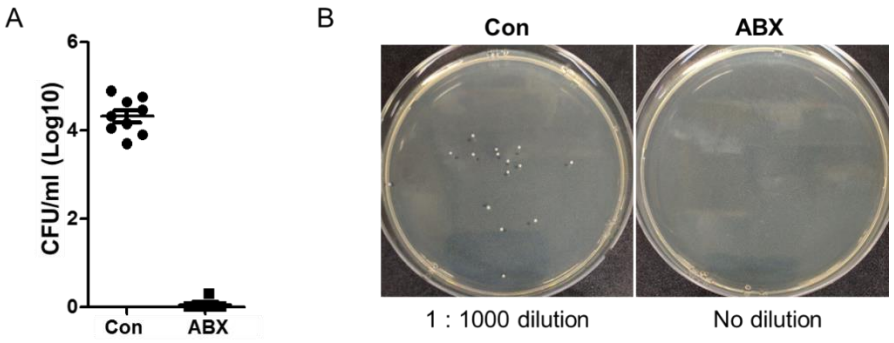


Figure 2. Elimination of gut microbiota in chickens treated with antibiotics. Chickens were treated with distilled water (control, Con) or cocktail of antibiotics (ABX, 1:10) for 7 days. Nine ceca were taken and cecal contents diluted with autoclaved distilled water were plated on BHI agar plate for 12 hr at 37C°. (A) CFU was determined by counting the number of colonies on the plate. (B) One representative picture from ten similar results is shown.

Table 1. Elimination of gut microbiota in chickens administered with various concentration of antibiotics in drinking water for 7 days.

DF	A	G	M	N	V	Unit	Elimination of microbes (%)
1:1	1	1	1	1	0.5	g/L (mg/ml)	99 >
1:2	500	500	500	500	250		99 >
1:10	100	100	100	100	50	mg/L (μg/ml)	99 >
1:20	50	50	50	50	25		97 >

* DF: Dilution factor, A: Ampicillin, G: Gentamycin, M: Metronidazole, N: Neomycin, V: Vancomycin

2) Verification of physiological alteration in ABX-treated chickens

It is critical that no side effects or physiologic changes are observed after the elimination of gut microbiota in chickens. No significant differences on body weight, and the length of distinct regions of small intestine (duodenum, jejunum and ileum) and large intestine (Fig. 3A-B) were observed. Amount of glucocorticoid in serum, as a stress marker, was not changed (Fig. 3C). Furthermore, the weight of major organs including spleen, bursa and liver was not altered (Fig. 3D). Taken together, ABX treatment in chicken model in the present study did not alter physiological traits.

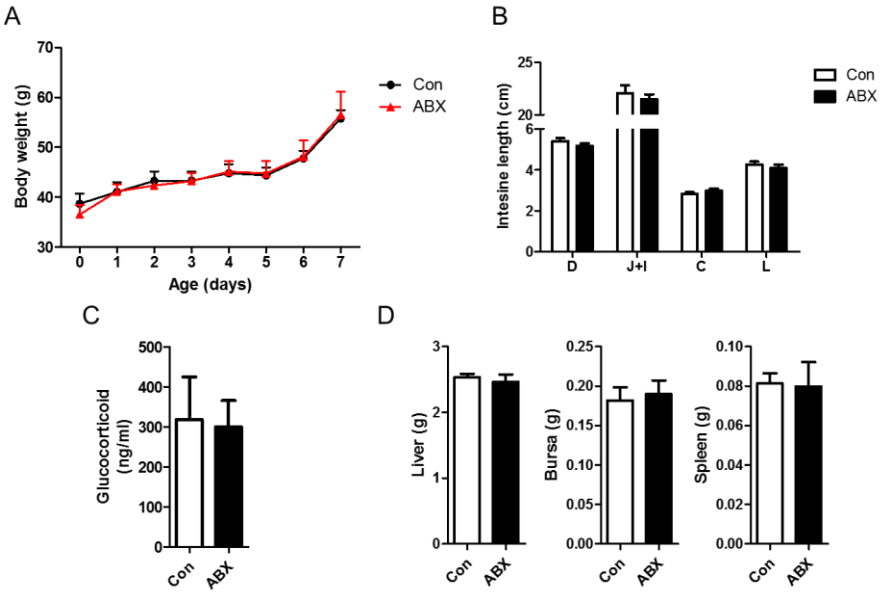


Figure 3. No physiologic changes in ABX-treated chickens. ABX in drinking water was treated to chickens at hatching for 7 days. (A) Body weight was measured daily. (B) The length of intestine (D: duodenum, J: jejunum, I: ileum), (C) amount of glucocorticoid by ELISA and (D) the weight of major immune organs were measured.

3) Change of B cells and macrophages in ABX-treated chickens

I examined whether the elimination of gut microbiota affects the population of B cells ($MHC2^{+}Bu\text{-}1^{+}$ cells) and macrophages ($KUL01^{+}$ cells) in cecal tonsils and spleen in chickens. No significant changes on the percentage and absolute number of B cells and macrophages in cecal tonsils (Fig. 4A-D) and spleen (Fig. 4E-H) were observed. Furthermore, MHC class II (MHC2) expression on B cells and macrophages in cecal tonsils (Fig. 4I and J) and spleen (Fig. 4K and L) was not significantly changed. Taken together, population and expression of MHC2 of B cells and macrophages did not alter by the elimination of gut microbiota.

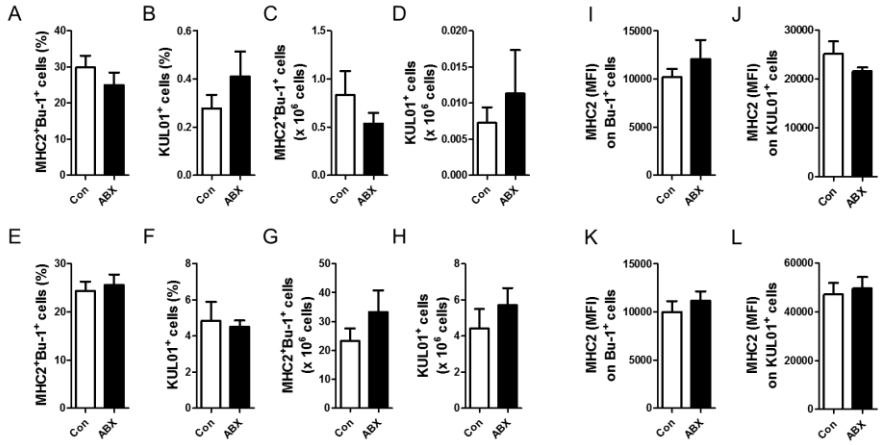


Figure 4. No changes of B cells and macrophages in cecal tonsils and spleen in ABX-treated chickens. Chickens, treated with antibiotics for 7 days, were sacrificed, and then (A-D and I, J) cecal tonsils and (E-H and K, L) spleen were taken. Single cells produced from each organ were stained with anti-chicken MHC class II (MHC2), KUL01 (for macrophages), and Bu-1 (for B cells) antibodies. The percentage of (A and E) B cells and (B and F) macrophages, and absolute number of (C and G) B cells and (D and H) macrophages was calculated from their percentages. MFI of MHC2 on (I and K) B cells and (J and L) macrophages were examined using flow cytometry.

4) Change of $CD4^+$ T cells in ABX-treated chickens

To examine the percentage and absolute number of $CD4^+$ subtype T cells in cecal tonsils, flow cytometry analysis after the staining of the cells with anti-chicken TCRγδ, CD3, CD4, CD8α, and CD25 antibodies was performed. $CD3^+\gamma\delta TCR^-$ cells were pre-gated, and then, $CD4^+$ T cells were divided into $CD4^+CD8^-$ and $CD4^+CD8^+$ T cells. Finally, $CD25^+$ cells were analyzed (Fig. 5). Total cell number of cecal tonsils showed no significant changes in ABX-treated chickens (ABX) when compared to control (Fig. 6A). Furthermore, there were no changes on αβ T cells (Fig. 6B and G), and $CD4^+CD8^-$ (Fig. 6C and H) and $CD4^+CD8^+$ (Fig. 6D and I) T cells. Interestingly, in $CD4^+CD8^-CD25^+$ (Fig. 6E and J) and $CD4^+CD8^+CD25^+$ T cells (Fig. 6F and K) T cells from cecal tonsils were significantly reduced in ABX compared with those of control (Con). Interestingly, however, no significant changes on $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells were observed in spleen (Fig. 7).

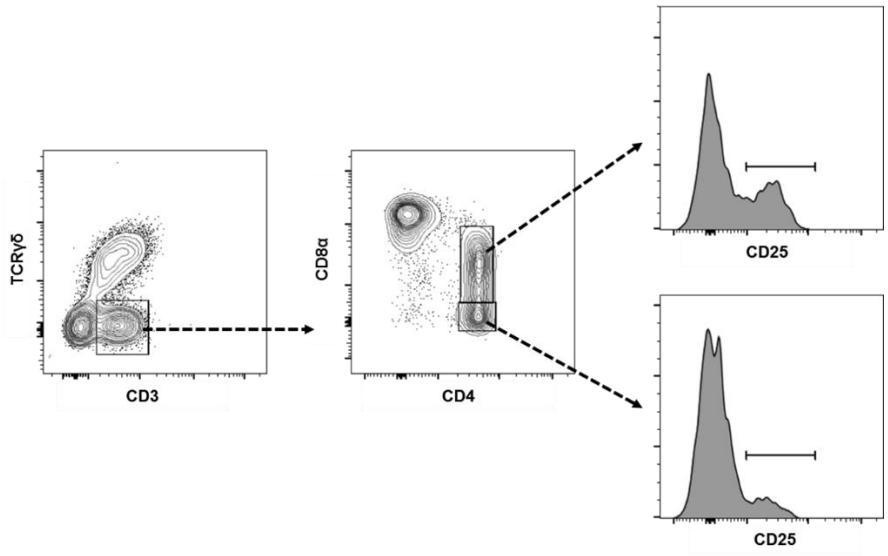


Figure 5. Gating strategy to analyze subtype of CD4$^+$ T cells. Chickens at hatching were given water containing antibiotics for 7 days and cecal tonsils were taken. Single cells produced from cecal tonsils were, then, stained with anti-chicken TCRγδ, CD3, CD4, CD8α, and CD25 antibodies. CD3$^+$TCRγδ$^-$ cells gated were regarded as T cells, and then, CD4$^+$CD8$^-$CD25$^+$ and CD4$^+$CD8$^+$CD25$^+$ T cells.

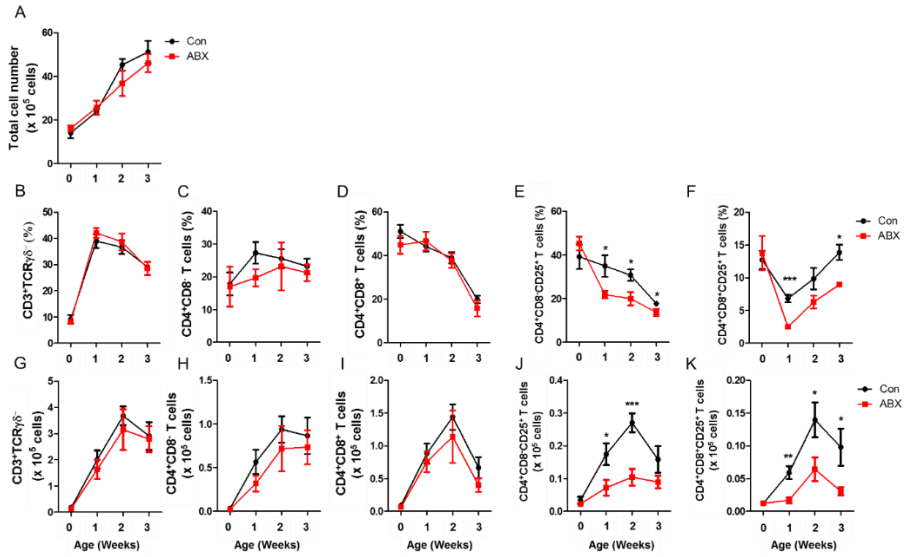


Figure 6. $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells were reduced in cecal tonsils from ABX-treated chickens. Chickens at hatching were given water containing antibiotics for 7 days and cecal tonsils were taken. Single cells from cecal tonsils were, then, stained with anti-chicken TCRγδ, CD3, CD4, CD8α, and CD25 antibodies. (A) Total number of cells in cecal tonsils is shown. The percentage of (B) $CD3^+\gamma\delta TCR^-$ cells, (C) $CD4^+CD8^-$ T cells, (D) $CD4^+CD8^+$ T cells, (E) $CD4^+CD8^-CD25^+$ and (F) $CD4^+CD8^+CD25^+$ T cells, and absolute number of (G) $CD3^+\gamma\delta TCR^-$ T cells, (H) $CD4^+CD8^-$ T cells, (I) $CD4^+CD8^+$ T cells, (J) $CD4^+CD8^-CD25^+$ and (K) $CD4^+CD8^+CD25^+$ T cells was calculated with the percentage of these cells. Significant differences were shown as asterisks between Con and ABX at $P \leq 0.05$.

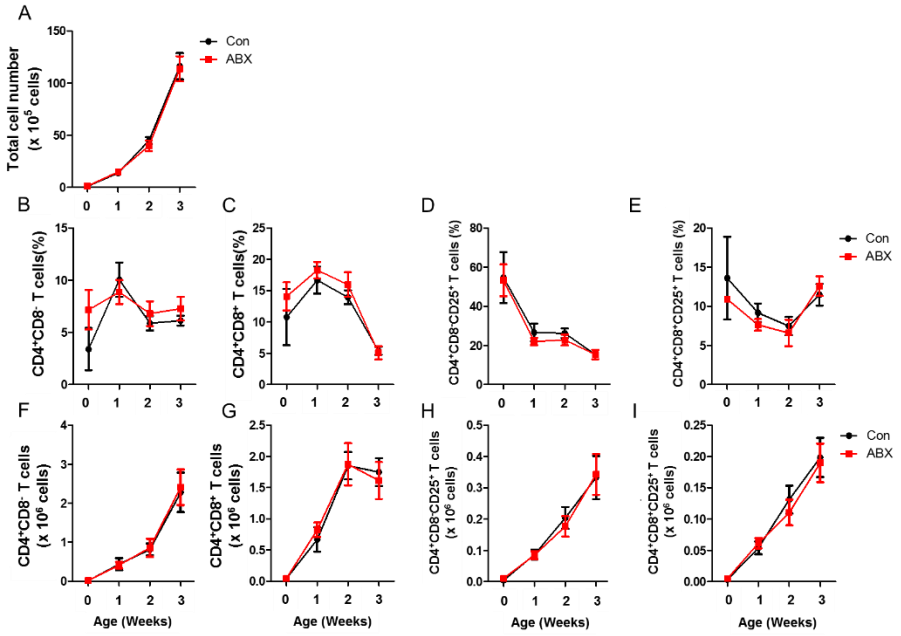


Figure 7. $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells were not changed in spleen from ABX-treated chickens. Chickens at hatching were given water containing antibiotics for 7 days and spleens were taken. Single cells from spleens were, then, stained with anti-chicken CD4, CD8α, and CD25 antibodies. (A) Total number of cells in spleen is shown. The percentage of (B) $CD4^+CD8^-$ T cells, (C) $CD4^+CD8^+$ T cells, (D) $CD4^+CD8^-CD25^+$ and (E) $CD4^+CD8^+CD25^+$ T cells, and absolute number of (F) $CD4^+CD8^-$ T cells, (G) $CD4^+CD8^+$ T cells, (H) $CD4^+CD8^-CD25^+$ and (I) $CD4^+CD8^+CD25^+$ T cells was calculated with the percentage of these cells.

5) Change of IL-10 and IFN-γ from subtype of $CD4^+$ T cells in ABX-treated chickens

Chicken $CD4^+CD25^+$ T cells expressed high IL-10 and played a role as Tregs [10]. I examined whether the elimination of gut microbiota affects mRNA expression of cytokines in subset of $CD4^+$ T cells. Interestingly, both *IL-10* (Fig. 8A) and *IFN-γ* (Fig. 8B) mRNA in $CD4^+CD8^-CD25^+$ T cells were significantly reduced in ABX (Fig. 8).

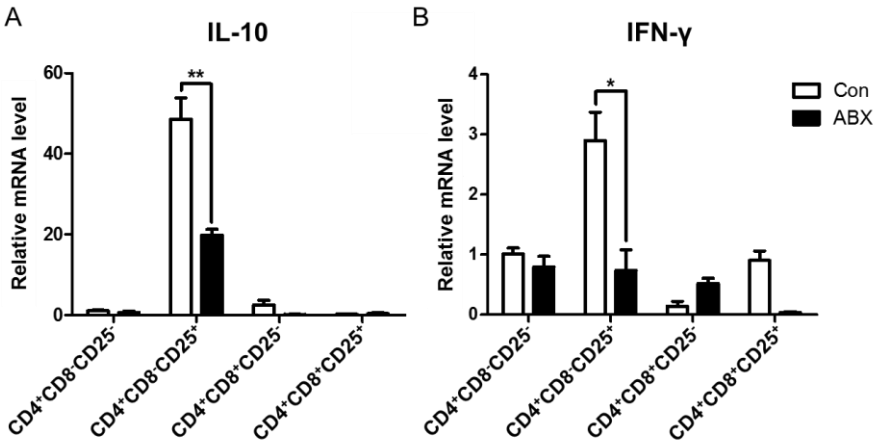


Figure 8. Expression of IL-10 and IFN-γ mRNA among $CD4^+$ T cell subsets in cecal tonsils from ABX-treated chickens. Chickens at hatching were given water containing antibiotics for 7 days and cecal tonsils were taken. Single cells from cecal tonsils were, then, stained with anti-chicken CD4, CD8α and CD25 antibodies. Each subset of $CD4^+$ T cells was sorted by using ARIA II FACS sorter. The mRNA was extracted from each subset and the level of (A) IL-10 and (B) IFN-γ was determined by RT-qPCR. Relative quantification of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to β–actin mRNA level. Significant differences were shown as asterisks between Con and ABX at $P \leq 0.05$.

6) Direct effect of antibiotics on the change of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells

In order to examine the possibility for the change of these T cells by direct effect of antibiotics, I performed *in vitro* experiment where pre-determined (data not shown) each antibiotic or combination was treated to splenocytes for 24 h. There were no significant differences on the cell number (Fig. 9A) and the proportion of these cells (Fig. 9B) when compared with control. These results suggested that the reduction of $CD4^+CD8^-CD25^+$ T cells in ABX-treated chickens was not be directly affected by antibiotics.

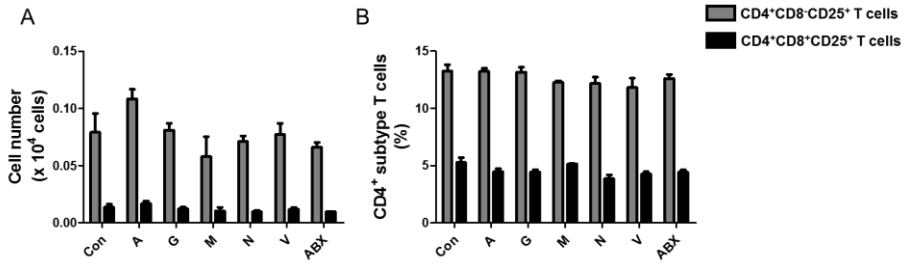


Figure 9. No changes of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in chicken splenocytes treated with antibiotics. Spleens were taken from two week-old chickens and splenocytes were treated with pre-determined concentration of each antibiotic or mixed antibiotics (ABX). (A) Cell number and (B) proportion of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells were examined using anti-chicken CD4-FITC, CD8α-PE and CD25-Alexa647 antibodies by flow cytometry. Con, non-treatment; A, ampicillin (100 μg/ml); G, gentamycin (100 μg/ml); M, metronidazole (100 μg/ml); N, neomycin (100 μg/ml); V; vancomycin (50 μg/ml); and ABX, antibiotics cocktail as mentioned in the Materials and Methods.

7) Changes of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in periphery of ABX-treated chickens

I further examined the reduction of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in periphery organs in ABX-treated chickens. It has been suggested that $CD5^{hi}CD4^+CD25^-Foxp3^-$ T cells preferentially develop into peripheral $Foxp3^+$ Tregs in mice [109]. The present results showed that $CD5^{hi}$ cells were deceased in both $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in cecal tonsils of ABX-treated chickens (Fig. 10).

$CD4^+CD25^+$ T cells migrate from thymus to cecal tonsils preferentially [110]. The reduction of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in cecal tonsils could be resulted by lesser migration from thymus. The results showed that $CD4^+CD8^+$ T cells are the major population of $CD4^+$ T cells in chicken thymus (Fig. 11A). Furthermore, there was no change on CD5 expression on$CD4^+CD8^+CD25^+$ T cells in the thymus from ABX-treated chickens (Fig. 11B). Taken together, the reduction of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in cecal tonsils of ABX-treated chickens was not affected from thymus.

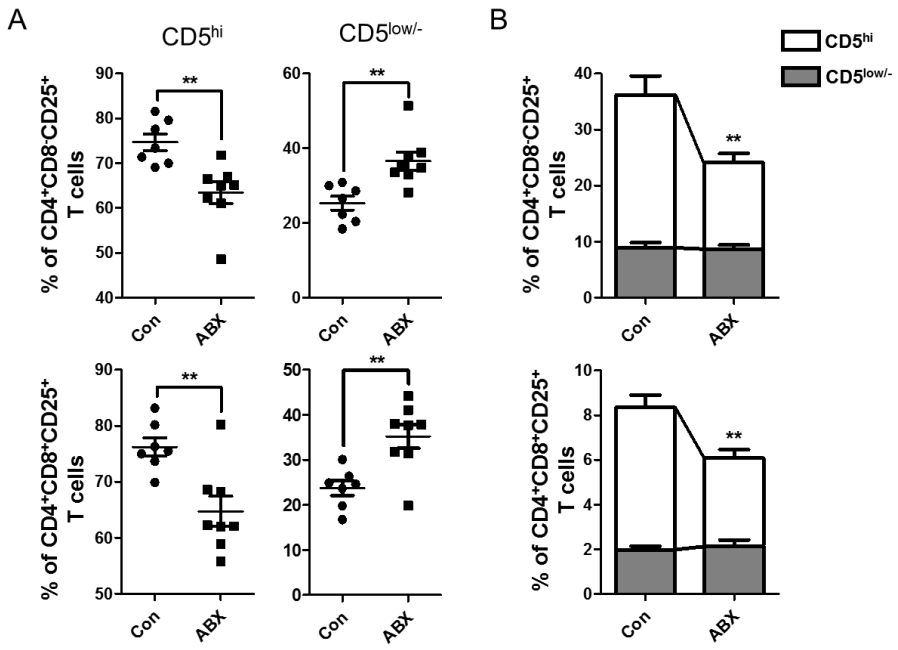


Figure 10. Reduction of $CD5^{hi}$ cells in $CD4^{+}CD8^{-}CD25^{+}$ and $CD4^{+}CD8^{+}CD25^{+}$ T cells in ABX-treated chickens. Chickens, at hatching, were given water containing antibiotics for 7 days and cecal tonsils were taken. Single cells from cecal tonsils were, then, stained with anti-chicken CD4, CD5, CD8α, and CD25 antibodies. (A) The percentage of $CD5^{hi}$ and $CD5^{low/-}$ cells was analyzed in $CD4^{+}CD8^{-}CD25^{+}$ and $CD4^{+}CD8^{+}CD25^{+}$ T cells in cecal tonsils by using flow cytometry. (B) $CD5^{hi}$ and $CD5^{low/-}$ cells in $CD4^{+}CD8^{-}$ and $CD4^{+}CD8^{+}CD25^{+}$ T cells were evaluated by Grandparents analysis by using FlowJo. Significant differences were shown as asterisks between Con and ABX at $P \leq 0.05$.

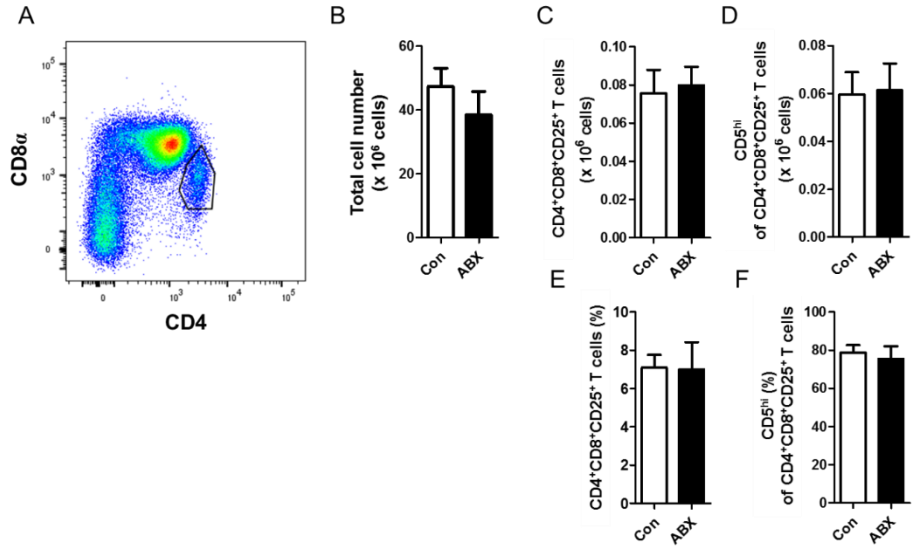


Figure 11. CD5^{hi} cells of CD4^{+}CD8^{+}CD25^{+} T cells were not changed in thymus of ABX-treated chickens. Chickens at hatching were given water containing antibiotics for 7 days and thymus was taken. Single cells from thymus were, then, stained with anti-chicken CD4, CD8α, and CD25 antibodies. (A) Thymocytes were analyzed by dot plot based on CD4 and CD8α expression. (B) Total cell number was obtained from a thymic lobe. Cell number of (C) CD4^{+}CD8^{+}CD25^{+} T cells and (D) CD5^{hi} of CD4^{+}CD8^{+}CD25^{+} T cells, and the percentage of (E) CD4^{+}CD8^{+}CD25^{+} T cells and (F) CD5^{hi} of CD4^{+}CD8^{+}CD25^{+} T cells are shown.

8) Suppressive function of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in ABX-treated chickens

The elimination of gut microbiota caused reduction of *IL-10* mRNA in $CD4^+CD8^-CD25^+$ T cells (Fig. 8A). It could be postulated that the reduction of IL-10 expression caused to change the function of $CD4^+CD8^-CD25^+$ T cells since it is known as an immune suppressive cytokine [111]. I examined whether the elimination of gut microbiota affected the suppressive function of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells. $CD4^+CD8^-CD25^-$ and $CD4^+CD8^+CD25^-$ T cells from ABX-treated chickens were proliferated more than those of Con (Fig. 12) suggesting that the elimination of gut microbiota caused a significant reduction of $CD4^+CD8^-CD25^-$ and $CD4^+CD8^+CD25^-$ T cells to suppress regular T cell function.

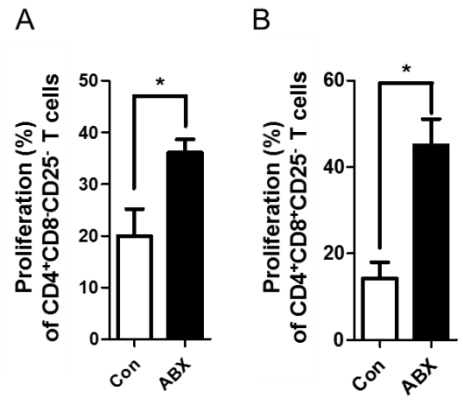


Figure 12. Elimination of gut microbiota caused reduction of suppressive ability of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells. Spleens were taken from two week-old chickens administered with water (Con) or water containing antibiotics (ABX) for two weeks. Splenic $CD4^+$ T cells were sorted by magnetic bead sorting. $CD4^+$ T cells, stained with CellTrace™ Violet (CTV) dye, were stimulated with anti-chicken CD3 and CD28 antibodies for 3 d. Proliferation of $CD4^+CD8^-CD25^-$ and $CD4^+CD8^+CD25^-$ T cells were determined by flow cytometry. Significant differences were shown as an asterisk between Con and ABX at $P \leq 0.05$.

9) Changes of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in ABX-treated chickens after co-housing with control chickens

$CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells were significantly reduced in ABX-treated chickens (Fig. 6). Therefore, the reconstitution of gut microbiota may concordant with recovery of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in ABX-treated chickens was examined after co-housing with wild type chickens. The CFU was observed as early as 6 h post co-housing and reached at the similar level as ABX-untreated control at 1 d post co-housing (Fig. 13A). Interestingly, the number of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells was gradually increased to the similar level as control at 7 days post co-housing (Fig. 13B) suggesting that gut microbiota influence the number and function of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells.

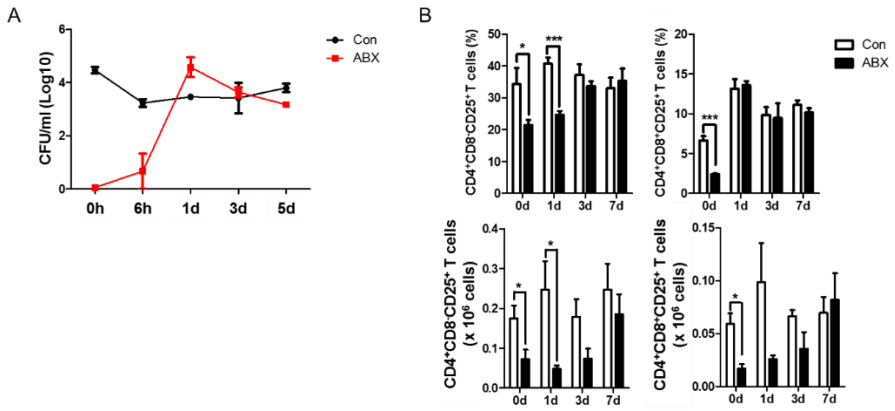


Figure 13. Changes of CFU, and $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in cecal tonsils from ABX-treated chickens after co-housing with control chickens. Chickens at hatching were treated with ABX for 7 days and then co-housed with ABX-untreated control (Con) chickens for 7 days at the normal condition. (A) CFU was measured from cecal contents (1 mg/ml) at 6 h, 1 d, 3 d and 5 d after co-housing. (B) Proportion and cell number of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in cecal tonsils were analyzed by flow cytometry after co-housing for 1 d, 3 d and 7 days. Significant differences were shown as asterisks between Con and ABX at $P \leq 0.05$.

10) Effect of Gram-positive or negative bacteria on the population changes of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells

Next, I examined whether Gram-positive or Gram-negative bacteria influenced the change of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells. Selective deletion of bacteria by using vancomycin (Van) for eliminating Gram-positive bacteria and polymyxin B (PolyB) for Gram-negative bacteria [2], was performed. The total CFU of Van and PolyB was slightly higher than that of Con (Fig. 14A). PolyB eliminated Gram-negative bacteria completely. Van eliminated Gram-positive bacteria from 33% to 7% (Fig. 14B). Surprisingly, $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells were significantly decreased by Van, but not PolyB treatment (Fig. 14C). In order to make sure the effect of Van, I have examined another group, ABX without vancomycin, Without Van, and the result showed no significant differences (Fig. 14D) indicating the change was caused by Gram-positive bacteria. Taken together, Gram-positive bacteria have a critical role to induce $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells.

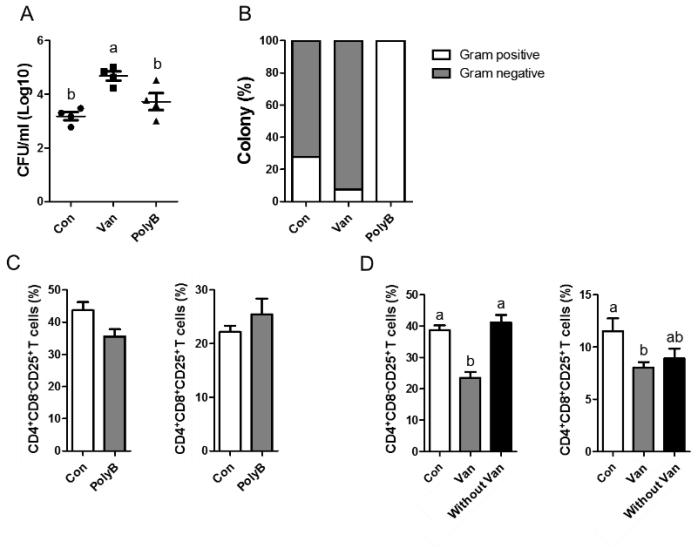


Figure 14. Elimination of Gram positive bacteria is responsible for the change of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in ABX-treated chickens. Chickens, at hatching, were treated with vancomycin (Van; 50 μg/ml), antibiotics without vancomycin (Without van; ampicillin 100 μg/ml, gentamycin 100 μg/ml, metronidazole 100 μg/ml, neomycin 100 μg/ml), or polymyxin B (PolyB; 10 μg/ml) for 7 days and co-housed with ABX-untreated control (Con) chickens for 7 days. (A) CFU of cecal contents was measured from Van and PolyB groups and, (B) The composition of colonies was averaged with Gram positive or negative colonies pre-determined by Gram staining. (C and D) Proportion of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in cecal tonsils were analyzed in chickens treated with vancomycin (Van), polymyxin B (PolyB), or antibiotics without vancomycin (Without Van) using flow cytometry and FlowJo. Significant differences were shown as a different alphabet at $P \leq 0.05$.

11) Effect of SCFAs on $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells

It has been suggested that short SCFAs are one of the factors to induce Tregs or Tr1 in mice [91]. We, therefore, examined whether SCFAs affect the population of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in chickens. It was intriguing that ABX-treated chickens administered with acetate recovered $CD4^+CD8^-CD25^+$ T cells in cecal tonsils (Fig. 15A). $CD4^+CD8^+CD25^+$ T cells showed a tendency of recovery without significant (Fig. 15B). Other SCFAs, butyrate and propionate, did not show such effect (Fig. 15C-F). GPR43 is known as a receptor for acetate [112]. GPR43 mRNA expression on $CD4^+CD8^-CD25^+$ T cells was significantly higher than other immune cells (Fig. 15G) strongly suggest that the recovery of $CD4^+CD8^-CD25^+$ T cells by acetate administration in ABX-treated chickens could be associated with high GPR43 expression on $CD4^+CD8^-CD25^+$ T cells.

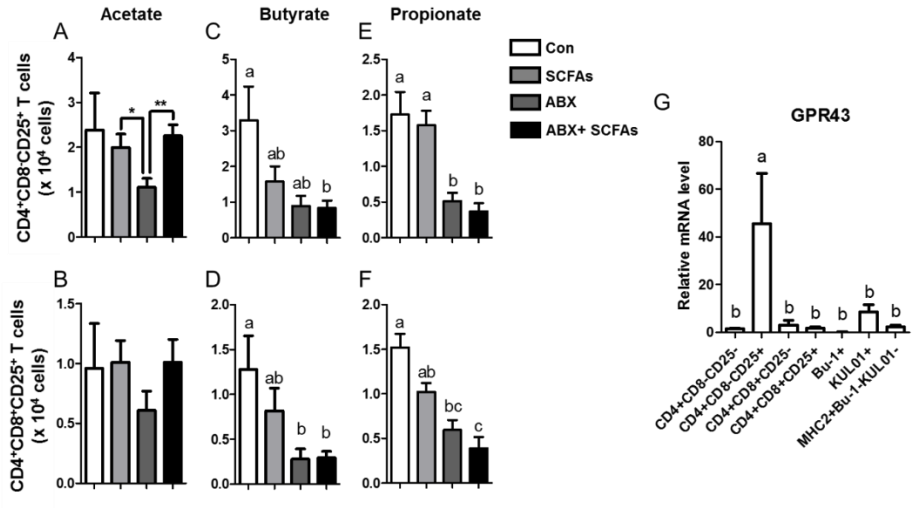


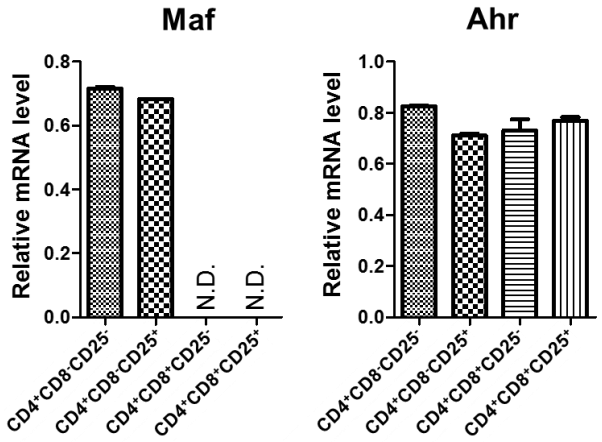
Figure 15. Changes of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in chickens administered with acetate. SCFAs (acetate 50 mM, butyrate 30 mM, propionate 10 mM) or ABX was treated to chickens at hatching with drinking water for 7 days. Cell number of (A, C and E) $CD4^+CD8^-CD25^+$ and (B, D and F) $CD4^+CD8^+CD25^+$ T cells of cecal tonsils was calculated with total cell number and proportion of $CD4^+$ subtype T cells. (G) Each subset of $CD4^+$ T cells, B cells ($Bu\text{-}1^+$) and APCs ($KUL01^+$, MHC class II $(MHC2)^+KUL01^-Bu\text{-}1^-$) were sorted by using ARIA II FACS sorter. The mRNA was extracted from each subset and the level of GPR43 was determined by RT-qPCR. Significant differences were shown as a different alphabet at $P \leq 0.05$.

V. Discussion

The purpose of the present study was to reveal the relationship gut microbiota and homeostasis in chicken. Chicken model was established to remove gut microbiota by antibiotics, as ABX-treated chickens. I demonstrated that proportion and absolute number of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells were significantly diminished in cecal tonsils of chickens after the elimination of gut microbiota. It was noting that there was no change on the $CD4^+CD8^+CD25^+$ T cells in thymus. Expression of IL-10 and IFN-γ, and suppressive function of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells were significantly decreased by the elimination of gut microbiota. Gram positive bacteria appeared to be responsible for the recovery of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells. Furthermore, $CD4^+CD8^-CD25^+$ T cells were induced by acetate administration. GPR43 was highly expressed on $CD4^+CD8^-CD25^+$ T cells.

I showed the inhibitory activity of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in cecal tonsils from ABX-treated chickens. I postulated that $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells may resemble Tr1 cells, because Tr1 cells are known as non-Foxp3 Tregs in human and mouse [8], and there is no Foxp3 gene in chicken [11]. Furthermore, $CD4^+CD8^-CD25^+$ T cells expressed IL-10 and IFN-γ (Fig. 7). Indeed, it has been demonstrated that Tr1 cells produce IL-

10 and IFN-γ much more than $Foxp^+$ Tregs in mouse [113]. I examined transcription factors associated Tr1 cells, namely cellular homolog of the avian virus oncogene musculoaponeurotic fibrosarcoma (c-Maf) and aryl hydrocarbon receptor (Ahr) [87]. There are homolog genes of c-Maf and AhR in chicken that are Gallus gallus v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (Maf) and Gallus gallus aryl hydrocarbon receptor (Ahr). There were no differences of Maf and Ahr mRNA level among $CD4^+$ subtype T cells (Suppl. Fig. 1). It has been suggested that the kinetics of both Maf and Ahr were increased coincident with Tr1 induction, TGF-β and IL-27 [114]. Molecular mechanisms of Maf and Ahr in $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells should be further investigated in chicken.



Supplementary Figure 1. No differences in Maf and Ahr gene expression levels among $CD4^+$ subtype T cells. Chickens at hatching were given water

containing antibiotics for 7 days and cecal tonsils were taken. Single cells from cecal tonsils were, then, stained with anti-chicken CD4, CD8α, and CD25 antibodies. Each subset of $CD4^+$ T cells was sorted by using ARIA II FACS sorter. The mRNA was extracted from each subset and the level of (A) Maf and (B) Ahr was determined by RT-qPCR.

There are a very few, if any, studies on the function of $CD4^+CD8^+$ T cells in chicken. Peripheral $CD4^+CD8^+$ T cells, analyzed in current study, are referred as $CD4^+CD8\alpha^+$ (double positive; DP) T cells in human and other chicken studies. DP T cells are very small population (< 3%) in blood of health people [115]. DP T cells secret IL-2 and IFN-γ and help the differentiation of B cells [116]. DP T cells express CD8α lower than $CD8^+$ cytotoxic T cells [117]. Interestingly, DP T cells are distributed in intestine abundantly when compared with those in blood in human [118, 119]. It has been shown that human intestinal DP T cells express IL-10 and IFN-γ, and no Foxp3 [120]. Human intestinal DP T cells are known to suppress proliferation of $CD4^+$ T cells [120]. In the lamina propria of IBD patients, DP T cells are significantly decreased [120]. The intestinal DP T cells expressed IL-10 or IFN-γ specifically when they were stimulated with *Faecalibacterium prausnitzii*, a *Clostridium* cluster IV strain [120]. In mice, DP intraepithelial lymphocytes (IELs) are known to produce IL-10 and prevent Type 1 helper T (Th1) cell-induced intestinal inflammation in a GATA3-dependent manner [120]. In chicken, DP T cells are

observed in the peripheral blood (20-40%), spleen (10-20%) and intestinal epithelium (5-10%) [121], whereas the function of DP T cells have not been studied in detail yet.

Reduction of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in cecal tonsil of ABX-treated chickens could be affected by low level of acetate. In mouse studies, induction and function of Tregs are affected by SCFAs [89-91] including acetate, propionate, and butyrate [122], which are generated especially by Firmicutes and Bacteroidetes, after fermenting undigested carbohydrates [84]. Activation of GPR43 using SCFAs promotes the number and function of $IL\text{-}10^+Foxp3^+$ Tregs, and propionate directly increases Foxp3 expression and IL-10 production [91].

Both butyrate and propionate are known to induce the differentiation of $Foxp3^+$ Tregs [91]. Interestingly, only acetate, not propionate and butyrate, induced $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in cecal tonsils. There are a few probable reasons for this. Firstly, propionate induced colonic $Foxp3^+$ Tregs via GPR43 *in vivo* [91], whereas no evidence has been reported on the induction of Tr1 cells. Secondly, butyrate showed the most efficient HDAC inhibitor activity and induced $Foxp3^+$ Tregs [89]. It stimulated the secretion of IL-10 and RA from DCs and macrophages via GPR109α, expressed in DCs and macrophages, not in T cells [98], to induce $Foxp3^+$ Tregs and Tr1 cells [123]. However, GPR109α gene does not exist in chicken [124]. Taken together, no effect of butyrate on $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells could be

caused by no GPR109α, which is the essential to induce Tregs.

Acetate can be produced by enteric bacteria and acetogens from H_2 and CO_2, or from formate via the Wood–Ljungdahl pathway [84, 125]. So far, about 2,000 acetogens have been characterized [126]. Acetate is transported readily to blood, whereas most butyrate is utilized by the epithelial cells [100, 127, 128]. The mouse studies suggested that the action of acetate appeared to mediate GPR41 and GPR 43 [129]. It was noting that mice, treated with antibiotics for gut microbiota-free condition, administered with acetate recovered colonic Tregs [89]. Acetate can induce the differentiation of naïve T cells to Tr1 cells directly with GPR43-independent pathway, whereas it acetylated p70 S6 kinase and phosphorylated of ribosomal protein S6 (rS6) as a HDAC inhibitor activity [100]. On the contrary, other study suggested that SCFAs can directly suppress HDAC in a GPR43-dependent manner [91]. Besides, the expression of GPR43 on the regulatory function of T cells has been controversial [91, 96, 112].

How $CD4^+CD8^-CD25^+$ T cells were affected by acetate is unclear. Acetate may play as a HDAC inhibitor via GPR43 [91] and activate mTOR-S6K signaling [100] to induce $CD4^+CD8^-CD25^+$ T cells. In human and mouse studies, GPR43 was highly expressed on myeloid cells [91], including monocytes [130]. Acetate decreased TNF-α and IFN-γ in human monocytes stimulated with LPS [131]. However, there is no evidence that monocytes treated with acetate result in the induction of Tregs.

There are a few studies on the effect of acetate on immune system in chicken. In acetylated starch feeding chickens, lesion score of necrotic enteritis and the number of *Clostridium perfringens* showed no differences compared with those of infected only control. Body weight of chicks infected with *Clostridium perfringens* and fed acetylated starch was significantly higher than infected chickens [132].

The other possibility for the reduction of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in ABX-treated chickens might be reduced migration of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in ABX-treated chickens. $CD4^+CD25^+$ T cells are shown to preferentially migrate to cecal tonsils [110]. I confirmed that there was no changes on $CD4^+CD8^+CD25^+$ T cells in thymus and $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in spleen. Collectively, migration is unlikely the mechanism for the reduction of cells $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells in ABX-treated chickens.

The present study demonstrated that $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells were affected by Gram positive bacteria. Probiotics *Bifidobacterium breve* and *B. longum* induced colonic Tr1 via $CD103^+$ DCs, and Tr1 cells ameliorated severe intestinal inflammation [9]. *Clostridium* cluster IV and XIVa produced abundant acetate as well as lesser butyrate [7]. *Bifidobacterium* and *Clostridium* as Gram positive bacteria likely affect $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells.

Reduction of Tregs causes susceptible status to induce inflammation in gut.

Pathogenic Th17 cells, which are producing IFN-γ and may cause gut inflammation, such as, colitis [133-135]. Tregs could prevent colitis by pathogenic Th17 cells [136]. Furthermore, gut inflammation was alleviated by generation of Tr1 cells in SCID gut inflammation model, which did not contain Tregs [9].

In germ-free mouse model, B cells showed a various changes in an organ-dependent manner. Although germinal center (GC) B cells and IgM^+ B cells in mesenteric lymph nodes were increased, no differences were found in spleen [137]. Furthermore, B cells in bone marrow were increased in germ-free mice [138]. The results in the present study showed that there was no change on the population of B cells (Bu-1^+ cells) in cecal tonsils and spleen of ABX-treated chickens. Furthermore, B cells in the bursa of Fabricius were decreased in ABX-treated chickens [139]. Chrząstek *et al*. reported that oral treatment of antibiotics to neonatal chickens decreased bursal B cells [140]. But there was no explanation at molecular mechanism.

Macrophages in ABX-treated chickens were not significantly changed in the present study. Similarly, macrophages in small intestinal lamina propria [141] and colon were not changed in germ-free mice [142]. In other study, macrophages were significantly reduced in germ-free mice [143]. No population changes on macrophages in cecal tonsils from ABX-treated chickens could be caused by sustaining recruitment of monocytes.

I established ABX-treated chicken model by removal of gut microbiota to

study gut homeostasis. It was noting that the ABX-treated chickens showed no changes on physiological traits including body weight, length of intestine, weight of a major organs, and glucocorticoid level in serum. In the present study, antibiotics was diluted 10-times more [108] and treated shorter time [2, 89] than those studies using ABX-treated mice [144-146]. The reasons could be that (1) born-free of microbes in chicks by the time of hatching [147], (2) unlike mammal, no interference of microbiota from the mother [148], (3) simple and easy control of microbiota at the initial stage as to provide a water containing ABX [147], (4) social differences in mouse including coprophagy and bruxing [149].

Antibiotics seemingly affect not only the population of microbiota but also a metabolism in the host. Although precise action mode of antibiotics for promoting growth in domestic animals is still unclear, it is widely accepted that antibiotics modulate gut microbiome and their products, such as short chain fatty acids [150] causing changes on the magnitude of host immunity. Of course, the suppression of enteric pathogens, for instance, *Escherichia coli*, *Salmonella ssp*., and *Clostridium perfringens*, by antibiotics would be a benefit for healthy intestinal epithelium [151]. However, how antibiotics target specifically those enteric pathogens, not common microbes, is yet to be defined and difficult to explain. Besides the dose of antibiotics used in the domestic animal feed industry was known to be a sub-therapeutic dose [152, 153]. It is likely that antibiotics, especially at the level used as a feed additive, would have

modulated host cells including primarily epithelial cells and intestinal immune cells.

Collectively, my study suggests that gut microbiota regulate both the population and the function of $CD4^{+}CD8^{-}CD25^{+}$ and $CD4^{+}CD8^{+}CD25^{+}$ T cells in cecal tonsils, and acetate plays as an important factor for gut immune homeostasis. It is likely that acetate producing Gram-positive bacteria can be applied to improve the gut health and used as probiotics. Furthermore, ABX-treated chicken model could be used for future studies on the relationship between gut homeostasis and microbes, including probiotics and synbiotics.

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VII. Summary in Korean

닭에서 장내 미생물 연구의 대부분은 성장 촉진 분야이며 장 항상성 관련 연구는 크게 조명 받지 못한 상태이다. 조절 T 세포는 $CD4^+$ T 세포의 한 종류로서 장 항상성 유지에 매우 중요한 역할을 한다고 알려져 있다. 장내 조절 T 세포는 *Clostridium* spp. cluster IV 와 XIVa strains, altered Schaedler flora, *Bacteroides fragilis* 같은 장내 미생물에 의해 유도 된다. 그러나 닭의 조절 T 세포($CD4^+CD25^+$ T 세포) 가 장내 미생물과 어떤 연관이 있는지는 거의 알려져 있지 않다.

본 연구에서는 닭 조절 T 세포와 장내미생물 간 관련성을 밝히기 위해서 다양한 농도의 항생제(ampicillin, gentamycin, neomycin, metronidazole, vancomycin) 조합하여 장내 미생물 제거 하였다. 특정 농도의 항생제 칵테일(ABX; ampicillin, gentamycin, neomycin, metronidazole 100 μg/ml 와 vancomycin 50 μg/ml)을 7일 간 음수처리 하였을 때, 맹장 내용물의 박테리아 colony forming unit (CFU)가 99% 이상 제거되었다. 이때 몸무게, 면역 장기(비장, 활액낭[bursa], 간) 무게 및 장 (십이지장, 공장, 회장, 대장) 길이에 변화는 없었다. 혈중 스트레스 호르몬[glucocorticoid] 수치도 변화 없었다. 나아가 비장 및 맹장 편도의 B 세포 및 대식세포의 비율과 MHC class II 발현 수치도 변하지 않았다. 이를 바탕으로 장내 미생물이 제거 된 ABX 처

리 닭 모델을 구축하였다.

다음으로 장내 미생물이 제거 되었을 때, $CD4^+$ T 세포 변화를 살펴보았다. ABX 처리 닭 모델의 맹장 편도 $CD4^+CD8^-CD25^+$ T 세포 및 $CD4^+CD8^+CD25^+$ T 세포 비율과 세포수가 유의적으로 감소하였다. 그러나 비장 $CD4^+CD8^-CD25^+$ T 세포 및 $CD4^+CD8^+CD25^+$ T 세포는 변하지 않았다. 사이토카인 발현 변화를 보았을 때, ABX 처리 닭 모델 유래 $CD4^+CD8^-CD25^+$ T 세포 및 $CD4^+CD^+CD25^+$ T 세포에서 IL-10 과 IFN-γ mRNA 발현이 유의적으로 감소하였다. 나아가 ABX 처리 닭 모델 유래 $CD4^+CD8^-CD25^+$ T 세포 및 $CD4^+CD8^+CD25^+$ T 세포의 $CD4^+CD25^-$ T 세포 증식 억제능이 유의적으로 감소하였다. 마우스 연구에서는 $CD5^{hi}$ $CD4^+Foxp3^-$ T 세포가 말단 조절 T 세포로 분화된다고 잘 알려져 있다. 이를 ABX 처리 닭 모델에서 확인해본 결과, 감소된 $CD4^+CD8^-CD25^+$ T 세포 및 $CD4^+CD8^+CD25^+$ T 세포는 대부분 $CD5^{hi}$ 세포였으며, 흉선 $CD4^+CD8^+CD25^+$ T 세포는 변하지 않았다. 종합하면 장내 미생물이 제거 되면서 맹장 편도 $CD4^+CD8^-CD25^+$ T 세포 및 $CD4^+CD8^+CD25^+$ T 세포도 감소되었으며, 억제능 또한 감소되었다.

다음으로 $CD4^+CD8^-CD25^+$ T 세포 및 $CD4^+CD8^+CD25^+$ T 세포 감소가 어떤 요인에 의해 발생된 것인지 확인하였다. 먼저, 장내 미생물이 회복 되었을 때, $CD4^+CD8^-CD25^+$ T 세포 및 $CD4^+CD8^+CD25^+$ T

세포가 회복하는지 확인하기 위해, ABX 처리 닭 모델을 일반 닭과 공동 사육하였다. 그 결과, 공동 사육 5일째, ABX 처리 닭 모델의 CFU 가 일반 닭과 비슷한 수준으로 회복하였으며, 7일 째, $CD4^+CD8^-CD25^+$ T 세포 및 $CD4^+CD8^+CD25^+$ T 세포가 회복되는 것을 확인하였다. 나아가 그람 양성균을 특이적으로 제거하는 항생제(vancomycin)와 그람 음성균을 특이적 제거하는 항생제(polymyxin B)를 음수 처리하는 실험을 통해 $CD4^+CD8^-CD25^+$ T 세포 및 $CD4^+CD8^+CD25^+$ T 세포는 그람 양성균에 의존적인 것으로 나타났다. 단쇄지방산 중 하나인 아세테이트를 음수 급이 하였을 때, 장내 미생물이 제거 되었음에도 불구하고 $CD4^+CD8^-CD25^+$ T 세포 및 $CD4^+CD8^+CD25^+$ T 세포가 회복되었다. 그 외 단쇄지방산 인 뷰틸레이트와 프로피오네이트는 효과를 보이지 않았다. 나아가 $CD4^+CD8^-CD25^+$ T 세포에서 아세테이트의 수용체로 알려진 GPR43 발현이 다른 면역세포와 비교하였을 때, mRNA 수준에서 유의적으로 높게 나타났다.

종합하면, 그람 양성균 의해 $CD4^+CD8^-CD25^+$ T 세포와 $CD4^+CD8^+CD25^+$ T 세포의 군집 및 기능이 조절되며, 특히 $CD4^+CD8^-CD25^+$ T 세포는 GPR43 에 매개하여 아세테이트에 의해 유도 되는 것으로 사료된다.