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«Reseacrh Note»

Chicken Interleukin-5 is Expressed in Splenic Lymphocytes and Affects Antigen-Specific Antibody Production

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Vaccination is important for reducing disease incidence in the poultry industry. To enhance immunity and vaccine efficacy, chicken cytokines associated with antibody production must be identified. In this study, we focused on interleukin-5 (IL-5), involved in antibody production in mice, measuring its expression and effects on antibody production. Concanavalin A-stimulated splenocytes were used for RT-PCR to clone *IL5* cDNAs. Recombinant IL-5 was prepared from the clone and administered to chickens with antigen via the ocular-topical route twice every alternate week. IL-5 enhanced antigen-specific IgY and inhibited antigen-specific serum IgA production in serum. Our findings suggest that IL-5 plays an important role in chicken antibody production, with possible unique functions.

Key words: antibody production, IgA production, interleukin-5, splenic lymphocyte

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Introduction

Chicken meat and eggs are important protein sources. Infectious diseases often cause significant economic losses to the poultry industry and require control through vaccination. Choosing vaccination routes is important for eliciting effective immune responses. Mucosal vaccination is a practical method that not only induces serum and mucosal antibody production[1], but also reduces the risk of accidents because it does not require injection. Ocular-topical vaccination using a zymosan A adjuvant enhances antigen-specific serum IgA antibody production[2]. However, unlike in mammals, the detailed mechanism underlying antibody production in chickens is less well-characterized. The identification of chicken cytokines that regulate antibody production is indispensable for enhancing vaccination efficacy.

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In mammals, interleukin-5 (IL-5) is a multifunctional cytokine primarily produced by mast cells and T-cell subsets, particularly activated T_h2 cells[3–5]. It acts on B cells to induce their proliferation and differentiation to produce immunoglobulins[6]. Among IL-5-responsive B-cell subsets, B-1 cells are thought to provide innate immunity by producing natural antibodies. Most B-1 cells express the IL-5 receptor, which is composed of a specific α chain specific to IL-5 (IL-5Rα) and a common β chain; it plays a key role in supplying IgA-producing plasma cells to the mucosa[6,7]. B-2 cells (conventional B cells) in the peripheral blood and spleen acquire IL-5-responsiveness when stimulated by CD38 and subsequently switch from IgM to IgG1 production[8,9].

In the 2000s, multiple chicken genes encoding cytokines and chemokines were identified. Although the chicken genes encoding T_h2 cytokines, such as IL-3, IL-4, IL-5, and granulocyte macrophage colony-stimulating factor (GM-CSF), have been mapped based on synteny[10], the chicken *IL5* gene lacks recognizable regulatory regions, such as transcription factor-binding sites and instability regions, and its cDNA has not been cloned. In contrast, the gene encoding chicken IL-5Rα possesses highly conserved motifs[11]. Chicken *IL5RA* is expressed at high levels in the lungs, spleen, and bone marrow; it binds specifically to chicken but not to mouse IL-5, suggesting that it is functional. However, chicken IL-5 expression and function have not yet

been elucidated.

In the present study, we investigated the expression of chicken IL-5 in splenic lymphocytes and cloned them. We also examined whether co-administration of recombinant chicken IL-5 and the antigen via the ocular-topical route affected antigen-specific antibody production.

Materials and Methods

Experimental animals and husbandry

Freshly laid and fertilized White Leghorn (WL) eggs and chickens, respectively, were purchased from Akita Co. (Fukuyama, Japan) and maintained in an isolation facility in the Animal Farm of Hiroshima University. All experimental methods were approved by the Animal Use and Care Committee of Hiroshima University (No. C22-33-2). Spleens were collected from one- and two-month-old chickens.

Splenocyte collection and culture

Chicken spleens were dissociated, followed by density-gradient centrifugation using Ficoll-paque™ PLUS (GE healthcare, Chicago, IL, USA) at 400 × *g* for 15 min. Lymphocytes were washed with PBS, seeded at a density of 4×10^7 per mL in 24-well plates, and cultured in Iscove's Modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO_2. Concanavalin A (ConA), a mitogen of T cells, was added to the cultured lymphocytes at a final concentration of 2 μg/mL. After stimulation for 24 and 48 h, the cells were collected and used for RT-PCR to detect chicken IL-5 levels.

RT-PCR

Total RNA was extracted from lymphocytes cultured with or without ConA using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), treated with DNase I (Thermo Fisher Scientific) and used to synthesize cDNA with oligo$(dT)_{20}$ primer and SuperScript® III reverse transcriptase (Thermo Fisher Scientific). Primers were designed to amplify the full-length *IL5* coding sequence, as per the NCBI NC_052544.1; *Gallus gallus* isolate bGalGal1 chromosome13, bGalGal1.mat.broiler.GRCg7b, whole genome sequence 16509448 to 16513401).

PCR was performed using Ex Taq DNA polymerase (TaKaRa Bio, Shiga, Japan) and the primers (5′-CTCTGAGCACATCAGGACC-3′) and (5′-GCTTGTTTAAGCTTACGGTG-3′), under the following conditions: 5 min at 95°C; 40 cycles of 10 s at 98°C, 30 s at 60°C, and 1 min at 72°C; and 10 min at 72°C. PCR products were subjected to electrophoresis on a 3% agarose gel.

Sequencing

PCR products were assembled into the pCR2.1-TOPO™ TA vector (Thermo Fisher Scientific) using TA cloning, and the cloned vectors were transformed into DH5α competent cells (Toyobo Co., Ltd., Osaka, Japan). Colony PCR was performed using primers M13-20 (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-GGAAACAGCTATGACCATG-3′). The amplified insert DNA was sequenced using the Big Dye Terminator Sequencing Kit (version 3.1; Applied Biosystems, Waltham, MA, USA). The cDNA sequences were aligned with GenBank AB618613.1.

Production of recombinant IL-5

The *IL5* cDNA was subcloned into the pcDNA4/myc-His B vector (Thermo Fisher Scientific). The coding region was PCR amplified using KOD Fx Neo DNA polymerase (Toyobo) and the primers: chIL-5 *Kpn*I F (5′-TAAGGTACCATGAGGACCCATC-3′) and chIL-5 *Age*I R (5′-CATACCGGTCACTCTTGCATC-3′). HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific), supplemented with 10% FBS, at 37°C under 5% CO_2. The expression vector was transfected into them using Lipofectamine3000 reagent (Thermo Fisher Scientific) according to the manufacturer's protocol, then the culture medium was replaced with FreeStyle293 expression medium. After transfection, recombinant His-tagged IL-5 was detected in the supernatant of the transfected cells by western blotting using a mouse anti-His as the primary antibody and HRP-labeled goat anti-mouse IgG as the secondary antibody. The molecular weight of the protein was approximately 13 kDa, consistent with the expected size (Supplemental Figure S1). Recombinant IL-5 was isolated from the supernatant by electrophoresis on a 15% sodium dodecyl sulfate-polyacrylamide gel. The 13-kDa band was excised and protein was extracted overnight in 1% SDS, 20 mM Tris-HCl. Thereafter, the polyacrylamide was removed using a spin column and the protein was concentrated using an ultrafiltration column (NMWL; 5000 Da; Millipore, Burlington, MA, USA). An SDS-eliminant (ATTO, Tokyo, Japan) was used to remove SDS from the protein solution.

Preparation of dinitrophenylated keyhole limpet hemocyanin (DNP-KLH)

Briefly, 200 mg of K_2CO_3 was dissolved in 6 mL of distilled water, and 200 mg of KLH was added to the solution. Two hundred milligrams of 2, 4-dinitrobenzene-solfonic acid hydrate (DNBS) was dissolved in 4 mL of distilled water. The DNBS solution was then slowly added to the KLH solution. The mixture was stirred in the dark at room temperature for 24 h.

Immunization and serum collection

DNP-KLH and recombinant IL-5 were co-administered to chickens via the ocular-topical route once every other week, for a month. Recombinant IL-5 was mixed with DNP-KLH at three dilutions (1/1000, 1/500, and 1/100) and administered at 2 mg DNP-KLH/kg body weight (five or six chickens per goup). Serum was collected one week after administration.

Enzyme-linked immunosorbent assay (ELISA)

Anti-DNP IgM, IgY, and IgA antibodies were measured using ELISA as described previously[2]. Anti-DNP titers that were more than a 1.5-fold quartile range from the first or third quartile were treated as outliers (Supplemental Figure S2) and analyzed using Dunnett's test (n = 3–6). Microsoft Excel was used to graph the data, and R studio (2022.07.0) was used for identification of outliers and statistical analyses. Statistical significance was defined as $*p < 0.05$, $**p < 0.01$.

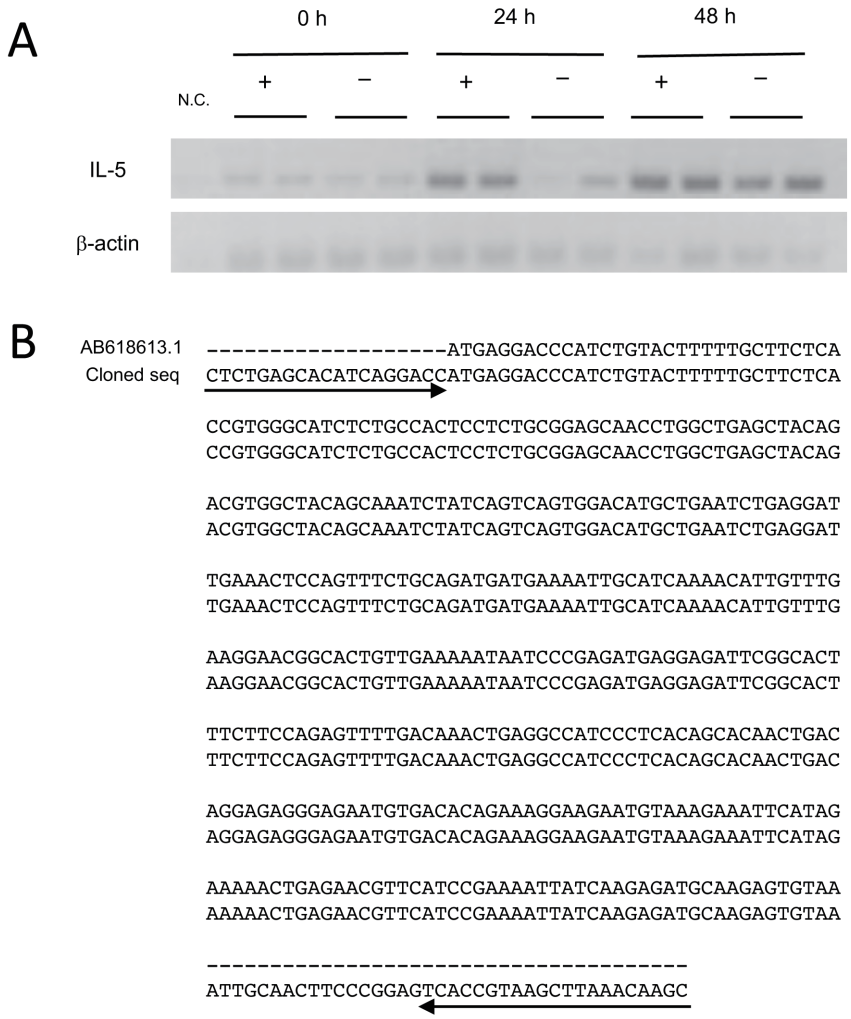

Fig. 1. **Expression and sequence of chicken *IL5*.** (A) RT-PCR measurements of mRNA expression. ConA-stimulated splenocytes were used for preparation of cDNA, which was used as a template in RT-PCR. (B) Sequence comparison of cloned *IL5* with that deposited in NCBI. Arrows show primers designed to amplify untranslated regions.

Results

Cloning of the chicken IL5 coding sequence

To evaluate chicken *IL5* expression, RT-PCR was performed on splenic lymphocytes cultured with and without ConA. The expression of *IL5* was consistently observed, regardless of ConA stimulation (Fig. 1A). The PCR amplicons were sequenced using our designed primer set. The full-length IL-5 coding sequence was 381 bp in length, consistent with GenBank AB618613.1 (Fig. 1B).

Recombinant IL-5 regulates antigen-specific Ig production

To examine the effects of chicken IL-5 on antibody production, chickens were treated with recombinant IL-5 and DNP-KLH via the ocular-topical route twice every other week. One week after each immunization, serum was collected and anti-DNP IgM, IgY, and IgA titers were measured.

Recombinant chicken IL-5 affected antigen-specific antibody production after co-administration with DNP-KLH. Low IL-5 levels significantly increased anti-DNP IgY titers after the second immunization compared with controls (Fig. 2B, $p < 0.01$). No significant differences in anti-DNP IgM titers were observed (Fig. 2A, $p = 0.084$). All groups that received IL-5 showed a significant decrease in anti-DNP IgA titers after the second dose compared to controls (Fig. 2C, $p < 0.05$).

Discussion

IL5 has been annotated in the chicken genome; however, no recognizable regulatory regions were identified[10]. IL-5Rα, a

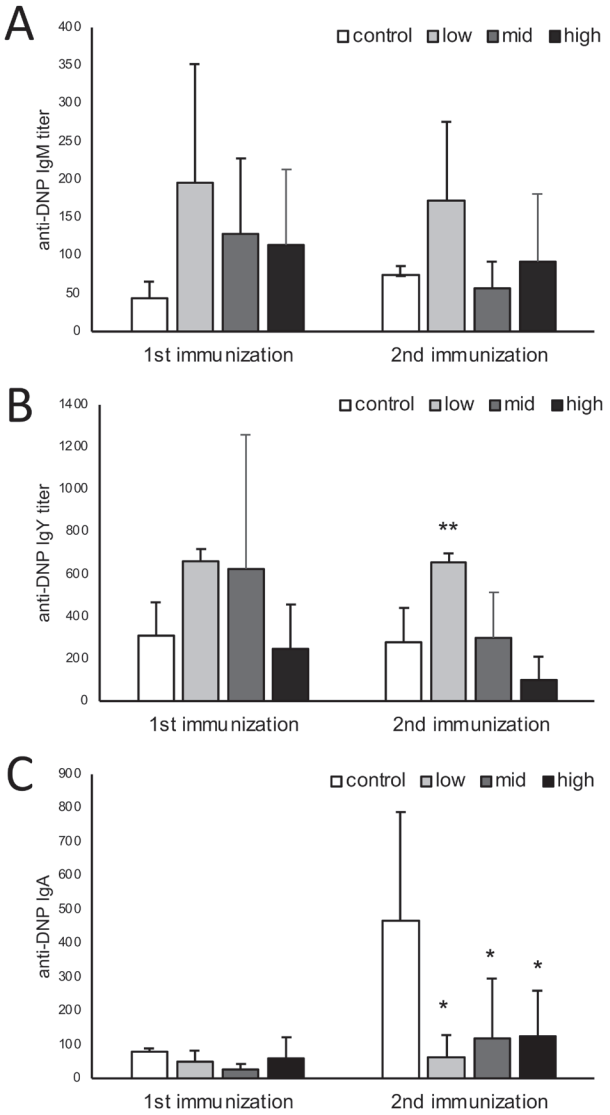

Fig. 2. **Titers of anti-DNP IgM, IgY, and IgA in the sera of immunized chickens.** Control chickens were immunized with 2 mg/kg DNP-KLH. Other groups were immunized with DNP-KLH (2 mg/kg body weight) with diluted recombinant IL-5 (low, 1/1000; medium, 1/500; and high, 1/100). Immunizations were performed once every other week, for a month. After each immunization, sera were collected for quantification of anti-DNP IgM, IgY, and IgA titers using ELISA.

receptor for IL-5, was identified and showed specific affinity for chicken IL-5[11], suggesting its potential for chicken IL-5 signal transduction. However, little progress has been made in understanding chicken IL-5 function. In this study, we showed that *IL5* is expressed in splenic lymphocytes and cloned its cDNA. When recombinant chicken IL-5 was administered via the ocular-topical route with the antigen, antigen-specific antibody production was affected, suggesting that IL-5 plays a role in chickens antibody production.

Chicken *IL5* mRNA expression in splenic lymphocytes was assessed by RT-PCR, and its expression was increased by ConA stimulation. This result is similar to the expression pattern of IL-5 in mammals[3,12], suggesting that IL-5 in chickens is a cytokine produced by T-cell subsets, similar to that in mammals. Chicken IL-5 may mediate lymphocyte interactions. To our knowledge the chicken *IL5* coding sequence had not yet been cloned; therefore, we confirmed the sequence in this study.

To evaluate the effects of chicken IL-5 on antigen-specific antibody production, recombinant IL-5 was co-administered with DNP-KLH via the ocular-topical route. After co-administration, DNP-specific antibodies were detected in the serum, suggesting that IL-5 affects antigen-specific antibody production.

Low-concentration IL-5 significantly enhanced anti-DNP IgY titers after the second immunization ($p < 0.01$). Mouse IL-5 participates in the regulation of B-cell differentiation into antibody-producing cells, such as the differentiation of anti-DNP IgG-producing and surface IgA^+ B-2 cells into IgA-producing cells[13–15]. IL-5 signaling plays a critical role in antibody production, as IL-5Rα-deficient mice show decreased serum IgM, IgG subclass, and IgA levels[7,16]. Therefore, our results suggest that chicken IL-5 regulates antibody-producing cells. The fact that the anti-DNP IgM titer tended to increase also supports this hypothesis. However, at medium and high concentrations, IL-5 reduced the titers of anti-DNP IgM and IgY. This suggests that it is important not to exceed an optimal IL-5 concentration for effective Ig production. Mammalian IL-5 acts on other immune cells including eosinophils and regulatory T cells, in addition to B cells[17–19]. The addition of exogenous IL-5 may cause an excessive immune response and disrupt the balance of antibody production.

Unlike the situation in mammals, IL-5 did not enhance anti-DNP IgA production. This suggests that IL-5 is involved in the class switch to IgY production but not in the switch to IgA production. Activated B cells are induced to switch or differentiate into antibody-producing cells. In the mouse, splenic B cells acquire responsiveness to IL-5 by CD38 stimulation; this response upregulates the expression of genes such as *Blimp1*, which promotes differentiation into antibody-producing cells, and *Aid* (also known as *Aicda*), which encodes an enzyme involved in class switching[15]. In chickens, IL-5 likely is involved in the differentiation and maturation of activated B cells. Our results suggest that IL-5 preferentially promotes class switching to IgY and differentiation into IgM- or IgY-producing cells, decreasing IgA production. Thus, chicken IL-5 may have two functions: the induction of a class switch to IgY and the regulation of antibody-producing cells. To more critically evaluate the function of chicken IL-5, we need to prepare highly purified recombinant IL-5. In addition, because IL-5 and IL-6 enhance rat tear IgA as adjuvants[20], we should focus on IgA-producing cells in tears and mucosal tissues. To understand the regulation of IgA production, it is essential to identify chicken IL-5Rα-expressing cells and determine the contribution of IL-5 to antibody production.

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Author Contributions

Tenkai Watanabe conducted experiments, analyzed data, and drafted the original manuscript. Tenkai Watanabe, Syuichi Furusawa, and Hiroyuki Horiuchi designed experiments. Tenkai Watanabe, Takumi Terada, Ryo Ezaki, Mei Matsuzaki, and Hiroyuki Horiuchi critically discussed the data and reviewed the manuscript draft.

Conflicts of Interest

The authors declare no conflicts of interest.

References

[1] Hikono H, Mase M, Matsuu A, Nakayama M and Saito T. Intraocular vaccination with an inactivated highly pathogenic avian influenza virus induces protective antibody responses in chickens. Vet Immunol Immunopathol, **151**: 83–89. 2013. https://doi.org/10.1016/j.vetimm.2012.10.005, PMID:23159237

[2] Abou Elazab MF, Inoue Y, Kamei H, Horiuchi H and Furusawa S. Zymosan A enhances humoral immune responses to soluble protein in chickens. J Vet Med Sci, **79**: 1335–1341. 2017. https://doi.org/10.1292/jvms.16-0636, PMID:28652560

[3] Cherwinski HM, Schumacher JH, Brown KD and Mosmann TR. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. J Exp Med, **166**: 1229–1244. 1987. https://doi.org/10.1084/jem.166.5.1229, PMID:2960769

[4] Plaut M, Pierce JH, Watson CJ, Hanley-Hyde J, Nordan RP and Paul WE. Mast cell lines produce lymphokines in response to cross-linkage of FcεRI or to calcium ionophores. Nature, **339**: 64–67. 1989. https://doi.org/10.1038/339064a0, PMID:2469965

[5] Mosmann TR and Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol, **7**: 145–173. 1989. https://doi.org/10.1146/annurev.iy.07.040189.001045, PMID:2523712

[6] Takatsu K. Interleukin 5 and B cell differentiation. Cytokine Growth Factor Rev, **9**: 25–35. 1998. https://doi.org/10.1016/S1359-6101(97)00034-8, PMID:9720754

[7] Moon B, Takaki S, Miyake K and Takatsu K. The role of IL-5 for mature B-1 cells in homeostatic proliferation, cell survival, and Ig production. J Immunol, **172**: 6020–6029. 2004. https://doi.org/10.4049/jimmunol.172.10.6020, PMID:15128785

[8] Mizoguchi C, Uehara S, Akira S and Takatsu K. IL-5 induces IgG1 isotype switch recombination in mouse CD38-activated sIgD-positive B lymphocytes. J Immunol, **162**: 2812–2819. 1999. https://doi.org/10.4049/jimmunol.162.5.2812, PMID:10072528

[9] Horikawa K, Kaku H, Nakajima H, Davey HW, Henninghausen L, Iwamoto I, Yasue T, Kariyone A and Takatsu K. Essential role of Stat5 for IL-5-dependent IgH switch recombination in mouse B cells. J Immunol, **167**: 5018–5026. 2001. https://doi.org/10.4049/jimmunol.167.9.5018, PMID:11673510

[10] Kaiser P, Rothwell L, Avery S and Balu S. Evolution of the interleukins. Dev Comp Immunol, **28**: 375–394. 2004. https://doi.org/10.1016/j.dci.2003.09.004, PMID:15062639

[11] Fukushima Y, Miyai T, Kumagae M, Horiuchi H and Furusawa S. Molecular cloning of chicken interleukin-5 receptor α-chain and analysis of its binding specificity. Dev Comp Immunol, **37**: 354–362. 2012. https://doi.org/10.1016/j.dci.2012.02.013, PMID:22391510

[12] Shilovskiy I, Andreev S, Mazurov D, Barvinskaia E, Bolotova S, Nikolskii A, Sergeev I, Maerle A, Kudlay D and Khaitov M. Identification of a novel splice variant for mouse and human interleukin-5. Heliyon, **6**: e03586. 2020. https://doi.org/10.1016/j.heliyon.2020.e03586, PMID:32211550

[13] Matsumoto R, Matsumoto M, Mita S, Hitoshi Y, Ando M, Araki S, Yamaguchi N, Tominaga A and Takatsu K. Interleukin-5 induces maturation but not class switching of surface IgA-positive B cells into IgA-secreting cells. Immunology, **66**: 32–38. 1989. PMID:15493259

[14] Sonoda E, Matsumoto R, Hitoshi Y, Ishii T, Sugimoto M, Araki S, Tominaga A, Yamaguchi N and Takatsu K. Transforming growth factor beta induces IgA production and acts additively with interleukin 5 for IgA production. J Exp Med, **170**: 1415–1420. 1989. https://doi.org/10.1084/jem.170.4.1415, PMID:2677210

[15] Horikawa K and Takatsu K. Interleukin-5 regulates genes involved in B-cell terminal maturation. Immunology, **118**: 497–508. 2006. https://doi.org/10.1111/j.1365-2567.2006.02382.x, PMID:16895557

[16] Yoshida T, Ikuta K, Sugaya H, Maki K, Takagi M, Kanazawa H, Sunaga S, Kinashi T, Yoshimura K, Miyazaki J, Takaki S and Takatsu K. Defective B-1 cell development and impaired immunity against Angiostrongylus cantonensis in IL-5R α-deficient mice. Immunity, **4**: 483–494. 1996. https://doi.org/10.1016/S1074-7613(00)80414-8, PMID:8630733

[17] Tran GT, Hodgkinson SJ, Carter NM, Verma ND, Plain KM, Boyd R, Robinson CM, Nomura M, Killingsworth M and Hall BM. IL-5 promotes induction of antigen-specific CD4+CD25+ T regulatory cells that suppress autoimmunity. Blood, **119**: 4441–4450. 2012. https://doi.org/10.1182/blood-2011-12-396101, PMID:22310911

[18] Verma ND, Plain KM, Nomura M, Tran GT, Robinson C, Boyd R, Hodgkinson SJ and Hall BM. CD4+CD25+ T cells alloactivated ex vivo by IL-2 or IL-4 become potent alloantigen-specific inhibitors of rejection with different phenotypes, suggesting separate pathways of activation by Th1 and Th2 responses. Blood, **113**: 479–487. 2009. https://doi.org/10.1182/blood-2008-05-156612, PMID:18827184

[19] Takatsu K and Nakajima H. IL-5 and eosinophilia. Curr Opin Immunol, **20**: 288–294. 2008. https://doi.org/10.1016/j.coi.2008.04.001, PMID:18511250

[20] Pockley AG and Montgomery PC. In vivo adjuvant effect of interleukins 5 and 6 on rat tear IgA antibody responses. Immunology, **73**: 19–23. 1991. PMID:2045126