A Thesis

for the Degree of Master of Science

Functional characteristics of spleen KUL01^{hi}MHCII^{lo} and KUL01^{lo}MHCII^{hi} monocyte/macrophage in chicken

두 군집의 닭 비장 단핵구/대식세포의 기능적 특성 연구

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농 학 석 사 학 위 논 문

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Summary

Monocytes/macrophages are found in diverse organs and have been reported to maintain tissue homeostasis and to act as the first line of defense in the host during steady state and pathogen-induced inflammation, respectively. Monocytes/macrophages have different nomenclature, phenotype, and functions in different organs. Most of monocyte/macrophage studies conducted in chickens are largely performed using cell lines, and subset of primary monocytes/macrophages, yet that is not well understood. The objective of the present study was to define characterization of the phenotype, function, and maintenance of spleen monocytes/macrophages during steady state and inflammation condition.

Spleen monocytes/macrophages can be identified as KUL01^{lo}MHCII^{hi} and KUL01^{hi}MHCII^{lo} cells based on their surface expression of KUL01, and MHCII. During steady state condition, KUL01^{lo}MHCII^{hi} cells are more frequent subset among KUL01^{+} cells. They expressed higher antigen presenting molecules (MHCII, MHCI, and CD80) than those on KUL01^{hi}MHCII^{lo} cells. On the contrary, KUL01^{hi}MHCII^{lo} cells are better phagocytic and migratory cells than KUL01^{lo}MHCII^{hi} cells. Furthermore,

KUL01^{hi}MHCII^{lo} cells are freshly infiltrated in the spleen and then becoming KUL01^{lo}MHCII^{hi} cells. During the inflammation situation induced by LPS administration via intraperitoneal (*i.p*) injection, the proportion and absolute number of KUL01^{hi}MHCII^{lo} cells are increased in the spleen. However, the major source of inflammatory cytokines (IL-1β, IL-6, and IL-12) was KUL01^{lo}MHCII^{hi} cells. Uniquely, inflammation downregulated the MHCII expression in KUL01^{hi}MHCII^{lo} cells, potentially via COX-2 production.

In conclusion, the present study defined phenotype, function, and maintenance of KUL01^{hi}MHCII^{lo} and KUL01^{lo}MHCII^{hi} monocytes/macrophages in spleen during steady state and inflammation condition. This study contributes to better understanding of primary monocytes/macrophages in relation to homeostasis and innate immunity in chicken.

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List of Abbreviations

APC	Antigen presenting cell
BM	Bone marrow
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
COX	Cyclooxygenase
CSF1R	Colony stimulating factor 1 receptor
CTV	Cell trace violet
CXCL	CXC chemokine ligand
DC	Dendritic cell
E	Embryonic
EAC	Ellipsoid associated cell
EAM	Ellipsoid associated macrophage
FDC	Follicular dendritic cell
FL	Fetal liver
GC	Germinal center
IDC	Interdigitating dendritic cell
IFN	Interferon
IL	Interleukin

iNOS	Inducible nitric oxide synthase
I.P	Intraperitoneal
I.V	Intravenous
LPS	Lipopolysaccharide
MARCO	Macrophage receptor with collagenous structure
MHC	Major histocompatibility complex
MMM	Marginal zone metallophilic macrophage
MZ	Marginal zone
MZM	Marginal zone macrophage
MFI	Mean fluorescent intensity
OVA	Ovalbumin
PALS	Periarteriolar lymphoid sheath
PBS	Phosphate Buffered Saline
PRR	Pattern recognition receptor
PWP	Peri-ellipsoidal white pulp
RP	Red pulp
TGF-β	Transforming growth factor - beta
TIM4	T cell Immunoglobulin and Mucin Domain 4
TLR	Toll like receptor
TNF-α	Tumor necrosis factor – alpha

I. Review of Literature

1. Anatomical structure and the function of the spleen

Spleen, an organ found virtually in all vertebrate, located in the left side of abdomen under the diaphragm [1]. Spleen is covered by fibrous capsule and trabecula emerged from fibrous capsule, both of them maintain spleen structure [1]. Spleen lacks lymphatic vessels meaning that only blood circulation system can enter and exit the spleen. Spleen can be largely divided into red pulp and white pulp. Each parts have their specific functions, therefore several types of immune cells are observed depending on the location.

Similar to mouse, chicken spleen is located at left side of abdomen. It is surrounded by fibrous capsule and trabecula and supplied fresh blood through spleen artery [2]. Unlike mouse elongated-shaped in mouse, chicken has an egg-shaped spleen. Importantly, chickens have a closed circulation system that, unlike mouse open circulation system, chicken penicillar capillaries are directly connected with venous sinuses [2]. Chicken and mouse spleens are generated at embryo stage, however, additionally developed within one week after the birth [3, 4]. During this period,

development of mouse spleen is regulated by lymphotoxin [3] whilst chicken spleen is known to be developed by exposure of antigens [4]. As shown in Table 1, unlike in mouse [5], chicken spleen cannot act as erythropoiesis organ and reservoir for erythrocytes, rather liver acts such role in chicken [6].

Table 1. Different characteristics between mouse and chicken spleen.

Characteristics	Mouse	Chicken
Shape	Elongated	Egg-like
Circulation	Open	Closed
Separation between white pulp and red pulp *	Marginal zone	Peri-ellipsoidal white pulp
Post-natal development mediator	Lymphotoxin	Antigen
Erythropoiesis and reservoir for erythrocytes	O	X

* Exact location is depicted in the Figure 1.

1.1 Red pulp

The red pulp consists of venous sinuses and cords (also known as cords of Billroth), occupies 75% of the splenic volume. The main function of red pulp is filtering the blood by removing foreign materials, damaged and old red blood cells [7], and antibodies secretion [8].

Various leukocytes exist in the red pulp, including monocytes, macrophages, dendritic cells (DC), granulocytes, and plasma cells, and macrophages are the most abundant among them [5]. Macrophages located in the cords are known as red pulp (RP) macrophages, expressing phagocytic receptors such as scavenger receptor (CD163) and mannose receptor (CD206), which recognize aged and abnormal erythrocytes in the cords. RP macrophages are also known to express signal regulatory protein α (SIRP-α), which inhibit the normal cell phagocytosis by interact with CD47. It has been also suggested that RP macrophages act to clear blood borne pathogens. For example, depletion of RP macrophage by clodronate liposome [9] or *Spic* knock out [10] in mouse showed increase in the infectivity of *Plasmodium chabaudi*.

Several types of lymphoid and non-lymphoid cells are found in the chicken RP. A large number of macrophages expressing KUL01 and MHCII are found in the RP [11]. A study with administration of various antigens demonstrated that most antigens in RP are phagocytosed by RP

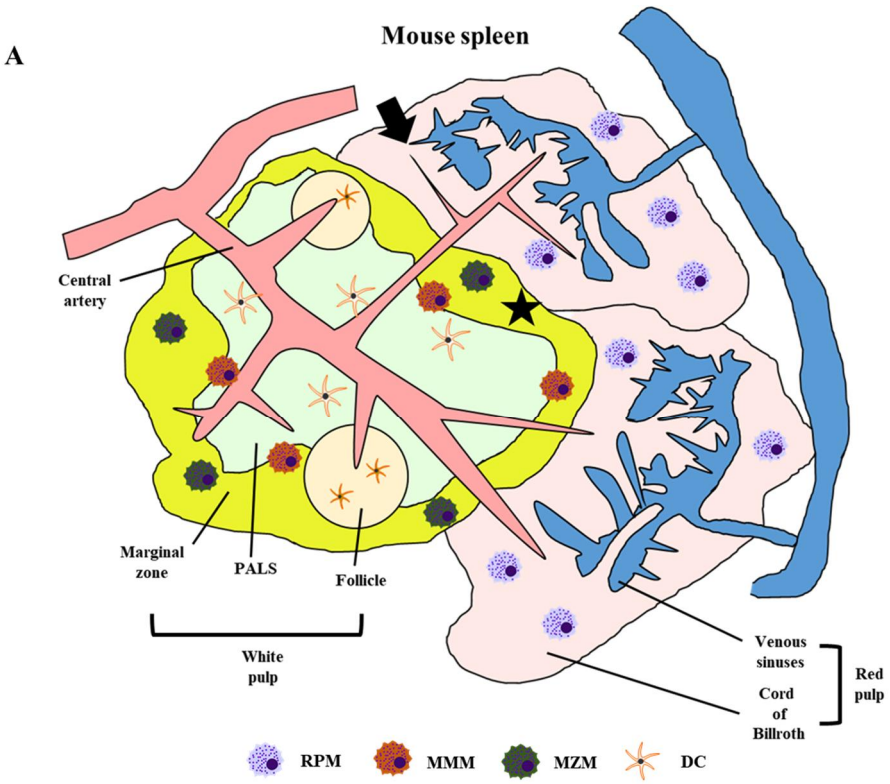
macrophages [12]. However, whether chicken RP macrophages can phagocytose old and abnormal erythrocytes is yet reported. Heterophils, homologous to neutrophils in mammal, are also frequently found [13]. Plasma cells expressing IgM and IgG are also observed [14]. Interestingly, unlike mouse red pulp, several T cells are found in red pulp and they are mostly $CD8^+$ and TCRγδ.

1.2 White pulp

In mouse, the white pulp is composed of periarteriolar lymphoid sheath (PALS), marginal zone and follicle. PALS surrounds the central arteriole where $CD4^+$ T cells are mainly located. Some $CD8^+$ T cells and DC migrated from marginal zone which present blood-borne antigens to T cells are also found [15]. In follicle, known as B cell zone, B cells took large proportion and follicular DC (FDC) also exist which presents antigen to B cells [15]. However, chicken white pulp has a distinct structure, consisting PALS, peri-ellipsoidal white pulp (PWP) and germinal center (GC). Similar to mouse, PALS surround the central arteriole and mainly $CD4^+$ T cells are localized [11]. Interdigitating DCs (IDCs) with KUL01 and MHCII expression are also observed [14]. GC is mainly composed of B cells and the formation of GC is related with FDC which is located near the PALS [11]. Marginal zone is an interface area between red pulp and white pulp and large number of antigen presenting cells (APCs) exist to recognize, clear

and deliver antigens to T cells and B cells [16-18].

Despite their similar component with mouse, chicken spleen has a distinct architecture. First of all, GCs which is similar to mouse follicles, are found at the initial site of central arteriole [19]. Furthermore, chicken spleen is lacking the marginal zone [20]. However, chicken spleen has a peri-ellipsoidal white pulp (PWP) whose function is similar with mouse marginal zone [2, 19]. Similar to mouse marginal zone, B cells and ellipsoid-associated macrophages (EAM) are found in PWP. While ellipsoid associated cells (EACs), which have no analogous cells in mouse, are also reported [20]. EACs composed a unique structure known as ellipsoid that is embedded in PWP [19], surround the penicillar capillaries and it has been suggested that EACs act as immune surveillance through the stomata of penicillar capillaries [21]. Antigens, delivered via intravenous (*i.v*) injection, are shown to be captured in ellipsoid by EACs [22]. Then, the EACs migrated to PALS and GC, and further differentiated into IDC and FDC respectively [19]. Capturing antigen by APCs and migrating to T and B cell area is the major function of the marginal zone in mouse spleen, therefore PWP in chicken is regarded as functionally analogous to spleen marginal zone in mouse. Structure of the spleen and distribution of mononuclear phagocytes in mouse and chicken are depicted in Fig. 1A and B, respectively.


A
Mouse spleen
Central artery
Marginal zone
PALS
Follicle
White pulp
Venous sinuses
Cord of Billroth
Red pulp
RPM
MMM
MZM
DC

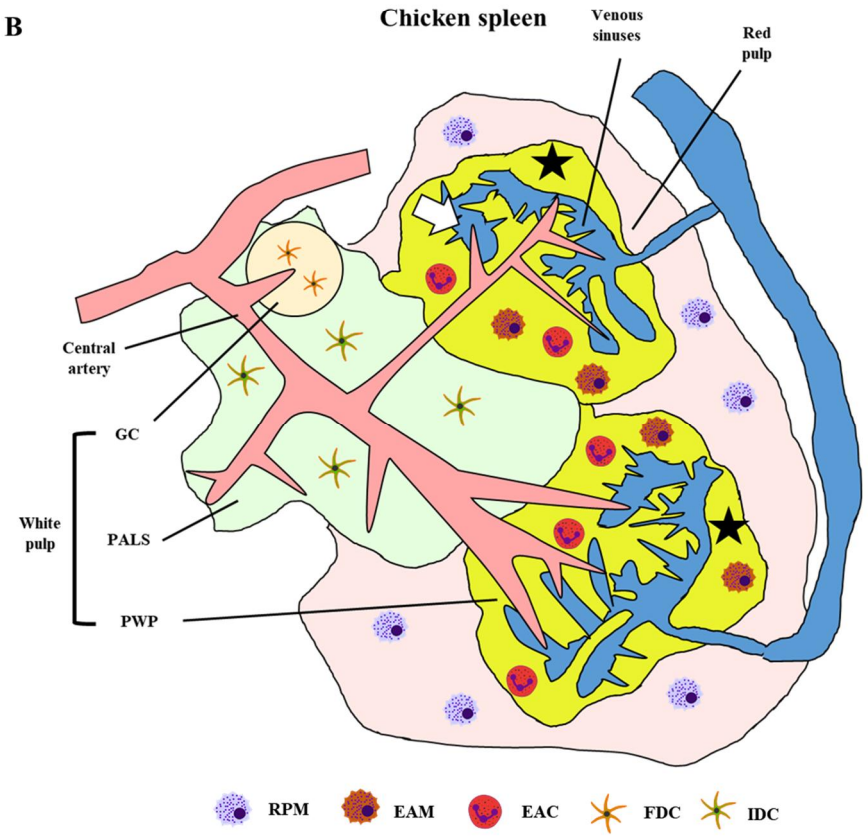

B
Chicken spleen
Venous sinuses
Red pulp
Central artery
GC
White pulp
PALS
PWP
RPM
EAM
EAC
FDC
IDC

Fig. 1 Structure of mouse and chicken spleens. Schematic diagram for the structure and immune cell distribution in spleens from (A) mouse and (B) chicken. A few significant differences between mouse and chicken spleen are the circulation system and separation of white pulp and red pulp. (A) Mouse has an open circulation system (indicated by filled arrow) and, on the other hand, (B) chicken has a closed circulation system (indicated by open arrow). Furthermore, white pulp and red pulp are separated by marginal zone in mouse and by PWP in chicken (indicated as stars). For easy understanding, distribution of mononuclear phagocytes only are depicted in the present figure. PALS, periarteriolar lymphoid sheath; RPM, red pulp macrophage; MZM, marginal zone macrophage; MMM, marginal zone metallophilic macrophage; GC, germinal center; PWP, peri-ellipsoidal white pulp; EAM, ellipsoid associated macrophage; EAC, ellipsoid associated cell; IDC, interdigitating DCs; FDC, follicular DC.

2. Mononuclear phagocytes

Mononuclear phagocytes, derived from bone marrow (BM) precursors, are composed of monocytes, DCs and resident macrophages [23]. Both mouse and chicken mononuclear phagocytes have similar features including morphology [24], plasticity [25], phagocytosis [26], and pathogen clearance [27].

In mouse, F4/80 a glycoprotein antigen is widely used as a marker for mononuclear phagocytes. Several monoclonal antibodies for chicken myeloid cells allow to identify subset of monocytes and/or macrophages [20, 28]. However, these antibodies seem to have some limitations such as unknown antibody binding target, reacting with thrombocyte [20, 29]. KUL01 targets the chicken mannose receptor (MRC1L-B), homologous mammalian mannose receptor [30], reacts only with monocytes and macrophages. therefore it is widely used for chicken mononuclear phagocytes [12].

2.1 Blood monocytes

In mouse, two subsets of monocyte exist in the blood circulation and classified as $Ly6C^{hi}$ classical monocytes and $Ly6C^{lo}$ non□classical monocytes, based on the surface expression of Ly6C [31]. Non-classical monocytes, also known as alternative or patrolling monocytes, reside and crawl the vascular endothelium in an integrin LFA-1-dependent manner [32].

This monocyte highly expresses CX3CR1 and rapidly extravasate to the site of infection than classical monocytes [32]. After infiltration, they act as early inflammation mediators whereas after 2-8 hours, they express genes involved in tissue remodeling and preferentially differentiated into M2 macrophages [32, 33]. Classical monocytes, on the other hand, migrate to the infection site through CCR2-dependent manner and produce inflammatory mediators to initiate the immune response [34]. These monocytes are known to mainly differentiate into M1 macrophages or DCs [35]. However, some studies suggested that classical monocytes are found in spleen during steady state condition and deliver antigens to the lymph node during infection without differentiated to DC [36, 37].

Chicken blood monocytes can be classified as $TIM4^+$ and $TIM4^-$ monocyte based on TIM4 expression which is involved in sensing apoptotic cells [38]. Both blood monocytes express homogeneous chicken monocyte/macrophage marker KUL01, MHCII and CSF1R. In addition, monocyte to macrophage differentiation related genes are elevated in $TIM4^+$ monocytes compared with $TIM4^-$, suggesting that $TIM4^+$ monocytes could be more differentiated form [38]. However, whether these monocytes further differentiated into different type of macrophages and act differently is yet to be elucidated.

2.2 Origin and the maintenance of tissue-resident

macrophages

Most of tissue resident macrophages are originated in prenatal stage, and maintain their populations through self-proliferation [39]. Yolk sac precursors are the primary precursor cells appear during the prenatal stage [40]. Then, the fetal liver (FL) precursors are generated by migration of erythro-myeloid precursors (EMPs) arise form yolk sac to the fetal liver [41]. The third developed precursor is fetal hematopoietic stem cell (HSC), further seed in fetal liver and fetal BM [42]. By using Cre-ER system[1], it has been reported that most of tissue resident macrophages are generated from fetal liver monocytes and fetal HSC, meanwhile microglia cells are derived from yolk sac progenitor [43].

Though, some tissue resident macrophages, including intestinal [44], dermal [45], and cardiac macrophages [46], are originated from prenatal precursors, with a greater or lesser extent can be replenished by infiltration of monocytes in steady state condition. For example, $CCR2^{-/-}$ mouse shows the decreased number (80% reduction) of dermal macrophages. Consistently, chimerism of dermal macrophages were increased in parabiosis mouse[2]. Whereas Langerhans cells, which are known as maintained by self-proliferation, didn't have a change in chimerism, suggesting dermal macrophages are greatly replenished by monocyte with a CCR2 dependent

[1] Transgenic system in which Cre recombinases are insert in target gene and activated by administration of tamoxifen.

[2] Surgically joined two mouse to share their blood circulation.

manner [45].

Similar to mouse macrophages, chicken macrophages are also arising from embryonic precursor and first founded at Hamburger-Hamilton stage (HH) 13, which is laying after 48-52 hours. This cells expressed CSF1R, which is important in development of mononuclear phagocytes and when differentiated this cells *in vitro* with CSF1, it showed phagocytic activity and expressed MHCII and KUL01 [47]. Though, whether diverse precursors, like mouse yolk sac, FL monocyte, and fetal HSC, exist in the chicken embryonic stage is not fully elucidated. Furthermore, whether tissue resident macrophages are maintained through self-proliferation or monocyte infiltration should be studied.

2.3 Function of tissue resident macrophages

Macrophages can be functionally classified as M1 and M2 macrophages, which is generated *in vitro* culture system (Fig.2). M1 macrophages can be generated by stimulating M0 macrophage with LPS and IFN-γ, whereas M2 macrophages by stimulating with IL-10 and IL-4 [48]. The main function of M1 macrophages is to induce inflammatory response to clear the invading pathogen, and signature markers are iNOS, CD80 and upregulation of MHCII. M2 macrophages can be further subdivided into several subsets, though their main functions are immunoregulation and tissue repair. Markers for M2 macrophages are CD206, PPAR- γ, and arginase.

Unfortunately, exact definition of M1 and M2 macrophages is not established in chicken macrophages. Though, induction of antibacterial capacity with increased MHCII and co-stimulatory molecules have been reported in BM-derived macrophages (BMM) by stimulating with pathogen-associated molecular pattern (PAMP) [49]. Furthermore, M2-likce macrophage have been reported in Newcastle disease virus (NDV) infected chicken macrophage cell line with increased IL-10 and PPAR- γ [50].

Tissue resident macrophages are found in diverse organs and highly heterogeneous by adapting to organ specific environment. However, most of tissue macrophages maintain tissue homeostasis by uptake the apoptotic and senescent cells [51], and production of anti-inflammatory mediators, such as TGF-β, IL-10, PGE2, which can repair the tissue damage [52]. Because of these features, tissue resident macrophages are sometimes classified as M2-like macrophages.

Several tissue resident macrophages are reported from mouse organs (i.e., spleen, brain, gut, liver, heart, lung, skin, bone etc.). Furthermore, depends on the organ and tissue, tissue macrophages have distinct surface marker expression which can represent their unique functions and different nomenclature. For example, Kupffer cells are the macrophages which reside in the sinusoid of the liver. As their signature marker, Kupffer cells express several complement receptor such as CR3 and CRIg [53, 54], and TIM4 which is involved in clearing dead cells [55]. This is because, various commensal microbes and food borne antigens are continuously infiltrated to

the liver and the liver is the major erythropoiesis organ.

Similarly, diverse tissue resident macrophages are reported in chicken by using CSF1R transgenic chicken [56]. Though because of few markers to distinguish monocyte and macrophages, most of tissue resident macrophages are referred as mononuclear phagocytes or monocytes/macrophages. Three types of mononuclear phagocytes are existing in chicken liver which can designated as $TIM4^{hi}$, $TIM4^{lo}$ and $TIM4^{-}$ cells [38]. Among them, $TIM4^{hi}$ cells are highly phagocytic and shared several mRNA signature with mouse Kupffer cells. On the other hand, $TIM4^{lo}$ and $TIM4^{-}$ cells are less phagocytic and expressed mRNAs associated with conventional DCs in mice. However, compare to mouse studies, very few studies about phenotype and function of tissue resident macrophages are conducted in chicken.

In mouse, during the invasion by pathogen, tissue resident macrophages can initiate the immunity. However, most of macrophages during inflammation are originated by blood circulating monocytes. These macrophages are M1-like macrophages and infiltrated to inflammation sites depend on several chemokine receptors such as CCR2, CCR1, and CCR5 [57].

Whether tissue resident macrophages or infiltrated monocyte derived macrophages are the source of inflammatory macrophage during inflammation is not studied in chicken. Moreover, several chemokines and it receptors have been reported, though which chemokine receptors are

important in monocyte and macrophages migration during inflammation is not fully elucidated, suggesting, primary monocytes/macrophages should be further conducted in chicken.

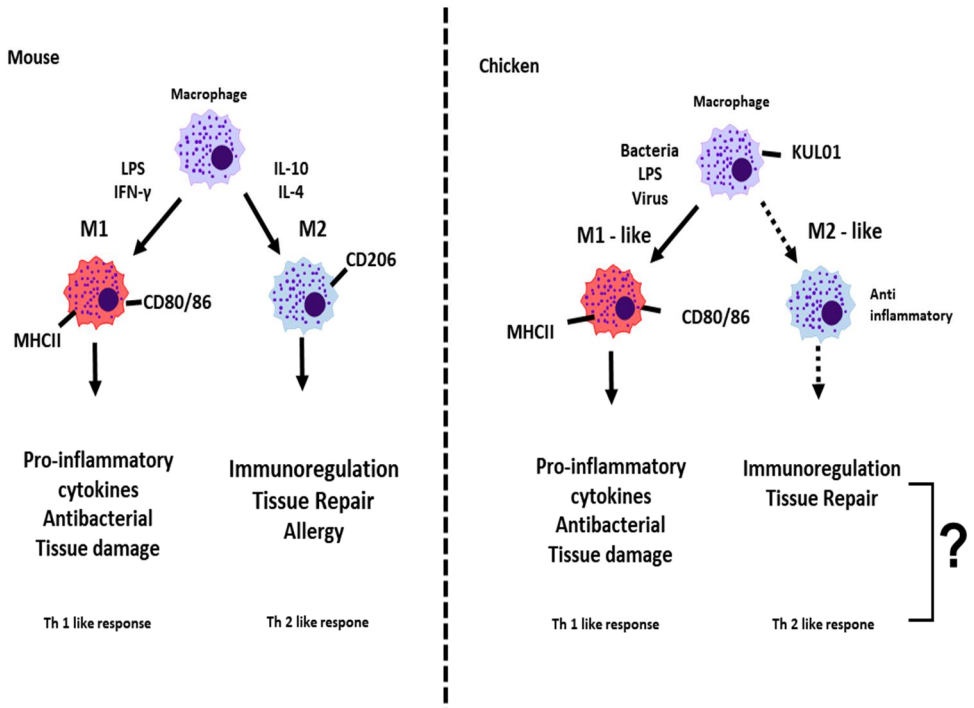


Fig.2 Schematic diagram for the concept of M1/M2 classification. In mouse, mouse macrophage can be largely divided into M1 and M2 macrophage depend on their function (left). By stimulating with LPS and IFN-γ *in vitro*, M0 macrophages can be polarized into M1 macrophage. M1 macrophages have Th 1 like response including antibacterial, inflammation induction, and tissue damage. Induction of MHCII, co-stimulatory molecules, and iNOS are the marker of M1 macrophages. Meanwhile, treating IL-4 and IL-10 induce M2 macrophages polarization from M0 macrophages which have Th2-like functions including immunoregulation,

tissue repair and involved in allergy response. CD206, arginase and IL-10 induction are the widely used M2 macrophage marker. However, M1 and M2 classification is not established in chicken (right), though M0 macrophages stimulated with PAMP induced inflammatory function of macrophage (M1-like) with induction of surface MHCII and co-stimulatory molecules in chicken. Broken lines indicate currently uncertain.

II. Introduction

Monocytes/macrophages are the major components of mononuclear phagocyte which are derived from bone marrow precursors [58]. Macrophages are seed in the various organs before the birth and maintained through self-proliferation and, to some extent, via the infiltration of circulating monocyte [43]. Thus, these cells exist in several types of tissues in steady state condition by clearing apoptotic and senescent cells [59, 60]. However, they are also rapidly recruited via chemokine signals and differentiated from circulating monocytes in response to inflammation or pathogen invasion [57].

Monocytes/macrophages are major component of innate immune system as the first line of defense in the host through various effector functions. These cells express several kinds of pattern recognition receptors (PRR), including Toll like receptors (TLR) and C-type lectin receptors (CLR) which allow to rapidly recognize and react to pathogens [61]. Then, they phagocytose and clear the pathogen by lysosomal acidification [62]. Activated monocytes/macrophages also release pro-inflammatory cytokines

such as IL-1β, IL-6, and IL-12 [63]. Furthermore, some of phagocytosed pathogens are processed and presented on major histocompatibility complex (MHC) molecules which can differentiate naïve T cell to pathogen-specific effector T cell [64], interconnecting innate and adaptive immunity.

Among lymphoid organs, the mammalian spleen is known to contain various type of mononuclear phagocyte subsets defined by phenotype, function and localization [65]. However, the chicken spleen differs from that of mammals in both structure and function [6]. In chicken, though, it has been reported that red pulp monocytes/macrophages in spleen express MHCII with a high phagocytosis ability in chicken which is similar to mammalian red pulp macrophages [12]. In addition, monocytes/macrophages are also found in chicken ellipsoid [12, 66], which is analogue to mammalian marginal zone.

Chicken mononuclear phagocytes also include monocyte, macrophage-like and DC-like cells [67]. The phenotype and function of macrophage- and DC-like cells are poorly defined because of a lack of specific immune reagents. However, *in vitro* culture of mononuclear phagocytes demonstrated that KUL01 ,which target the MRC1L-B [30], homologous mammalian mannose receptor, can be used as the representative marker for monocyte/macrophage lineage cells, whereas 8F2 (putative chicken CD11c) for DC-like cells [66]. Furthermore, the comparative profiling of gene expression on spleen mononuclear phagocytes was performed between chicken and mammals, and demonstrated that spleen $KUL01^+$ and $CD11c^+$

cells are distinct phagocytic population as macrophage and DC, respectively, analogue to mammalian counterparts [67].

Chicken monocytes/macrophages, which are stained with KUL01, are found to exhibit similar features including morphology [24], plasticity [25], and phagocytosis [26, 38] to that of mammals. Furthermore, chicken monocytes/macrophages expressed several TLRs and activation with TLR ligands induces bacterial lysis [27] and pro-inflammatory cytokine secretion [68], suggesting that chicken monocytes/macrophages also have similar response during inflammation compared with mammal. However, it was noting that most studies on functional aspect of chicken monocytes/macrophages are limited to the *in vitro* experiments using cell lines (i.e., HD11 and MQ-NCSU) and bone marrow- or monocyte-derived macrophages [69-72]. Moreover, few studies are addressed to define the function of chicken primary monocytes/macrophages.

Due to poorly developed lymphatic system and lack of draining lymph node, chicken spleen is considered as the most important secondary immune organs [11]. Therefore, characterization of spleen monocytes/macrophages will be helpful to investigate the regulation of systemic inflammation in chicken. However, how monocytes/macrophages exist and maintained in steady states and respond to danger signals provided by inflammation in the spleen was not well defined. Therefore, aim of present study was to define the phenotype, function, and maintenance of spleen monocytes/macrophages during steady state and inflammation condition.

III. Materials and Methods

Experimental animals and their treatment

Fertile eggs of White Leghorn chickens were obtained from Green-Bio Science and Technology (GBST, Seoul National University, Pyeongchang, Korea). Fertile eggs were incubated at 37.5–38°C incubator for 21 d. The hatched young chicks were housed under conventional conditions and were allowed free access to food and water. All experimental procedures using fertilized eggs and chickens were performed under the approval of the Institutional Animal Care and Use Committee of Seoul National University (IACUC No., SNU-150327-2).

In order to induce systemic inflammation, chickens were injected with

lipopolysaccharide (LPS, *Escherichia coli* serotype O111:B4) (from Sigma-Aldrich, St Louis, MO, USA) dissolved in PBS through intraperitoneal at a dose of 1 mg/kg. Control animals received with the same amount of phosphate buffered saline (PBS).

Single-cell isolation

Spleen, cecal tonsil, lung, thymus, and bursa of Fabricius were collected, minced, and filtered through a 70-μm nylon cell strainer (Corning Inc., Corning, NY, USA) and washed with RPMI-1640 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific) to obtain a single-cell suspension. To isolate peripheral blood mononuclear cells (PBMC), blood was collected through brachial wing vein by using 5 ml syringe equipped with 26-gauge (0.45 mm diameter) needle and stored the blood in BD Vacutainer coated with sodium heparin (Corning Inc.). Blood was diluted in PBS at a 1:1 ratio and layer the diluted blood on top of the Ficoll-Paque (Sigma Aldrich). Tube was centrifuged at 400 x*g* for 20 min at 25°C with the brake off. To collect the buffy coat, clean pipette was inserted through the plasma layer and obtain the interface layer. To obtain bone marrow cells, femurs and tibias were obtained after the removal of the surrounding muscles. Then, both ends of the bone were cut with scissors and the marrow was flushed out with RPMI-1640 media supplemented with 5% heat-inactivated FBS using a 10 ml syringe equipped with 26 gauge (0.45 mm diameter) needle. Clusters within the marrow cell suspension were disaggregated using a 70 μm nylon cell strainer. The single cells were suspended with media and centrifuged at 300–400 x*g* for 3 min at 4°C. Then, the pellet was treated with appropriate amount of ACK lysing buffer depends on their cell number, incubated for 7 min at 4°C, and centrifuged at 300–400 x*g* for 3 min at 4°C. The pellet was washed and resuspended with media and filtered through a 70 μm strainer.

Absolute number of the target cell population was calculated by multiplying the percentage of target cell with the total cell number of each organ examined.

Flow cytometry analysis

For the cell surface staining, single cells were stained with fluorochrome-conjugated monoclonal antibodies for 20 min at 4 °C in the dark. The primary antibodies used were the anti-chicken MHC class II-FITC (2G11), Monocyte/Macrophage-PE (KUL01), CD3-Pacific Blue (CT3), Bu-1-APC (AV20), MHC class I-Biotin (F21-2) (all from Southern Biotec, Birmingham, AL, USA), CD80 (IAH:F864:DC7) (Bio-RAD Laboratories, Hercules, CA, USA). After the incubation for 20 min and washing for three times with PBS, the cells were stained with streptavidin APC/Cy7, PE/Cy7 anti-mouse IgG1, or APC anti-mouse IgG2a (all from BioLegend, San Diego, CA, USA). After the incubation for 20 min, the cells were washed and the expression of surface markers was acquired by using flow cytometry (FACS Canto II, BD Biosciences). The acquired data were analyzed by using FlowJo software (Tree Star Inc., Ashland, OR, USA).

For the cell labeling a standard CellTrace™ Violet (CTV) cell proliferation kit was used while the cell death was assessed by annexin V (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and propidium iodide (PI; Sigma-Aldrich) staining. After the staining, the cells were washed and analyzed by using flow cytometry (FACS Canto II, BD

Biosciences) and the data were analyzed using FlowJo software (Tree Star Inc.). Cell sorting was performed using the FACSAria sorter (BD Biosciences) after sorted by magnetic beads. After the sorting of the two subsets, $KUL01^{hi}MHCII^{lo}$ and $KUL01^{lo}MHCII^{hi}$, the purity was > 98%.

$KUL01^{+}$ cell purification for RT-qPCR, phagocytosis and migration assay

Splenocytes were stained with mouse anti-chicken monocyte/macrophage (clone KUL01) antibody, purchased from Southern Biotec (Birmingham, AL, USA) for 15 min at 4°C. After washing with MACS buffer (PBS containing 0.5% BSA and 2 mM ETDA), the cells were incubated with anti-mouse IgG microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4°C in the dark. Then, the cells were washed with MACS buffer, the cell suspended with MACS buffer and was separated on a MACS LS column in the magnetic field of a MACS Separator (Miltenyi Biotec). The magnetic fraction of positively-selected cells was used in subsequent mRNA experiments, phagocytosis and migration assay.

RNA extraction and cDNA synthesis

Total RNA was extracted from total splenocytes, magnetic bead-sorted KUL01+ cells or aria-sorted $MHCII^{hi/lo}$ cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction or Qiagen RNeasy kit (Qiagen, Hilden, Germany). Briefly, the

cells (5 x 10^5 cells for FACSARIA-sorted cells and 5x10^6 cells for splenocytes) were treated with 1 ml of TRIzol. Total RNA was isolated with the addition of 200 µl of chloroform followed by centrifugation at 12,000 x*g* for 15 min at 4°C. The aqueous phase was transferred into a new tube and 500 µl of isopropanol was added. Then, the samples were incubated for 10 min at room temperature for RNA precipitation and centrifuged at 12,000 x*g* for 10 min at 4°C. The RNA pellet was obtained after washing with 75% ethanol, air drying for 5–10 min, and then it was resuspended with DEPC water. RNA concentration was quantified using a NanoDrop (Amersham Biosciences, Piscataway, NJ, USA) at 260 nm. Subsequently, 500 ng of purified RNA was reverse-transcribed to cDNA using M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer's instruction.

Quantitative real-time PCR

Quantitative real-time PCR was performed on cDNA using a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). SYBR® Green PCR Master Mix (Applied Biosystems) was used according to the manufacturer's specifications. The PCR reaction was carried out in a 96-well reaction plate with 10 µl of SYBR® green PCR master mix, 0.5 µl each of primers, 11 µl of cDNA template mixed with DEPC water. Each reaction involved a pre-incubation at 95°C for 10 min,

followed by 50 thermal cycles at 95°C for 15s, 55°C for 30s, and 72°C for 30s. 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 were designed using NCBI Primer-BLAST as shown in Table 2 and synthesized by Bioneer Inc. (Daejeon, Korea).

In vitro migration assay

Migration ability was measured by using a modified Boyden chamber (Corning Costar, Pittsburgh, PA, USA) containing a polycarbonate membrane filter (6.5 mm diameter, 24 well, 5 μm pore size). The inserts were pre-incubated with media (RPMI-1640 containing 5% heat-inactivated FBS) for 1h. Bead sorted spleen $KUL01^+$ cells (1×10^6) were placed in the upper chamber with 200 μl of media and lower chamber contained 500 μl media alone or media containing recombinant chicken CCL5 (Kingfisher Biotech, Saint Paul, MN, USA). The plates were incubated at 39°C in 5% CO_2 for 1 h. The cells that had migrated to the lower chamber, but attached to the insert, were collected by replacing the un-migrated cells and placing the bottom of the insert to 500 μl of TE. Dissociate cells from insert and lower chamber cells were stained with anti-chicken MHC class II and KUL01, further analyzed by flow cytometry.

Adoptive transfer assay

Chicken PBMC were stained with CellTrace^{TM} Violet (Thermo Fisher Scientific) for 20 min at 39°C in 5% CO_2. The cells were washed with PBS supplemented with 5% heat-inactivated FBS and centrifuged at 300–400 x*g* for 3 min at 4°C. Pellet was resuspended with PBS and then the cells ($1x10^7$) were injected to 3 weeks old chicken through the wing vein. For systemic inflammation, as previously described, LPS or PBS only were injected through intraperitoneal route.

Phagocytosis assay

For dead cell, CellTrace^{TM} Violet (Thermo Fisher Scientific) stained splenocytes were boiled at 56°C for 30 min by using heat block and confirmed the cell death by annenxin V and PI (Corning Inc.) staining according to the manufacturer's instruction. Dead cells were incubated with spleen KUL01$^+$ cells (1 x 10^6 cell/ml) at a 1:1 ratio for 1 h at either 37°C or 4°C to exclude adhesion dead cells. For ovalbumin (OVA) uptake, spleen KUL01$^+$ cells (1 x 10^6 cell/ml) were incubated with OVA-FITC (1-2 μg/ml) for 1h at 37°C or 4°C. The uptake was terminated by washing cells with PBS supplemented with 5% heat-inactivated FBS and centrifuged at 300–400 x*g* for 3 min at 4°C. The cells were stained with anti-chicken MHC class II and KUL01, further analyzed by flow cytometry.

Statistics analysis

Statistical analysis (one-way ANOVA with Tukey posttest or two-way ANOVA with Bonferroni posttest) was performed using the GraphPad Prism (version 7.03, GraphPad Software, San Diego, USA). Differences were considered significant if $*P < 0.05$, $** P<0.01$, or $*** P<0.001$.

Target Gene	Primer Sequence (5′ – 3′)	Accession No.
CCR5	F* - GTGGTCAACTGCAAAAAGCA R# - GCCCGTTCAACTGTGTCG	NM_001045 834.1
CCR2	F - ATGCCAACAACAACGTTTGA R - TGTTGCCTATGAAGCCAAA′	NM_001045835.1
CX3CR1	F - TCCAGAACGATCAAGCACAG R - CGGTGTTCAGTTCCACATTG	XM_418820.2
IL-10	F - CGCTGTCACCGCTTCTTCA R - CGTCTCCTTGATCTGCTTGATG	NM_001004414.2
TGF- β	F- GAGTCCGAGTACTACGCCAAAGA R- CACGTTAAAGCGGAACACATTG	NM_205454.1
COX-2	F - TGCTGGCCGCTCTCCTT R - GTCCTCGTGCAGTCACATTCA	NM_001167719.1
IL-1β	F - ACCCGCTTCATCTTCTACCG R - TCAGCGCCCACTTAGCTTG	NM_204524.1
IL-12p40	F - CCTGTGGCTCGCACTGATAA R - TCTTCGGCAAATGGACAGTA	NM_213571.1
IL-6	F - CGAGTGGGTGCTGTGTCAAA R - CATCCCTGAACGTGTATTTA	XM_015281283.2
iNOS	F - AGCAGCTGAGTGATGATCCA R - GGACCGAGCTGTTGTAGAGA	NM_204961.1

Table 2. Primer sequences used for RT-qPCR.

*F : Forward primer for PCR, #R : Reverse primer for PCR

IV. Results

1) Two subsets of spleen monocytes/macrophages showed different expression pattern of MHCII and KUL01

To investigate the distribution of the chicken primary monocytes/macrophages in different organs, several primary (bone marrow, thymus, and bursa of Fabricius) and secondary (spleen, cecal tonsil, and lung) immune organs from 3 weeks old chicken were taken, and analyzed for the phenotypes based on the expression of KUL01, a well-known chicken monocytes/macrophages marker [12] together with MHCII. All organs examined, except for spleen, were found to be comparable for their expression of KUL01 and MHCII (Fig. 3A). Interestingly, however, monocytes/macrophages from spleen showed two distinct populations with heterogeneous expression of MHCII and KUL01. Based on the expression pattern of KUL01 and MHCII, two populations were designated as $KUL01^{lo}MHCII^{hi}$ and $KUL01^{hi}MHCII^{lo}$ (Fig. 3B). In spleen, proportion and absolute number of $KUL01^{lo}MHCII^{hi}$ cells were higher than $KUL01^{hi}MHCII^{lo}$ cells at steady state condition (Fig. 3C and D). $KUL01^{lo}MHCII^{hi}$ cells exclusively outnumbered over $KUL01^{hi}MHCII^{lo}$

cells during the first 3 weeks of life (Fig. 3E). Taken together, these results suggested that, unlike other organs, spleen showed unique populations of monocytes/macrophages with distinct expression pattern of KUL01 and MHCII.

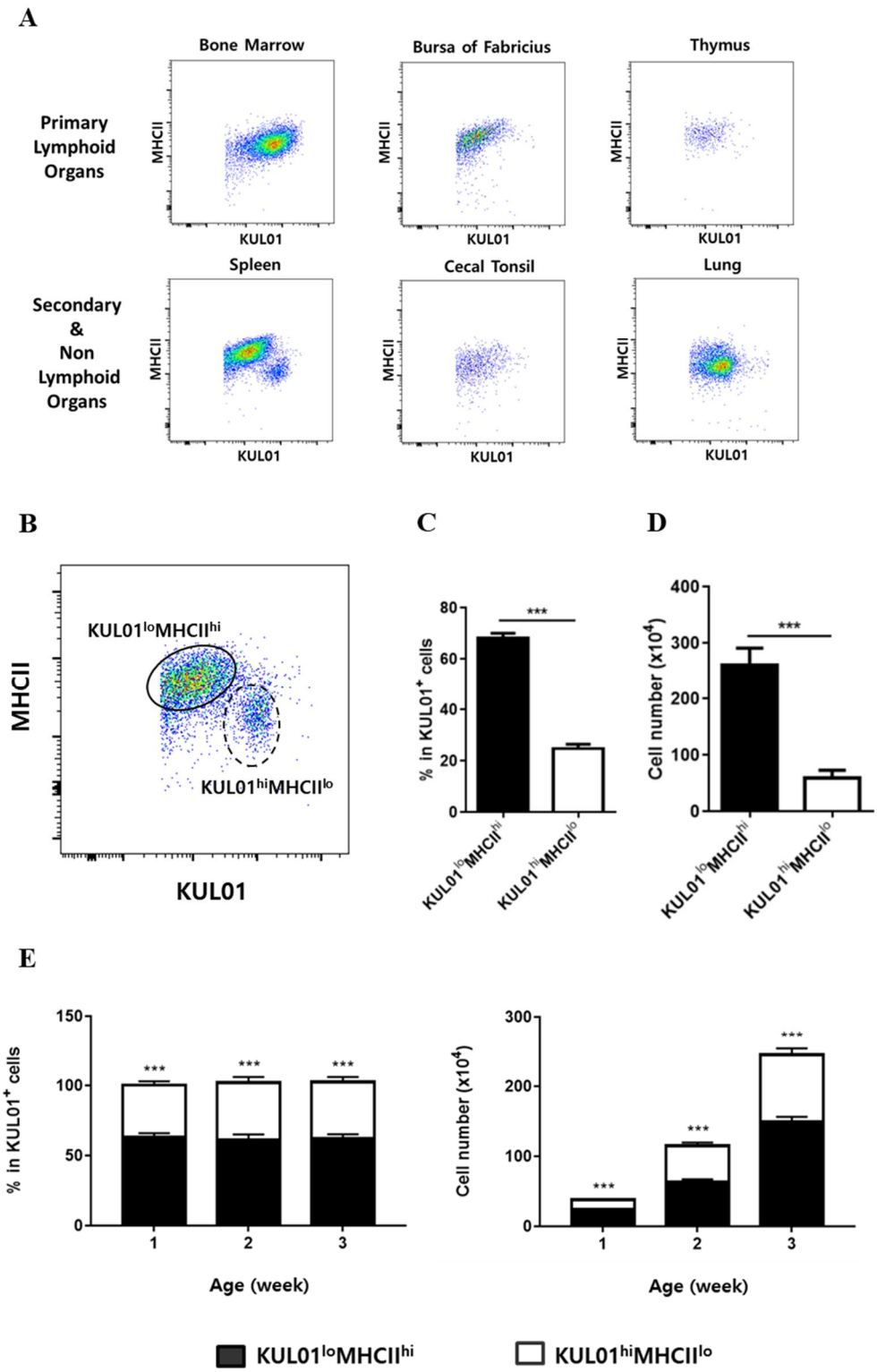

A
Bone Marrow
Bursa of Fabricius
Thymus
Primary Lymphoid Organs
Spleen
Cecal Tonsil
Lung
Secondary & Non Lymphoid Organs
MHCII
KUL01
B
KUL01loMHCIIhi
KUL01hiMHCIIlo
C
% in KUL01+ cells
D
Cell number (x10^4)
***
E
Age (week)
KUL01loMHCIIhi
KUL01hiMHCIIlo

Figure 3. Two distinct populations of monocytes/macrophages in spleen from chicken based on the expression of KUL01 and MHCII. Various organs were harvested from 3 weeks old chicken. Single cells were stained with anti-KUL01 and MHCII antibodies and analyzed by flow cytometry. (A) Representative dot plots of monocytes/macrophages in bone marrow, bursa of Fabricius, thymus, spleen, cecal tonsil, and lung. (B) Two distinct populations of spleen monocytes/macrophages are shown. The gating with solid line represents for $KUL01^{lo}MHCII^{hi}$ cells, and dashed line for $KUL01^{hi}MHCII^{lo}$ cells. (C) Proportion and (D) absolute number of $KUL01^{lo}MHCII^{hi}$ (filled) and $KUL01^{hi}MHCII^{lo}$ (open) cells in the spleen. ****P<0.001*. (E) Proportion and absolute number of spleen monocytes/macrophages from 1-week to 3-week old chicken. ****P<0.001*. The representative picture from three independent experiments with similar results is shown.

2) Two splenic monocytes/macrophages showed distinct phenotypic and functional characteristics during steady state situation

To characterize phenotypic features of the two subsets, cells were stained with the markers known to be involved in antigen presentation. The expression of MHCII, MHCI, and CD80 were higher on KUL01^{lo}MHCII^{hi} cells than KUL01^{hi}MHCII^{lo} cells (Fig. 4A) suggesting that KUL01^{lo}MHCII^{hi} cells might have better antigen presenting potency than KUL01^{hi}MHCII^{lo} cells.

Phagocytosis of foreign antigens including pathogens and aberrant cells is important task of antigen-presenting cells for the activation of immune system and the maintenance of homeostasis, respectively [58]. To examine the phagocytic activity, OVA labeled with FITC or dead cells labeled with CTV were used. Higher number of CTV^{+} cells were observed in KUL01^{hi}MHCII^{lo} cells than KUL01^{lo}MHCII^{hi} cells (Fig. 4B), suggesting higher dead cell uptake. Similarly, OVA uptake was also higher in KUL01^{hi}MHCII^{lo} cells than KUL01^{lo}MHCII^{hi} cells (Fig. 4C). These results suggested that KUL01^{hi}MHCII^{lo} cells have superior phagocytosis properties to KUL01^{lo}MHCII^{hi} cells.

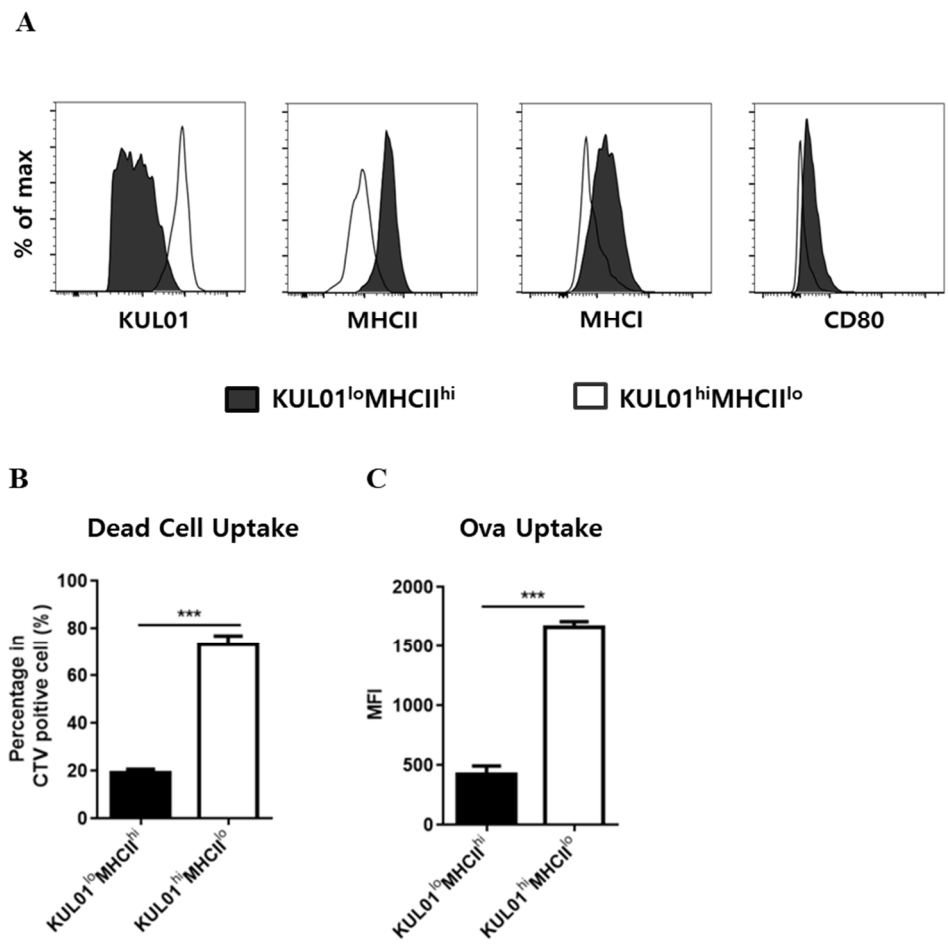


Figure 4. Expression of antigen presenting molecules and uptake ability of two distinct subpopulations of splenic monocytes/macrophages. Single cells prepared from spleen were stained with anti- KUL01, MHCII, MHCI, and CD80 antibodies. (A) Representative histograms presenting the surface expression of KUL01, MHCI, MHCII and CD80 on the two subsets. The representative histogram from four independent experiments with similar results is shown. For *in vitro* phagocytosis assay, $KUL01^{+}$ cells were stained with anti-KUL01, sorted from splenocytes by positive selection and

treated with CTV-labeled dead cells or OVA-FITC. Bothe two experiments are analyzed by flow cytometry. Bead sorted $KUL01^{+}$ cells (1 x 10^{6} cell/ml) were incubated with (B) CTV-labeled dead cells (1 x 10^{6} cell/ml) or (C) OVA-FITC (1 ng/ml) for 1 hour at 39℃ or 4℃. Data obtained at 39℃ are normalized with results from 4℃. The data represent (B) the percentage of CTV-positive cells, and (C) mean fluorescent intensity (MFI) of OVA-FITC in $KUL01^{lo}MHCII^{hi}$ (filled) and $KUL01^{hi}MHCII^{lo}$ (open) cells. ***P<0.01 ***P<0.001.*

3) KUL01^{hi}MHCII^{lo} cells showed higher migration capacity than KUL01^{lo}MHCII^{hi} cells

Monocytes/macrophages possess migrating capacity that is important to regulate infection and inflammation [57, 73] and to maintain organ homeostasis, even at steady state [44, 45]. To evaluate the migration capacity, the two subsets were examined for their transcriptional expression of chemokine receptors. Transcription of CCR5 was significantly upregulated in KUL01^{hi}MHCII^{lo} cells in comparison to KUL01^{lo}MHCII^{hi} cells (Fig. 5A). However, comparable expression of CCR2 (Fig. 5B) and CX3CR1 (Fig. 5C) on both subsets was found.

Next, the migration assay for the two populations using recombinant chicken CCL5, a ligand for CCR5, was performed. Due to high proportion within KUL01^{+} population (Fig. 3C), KUL01^{lo}MHCII^{hi} cells were observed with high frequency in the absence of CCL5 (i.e., spontaneous migration). However, when CCL5 was placed under the trans-well, higher percentage (Fig. 5D) and number (Fig. 5E) of the KUL01^{hi}MHCII^{lo} cells than KUL01^{lo}MHCII^{hi} were observed, highly likely because of high CCR5 expression on KUL01^{hi}MHCII^{lo} cells. Taken together, KUL01^{hi}MHCII^{lo} cells showed CCR5-dependent migratory pattern.

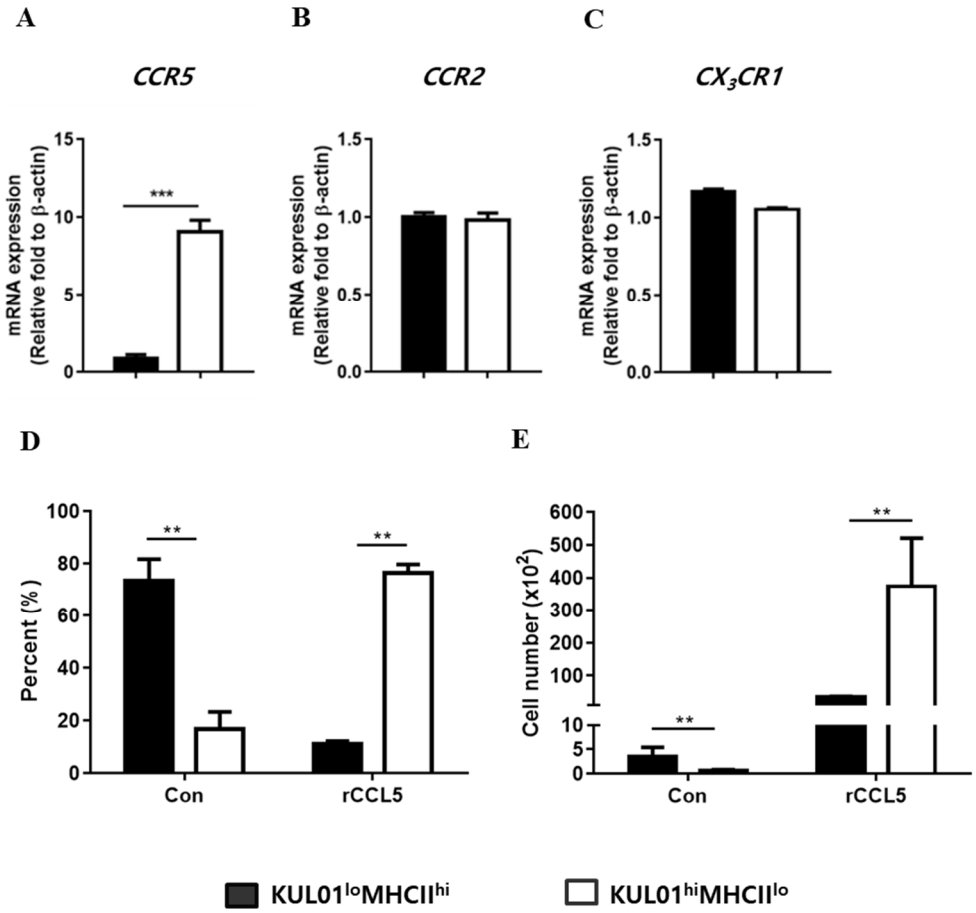


Figure 5. KUL01^{hi}MHCII^{lo} cells migrate better than KUL01^{lo}MHCII^{hi} cells towards CCL5. (A-C) Chicken splenocytes were sorted into KUL01^{lo}MHCII^{hi} (filled) and KUL01^{hi}MHCII^{lo} (open) cells using a cell sorter. The transcriptional level of chicken chemokine receptors, (A) CCR5, (B) CCR2 and (C) CX_3CR1 was analyzed by RT-qPCR. The expression was normalized to β-actin mRNA level. ****P<0.001*. (D, E) For *in vitro* migration assay, bead sorted KUL01$^+$ cells (1 x 10^6 cell/ml) with 200 μl of media were placed at the insert of trans-well plate. Lower compartment was

filled with media in the absence or presence of chicken recombinant CCL5 (50 ng/ml). After 1 hour, (D) percentage and (E) absolute number of migrated cells at the low compartment were quantified by flow cytometry (n=6). *$P<0.05$, **$P<0.01$.

4) Inflammation caused increase of KUL01^{hi}MHCII^{lo} cells in proportion and absolute number

Next, to further characterize spleen monocytes/macrophages upon the inflammation condition *in vivo*, chickens were treated with LPS through intra-peritoneal route. No obvious differences on the composition of immune cells including KUL01^{+} cells, B cells, and T cells in the spleen were found at 4 hours after the stimulation (Fig. 6A). While both sub-populations of monocytes/macrophages were seen, their proportion and absolute number were greatly altered (Fig. 6B). As shown in Fig. 6B, KUL01^{lo}MHCII^{hi} cells are the major population (over 65%) among monocytes/macrophages in the spleen in PBS-treated control group, which was similar to the steady state (Fig. 3B). However, at 4 hours post-LPS stimulation, proportion (Fig. 6C) and absolute number (Fig. 6D) of KUL01^{hi}MHCII^{lo} cells were greatly increased, resulting the ratio of the two population reversed. Collectively, LPS-induced inflammation caused a change in proportion and absolute number of the subsets of monocytes/macrophages.

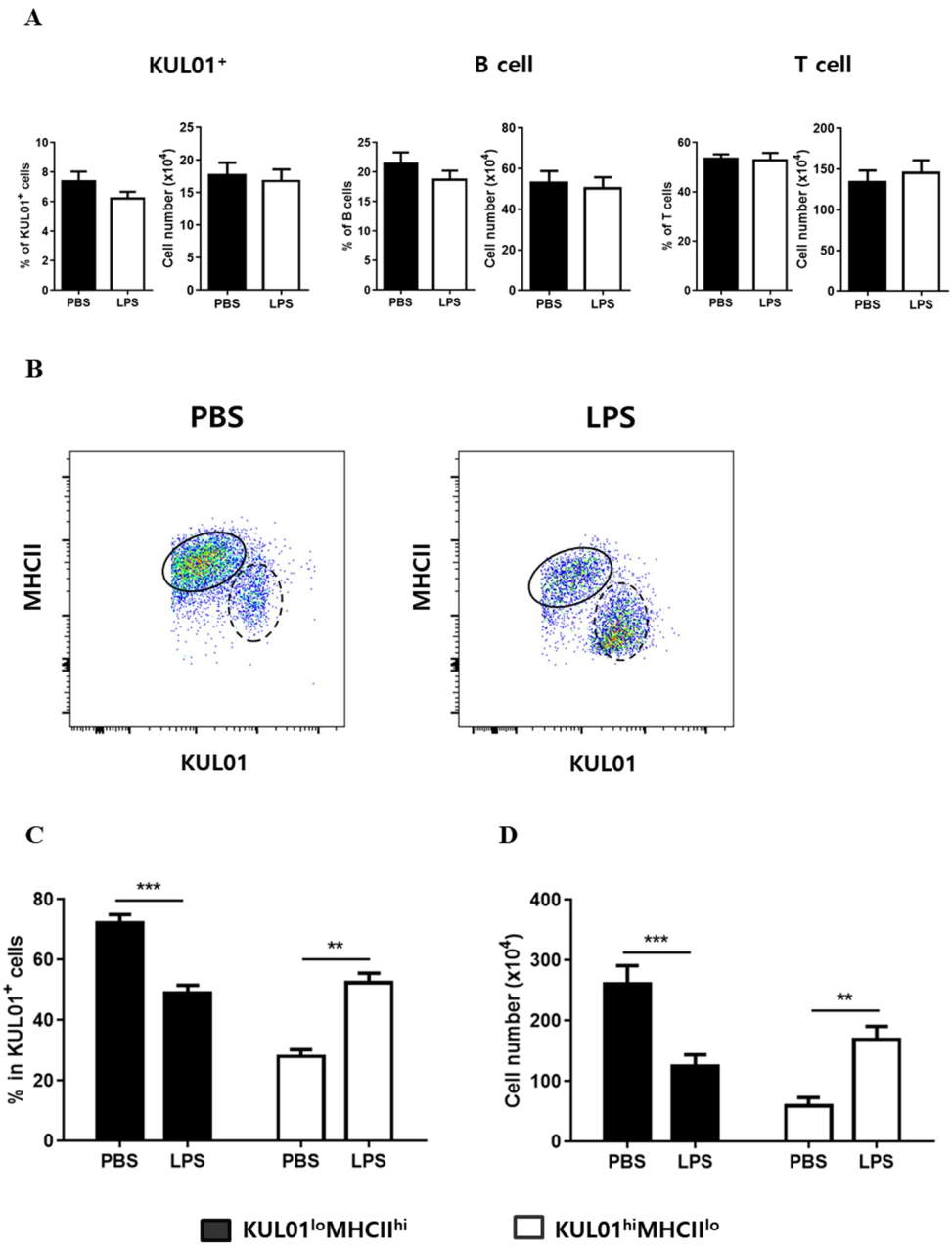


Figure 6. LPS-induced inflammation in chickens showed the proportional and number changes in the two populations of

monocytes/macrophages. Chickens were administered with PBS or LPS (1 mg/kg BW) via intraperitoneal route. After 4 hours, spleens were taken and splenocytes were analyzed for the number and proportional changes of subtypes of monocytes/macrophages by flow cytometry. (A) The bar graph represents proportion and absolute number of spleen leukocyte at 4 hours post-LPS or PBS treatment. (B) Representative dot plots of monocytes/macrophages from the spleen of chicken administered with PBS or LPS. Gating with solid line represents $KUL01^{lo}MHCII^{hi}$ cells, whereas dashed line represents $KUL01^{hi}MHCII^{lo}$ cells. The bar graph represents (C) proportion and (D) absolute number of the two subsets in PBS or LPS administration group. ***P<0.01, ****P<0.001*.

5) Inflammation triggered a reduction of MHCII expression in monocytes/macrophages.

In mammal, LPS can trigger the phenotype changes by activating mononuclear phacotyes both *in vivo* and *in vitro* [74]. Chicken macrophage cell line treated with LPS showed a similar response [49]. In the present study, phenotype of the two subsets was analyzed in chicken administered with LPS via *i.p*. The expression of KUL01 on KUL01^{lo}MHCII^{hi} cells was further decreased, whereas there was no significant decrease in KUL01^{hi}MHCII^{lo} cells (Fig. 7A). Granularity (indicated by side scatter, SSC) of KUL01^{hi}MHCII^{lo} cells was greatly increased at 4 hours after the LPS administration (Fig. 7B). Interestingly, MHCII expression on KUL01^{hi}MHCII^{lo} cells was declined (Fig. 7C) at 4 hours post-LPS stimulation. Whilst a minimal, if any, changes were observed in granularity and MHCII expression on KUL01^{lo}MHCII^{hi} cells (Fig. 7B and C).

A few factors that known to reduce the expression of MHCII include TGF-β [75], IL-10 [76], and COX-2 [77, 78]. To elucidate the potential mediators which negatively regulate MHCII expression during the inflammation, transcription level of TGF-β, IL-10, and COX-2 on splenocytes during the inflammation were analyzed by RT-qPCR. The mRNA expression levels of IL-10 (Fig. 7D) and TGF-β (Fig. 7E) were

similar between PBS and LPS group. Interestingly, however, COX-2 was greatly elevated in LPS group compared to PBS group (Fig. 7F) suggesting that COX-2 might be the potential mediator, responsible for the down-regulation of MHCII expression.

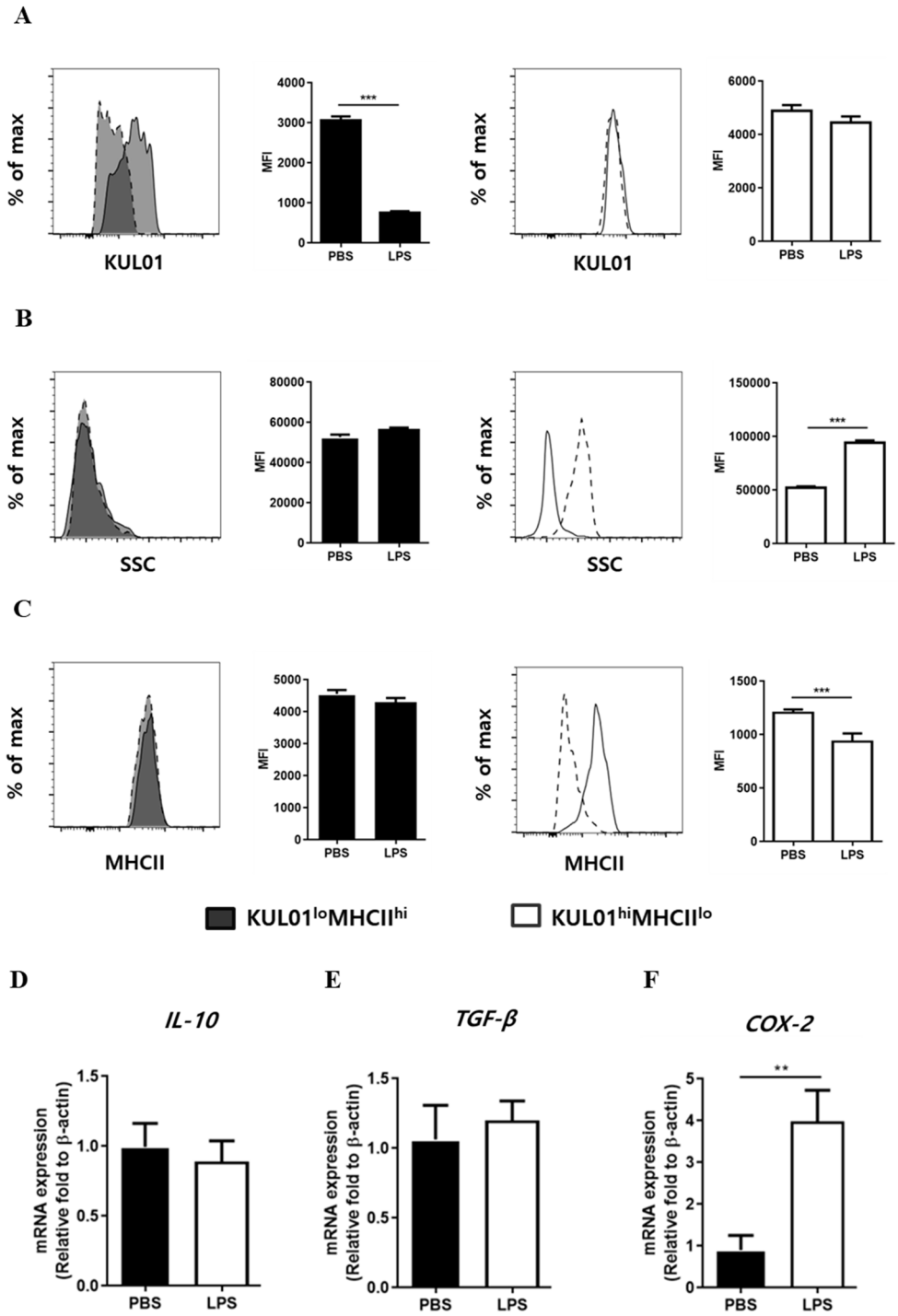

A
% of max
KUL01
MFI
PBS
LPS
***
B
SSC
C
MHCII
KUL01loMHCIIhi
KUL01hiMHCIIlo
D
IL-10
E
TGF-β
F
COX-2
mRNA expression
(Relative fold to β-actin)
**

Figure 7. LPS-induced inflammation in chicken decreased the MHCII expression on KUL01^{hi}MHCII^{lo} cells. Chickens were administered with PBS or LPS (500 μl of 1 mg/kg body weight) via *i.p.* for 4 hours. Then, spleen was taken and analyzed (A-C) for their phenotype and (D-F) mRNA levels of potential mediators through flow cytometry and RT-qPCR, respectively. Representative histograms showed the changes of (A) KUL01, (B) SSC, and (C) MHCII expression on two subsets at 4 hours after the LPS injection. Solid histograms represented PBS group and dashed histograms showed LPS group. The Bar graph represented the MFI of expression. ***P<0.01, ***P<0.001*. Transcriptional level of (D) IL-10, (E) TGF-β, and (F) COX-2 in splenocytes was examined by RT-qPCR. The expression was normalized to β-actin mRNA level. ***P<0.01*.

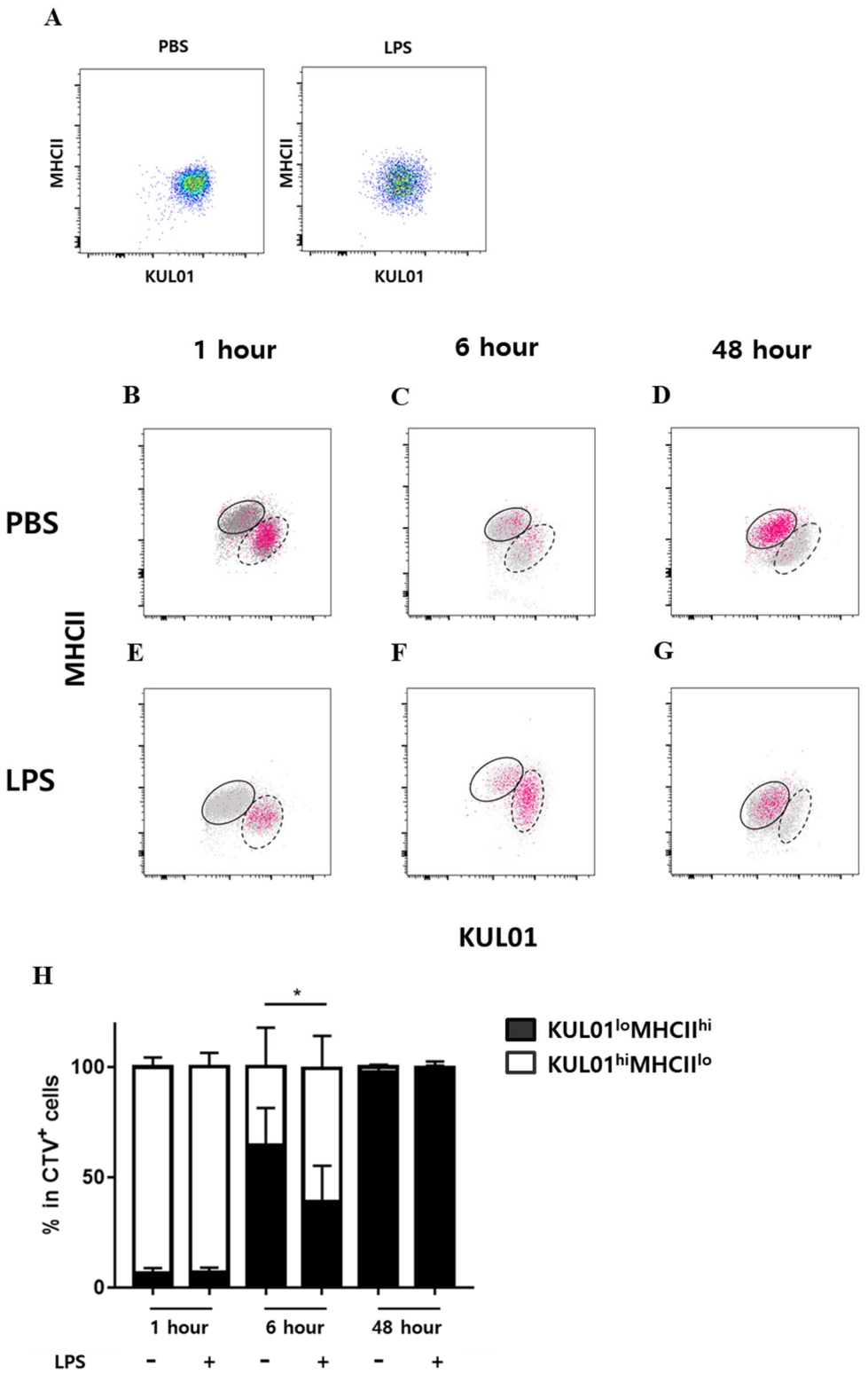
6) Two subsets of monocytes/macrophages showed distinct phenotypic changes during LPS-induced inflammation.

In mammal, tissue resident macrophages are seeded in the pre-natal stage and maintained their population by self-renewal [39] and/or replenished by infiltration of monocytes [45, 79]. To elucidate whether the two subsets of spleen monocytes/macrophages were replenished from circulating monocytes, CTV-labeled PBMCs were adoptively transferred and kinetic changes on the two subsets in the spleen were analyzed. Similar to other organs, monocytes from the PBMC had homogenous expression of KUL01 and MHCII in both PBS and LPS treated group (Fig. 8A). One hour post-adoptive transfer, most of the CTV-positive cells were found in $KUL01^{hi}MHCII^{lo}$ population (Fig. 8B and H), suggesting $KUL01^{hi}MHCII^{lo}$ cells are freshly infiltrated cells. However, at 6 and 48 hours post-adoptive transfer, CTV-positive cells were gradually increased within $KUL01^{lo}MHCII^{hi}$ population (Fig. 8 C, D, and H). Collectively, the two subsets were replenished by monocytes and, $KUL01^{lo}MHCII^{hi}$ cells were derived from $KUL01^{hi}MHCII^{lo}$ cells.

To examine how $KUL01^{hi}MHCII^{lo}$ cells were increased during the LPS-induced inflammation (Fig. 6C and D), I hypothesized two possibilities; ⅰ) delayed change from $KUL01^{hi}MHCII^{lo}$ cells to $KUL01^{lo}MHCII^{hi}$ cells ⅱ) phenotype change of $KUL01^{lo}MHCII^{hi}$ cells to $KUL01^{hi}MHCII^{lo}$ cells.

Thus, CTV-labeled PBMCs were adoptively transferred via wing vein in chickens at the same time as LPS injection through intraperitoneal route. At one hour post-adoptive transfer, CTV-labeled cells were readily observed within $KUL01^{hi}MHCII^{lo}$ population (Fig. 8E and H), similar to its control (Fig. 8B and H). However, at 6 hours post-adoptive transfer, CTV-positive cells were still highly observed within $KUL01^{hi}MHCII^{lo}$ population in LPS group (Fig. 8F and H), compared to those of PBS control (Fig. 8C and H), indicating LPS-induced inflammation delayed appearance of the donor cells in the $KUL01^{lo}MHCII^{hi}$ cell gate. Notably, in LPS group at 48 hours post-adoptive transfer, most of CTV^{+} cells were localized at $KUL01^{lo}MHCII^{hi}$ cell population (Fig. 8G and H), which was closely related with those of PBS group at 48 hours post-adoptive transfer (Fig. 8D and H).

Another possibility for the increase of $KUL01^{hi}MHCII^{lo}$ cells during LPS-induced inflammation could be phenotypic change of $KUL01^{lo}MHCII^{hi}$ cells into $KUL01^{hi}MHCII^{lo}$ cells. To investigate whether $KUL01^{lo}MHCII^{hi}$ cells changed their phenotype during the inflammation condition, CTV-labeled $KUL01^{lo}MHCII^{hi}$ cells were adoptively transferred via wing vein at the same time as *i.p.* injection of LPS. At 6 hours post-adoptive transfer, almost all CTV-positive cells were found within $KUL01^{lo}MHCII^{hi}$ gating (Fig. 8I), suggesting $KUL01^{lo}MHCII^{hi}$ cells do not change their phenotype in LPS-induced inflammation condition. Collectively, increased $KUL01^{hi}MHCII^{lo}$ cells during inflammation was caused by delayed change from $KUL01^{hi}MHCII^{lo}$ cells $KUL01^{lo}MHCII^{hi}$ cells.


A
PBS
LPS
MHCII
KUL01
1 hour
6 hour
48 hour
B
C
D
PBS
MHCII
E
F
G
LPS
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H
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% in CTV+ cells
100
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I

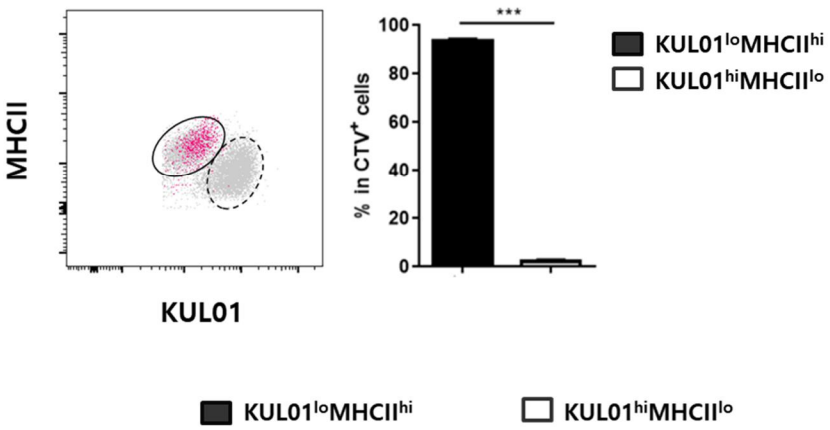

Figure 8. Spleen monocytes/macrophages are infiltrated from monocytes and have distinct phenotypic changes between steady state and LPS-induced inflammation. Chicken PBMCs were isolated from the blood by density gradient centrifugation and labeled with CTV and 200 µl of 1 x 10^7 cells were delivered through wing vein. At the same time, LPS (1 mg/kg) or PBS were administered through intraperitoneal route. After 6 hours, chicken PBMCs were isolated from the blood by density gradient centrifugation and stained with anti- KUL01 and MHCII then analyzed by flow cytometry. (A) Representative dot plots of monocytes from the PBMC of chicken administered with PBS or LPS. The CTV^+ cells were analyzed within $KUL01^{lo}MHCII^{hi}$ (filled) and $KUL01^{hi}MHCII^{lo}$ (opened) cell population in the spleen from recipient chicken. Representative dot plot of (B and E) 1, (C and F) 6, (D and G) 48 hours post- adoptive transfer. (H) The bar graph represents the proportion of CTV^+ cells in spleen at 1, 6, and

48 hours post-adoptive transfer. KUL01^{lo}MHCII^{hi} cells, sorted and labeled with CTV, were transferred (200 µl of 5 x 10^6 cells) into the recipient chicken through wing vein. At the same time the chicken was injected with LPS (1 mg/kg BW) via *i.p.* injection. The CTV$^+$ cells were analyzed within KUL01^{lo}MHCII^{hi} (filled) and KUL01^{hi}MHCII^{lo} (opened) cell population in the spleen from recipient chicken. (I) Representative dot plot of 6 hours post-adoptive transfer. The bar graph represents the proportion of KUL01^{lo}MHCII^{hi} cells (filled) and KUL01^{hi}MHCII^{lo} cells (open) of donor cells. Solid gating represents KUL01^{lo}MHCII^{hi} cells, whereas dashed gating represents KUL01^{hi}MHCII^{lo} cells. Pink dots represent donor cells and, gray dots recipient cells. * P<0.05, *** P<0.001.

7) KUL01^{lo}MHCII^{hi} cells are prone to cytokine secretion compared to KUL01^{hi}MHCII^{lo} cells

Monocytes/macrophages are the major source of inflammatory mediators during inflammation [63]. To investigate the ability to produce inflammatory mediators, the two subsets were sorted from spleen after the LPS administration and examined the mRNA expression of IL-1β, IL-6, IL-12p40, and iNOS. Transcription level of pro-inflammatory cytokines such as IL-1β (Fig. 9A), IL-6 (Fig. 9B) and IL-12p40 (Fig. 9C) were higher in KUL01^{lo}MHCII^{hi} cells than those in KUL01^{hi}MHCII^{lo} cells at both 2 and 4 hours post-LPS injection. No significant difference was found in iNOS mRNA expression at 4 hours post-LPS injection, though iNOS mRNA expression in KUL01^{hi}MHCII^{lo} cells was higher than KUL01^{lo}MHCII^{hi} cells at 2 hours post-LPS injection (Fig. 9D). Together, KUL01^{lo}MHCII^{hi} cells have a high potency to produce inflammatory cytokines than those of KUL01^{hi}MHCII^{lo}cells.

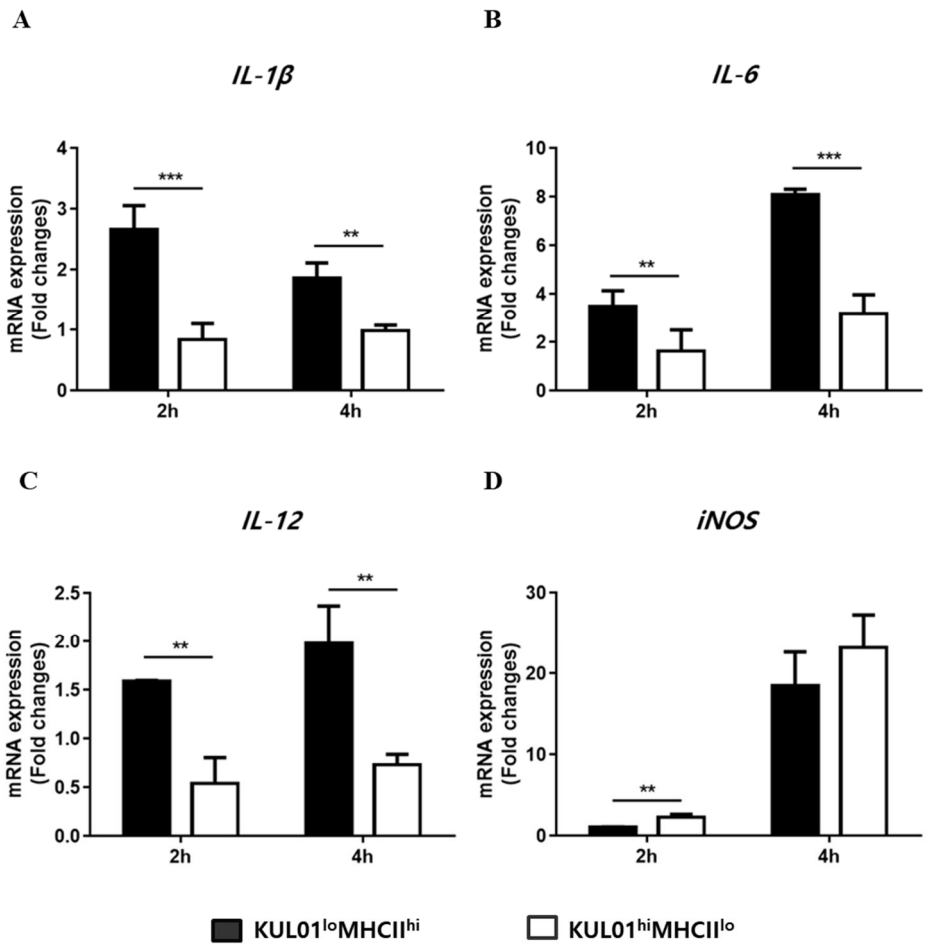

Figure 9. KUL01^{lo}MHCII^{hi} cells are the main source of both inflammatory and anti-inflammatory mediators. The mRNA expression for (A) IL-1β, (B) IL-6, (C) IL-12, and (D) iNOS on FACS-sorted KUL01^{lo}MHCII^{hi} (filled) and KUL01^{hi}MHCII^{lo} (open) cells from spleen in chickens at 2 and 4 hours post-LPS injection. Data are normalized with PBS group. ***P<0.01, ***P<0.001.*

V. Discussion

In chicken, a very few studies have been conducted on the function of primary mononuclear phagocytes. Though, $CD11c^{+}$ and $KUL01^{+}$ cells, analogue to mammalian DC and monocytes/macrophages, respectively, from spleen were reported in chicken [67]. In the present study, chicken spleen monocytes/macrophages were further divided into two subsets based on their surface expression of MHCII and KUL01, and analyzed to characterize the phenotype, function, and response to inflammation (Table 3).

Phagocytosis of dead cell is one of the important functional feature of tissue resident macrophages to balance the tissue homeostasis [59]. Several receptors, such as T cell immunoglobulin- and mucin-domain–containing molecule 4 (TIM4) [80], receptor tyrosine kinase Mer (MerTK) [81], and mannose receptor [82], are known for having an important role in recognition and phagocytosis of apoptotic cells in mouse. Similarly, the role of TIM4 to phagocytose apoptotic cells is reported in chicken [38]. In the present study, $KUL01^{hi}MHCII^{lo}$ cells showed higher phagocytic capacity than $KUL01^{lo}MHCII^{hi}$ cells, which is consist with the expression level of KUL01, a chicken mannose receptor [30].

Compared to $KUL01^{hi}MHCII^{lo}$ cells, $KUL01^{lo}MHCII^{hi}$ cells have a high potency to produce pro-inflammatory cytokines (IL-1β, IL-6, and IL-12)

during LPS-induced inflammation, suggesting that KUL01^{lo}MHCII^{hi} cells are

Table 3. Phenotypic and functional characteristics of spleen KUL01^{lo}MHCII^{hi} and KUL01^{hi}MHCII^{lo} monocyte/macrophage during steady stat and inflammatory condition.

	KUL01^{lo}MHCII^{hi}	KUL01^{hi}MHCII^{lo}
Phenotype	High MHCII, MHCI, CD80	High KUL01
Phagocytosis	↑	↑↑
Migration	↑	↑↑
Proportion	Decreased	Increased
Phenotype change	Decreased KUL01	Increased granularity Decreased MHCII
Cytokine	↑↑	↑

more prone to cytokine secretion than KUL01^{hi}MHCII^{lo} cells. The reason why KUL01^{hi}MHCII^{lo} cells with low potency to produce pro-inflammatory cytokines might be in close relation with their phagocytic activity (Fig. 4B), as uptake of apoptotic bodies repress the production of inflammatory mediators [83]. Moreover, red pulp macrophages with phagocytic activity against apoptotic cells in mouse showed a low IL-1β mRNA expression [84]. Therefore, KUL01^{lo}MHCII^{hi} cells are prone to cytokine secretion compared to KUL01^{hi}MHCII^{lo} cells whereas the uptake of apoptotic cells were high in KUL01^{hi}MHCII^{lo} cells.

Adoptive cell transfer technique has been widely used to demonstrate the origin [45, 85] and function [86, 87] of mononuclear phagocytes in mouse studies whereas a very few studies in chicken. To the best of my knowledge, this is the first study with transfer of post-natal PBMC into chicken, and it is useful method to study the replenishment of tissue resident macrophages by monocyte infiltration. In the present study, adoptive transfer *i.v.* with CTV-labeled PBMC in chickens demonstrated that KUL01^{hi}MHCII^{lo} cells are freshly infiltrated into the spleen and then their phenotype switched into KUL01^{lo}MHCII^{hi} cells as time goes by. Unlike mammalian tissue resident macrophages, which self-maintained with less monocyte contribution [79], the present data suggest that chicken spleen monocytes/macrophages are replenished by monocyte infiltration. Similarly, phenotype change has been

reported in EAC that exist in the spleen ellipsoid and migrate to PALS and GC in chicken, changing their phenotype to become IDC and FDC, respectively [14]. However, whether phenotypic change of KUL01^{hi}MHCII^{lo} cells is also occurred during the migration is yet to be known. Therefore, as a further study, immunohistochemistry or live imaging approaches should be conducted to demonstrate the location and timing of the phenotypic change of two subsets.

MHCII, mainly expressed on antigen presenting cells, is considered as the activation marker together with co-stimulatory molecules (CD86 and CD80) in DCs and macrophages. For example, when bone marrow-derived immature DCs and M0 macrophages are treated with LPS or IFN-γ, surface expression of MHCII and co-stimulatory molecules are elevated and, immature cells are differentiated to the activated status of mature DCs and M1 macrophages, respectively [74]. Furthermore, treatment with several TLR ligands also induced the upregulation of MHCII expression along with increased co-stimulatory molecules in chicken macrophage cell line [49]. However, in the present study, unlike the expectation that LPS injection would induce the elevation of MHCII expression, LPS-induced inflammation caused to decrease the MHCII expression on KUL01^{hi}MHCII^{lo} cells. Interestingly, however, no significant difference was observed in MHCII expression on KUL01^{lo}MHCII^{hi} cells.

It has been well-known mammal that transcription of MHCII is tightly regulated by master co-activator, class II major histocompatibility complex

trans-activator (CIITA) [88]. The exact mechanism of MHCII downregulation during LPS-induced inflammation was not defined in the current study, although, the factors known to regulate MHCII expression were measured in LPS stimulated spleen. It has been reported that in mammal, COX-2, increased at inflammation condition, can reduce the MHCII expression by regulating CIITA in a prostaglandin-dependent cAMP production [89]. In the present study, COX-2 showed a remarkable elevation of its transcription in LPS-stimulated spleen, suggesting COX-2 may potential MHCII regulator during LPS-induced inflammation.

Finally, transcriptional level of iNOS was higher in KUL01^{hi}MHCII^{lo} cells than KUL01^{lo}MHCII^{hi} cells at 2 hours post LPS-induced inflammation. The iNOS is produced in activated macrophages and involved in the generation of nitric oxide (NO), an unstable free radical that has an antimicrobial activity [90], suggesting KUL01^{hi}MHCII^{lo} cells may have a role in pathogen clearance.

In conclusion, current study demonstrated that two subsets of spleen monocyte/macrophages in chicken have different phenotype and function both in steady state and inflammation situation, able to better understanding of primary monocytes/macrophages in relation to homeostasis and innate immunity in chicken. At the best of my knowledge, this is the first study to demonstrate two subsets of spleen monocyte/macrophages and it will be useful to understand the systemic innate immunity of avian immune system.

VI. Literature cited

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Summary in Korean

단핵구/대식세포들은 단핵식세포들의 골수 전구체로부터 유래된 단핵식세포들로 조직과 세포의 항상성 유지 뿐만 아니라 병원균이 침투 시 이에 대응하는 선천셩 면역의 주를 이루는 면역세포들이다. 단핵구/대식세포들은 여러 조직 및 장기에 존재를 하며 다양한 기능을 수행한다. 예를 들어, 평상 시에는 사멸 세포를 대식하여 치우거나 조직의 생성에 관여하는 물질들을 생성함으로 조직의 항상성을 유지를 주로 수행한다고 알려져 있지만, 병원균의 침투 시에는 병원균을 대식하여 사멸을 유도하며 T 세포에게 항원을 제시하여 선천성 면역과 후천성 면역을 이어주는 중요한 역할을 수행한다. 더 나아가, 다양한 염증성 사이토카인들을 분비하여, 선천성 면역 시스템의 촉진을 유도하기도 한다.

단핵구/대식세포들은 주위 환경 및 상황에 잘 적응한다고 알려져 있으며 각 조직에 존재하는 단핵구/대식세포들의 역할이 조금씩 다르다는 것 또한 잘 알려져 있다. 조류의 경우에도 다양한 조직에 단핵구/대식세포들이 존재를 한다고 보고가 되었지만, 바이

오마커의 부재로 이러한 단핵구/대식세포들의 표현형 및 기능들에 대한 연구가 매우 적은 상태이다.

이러한 이유로 본 연구에서는 조류의 조직에 존재하는 단핵구/대식세포들에 대한 연구를 진행하고 하였으며, 놀랍게도 비장에 존재하는 단핵구/대식세포들은 다른 장기 (활액낭, 골수, 흉선, 폐. 맹장 편도선, 혈액)들과 달리 두 종류의 단핵구/대식세포들이 존재함을 확인하였으며 이 세포의 표현형 및 기능적 특성에 대하여 연구를 진행하였다.

조류의 비장에 존재하는 두 종류의 단핵구/대식세포들의 조류 단핵구/대식세포들 마커인 KUL01과 MHCII의 표현 양상이 다름을 확인하였고 이를 통해 두 군집을 $KUL01^{hi}MHCII^{lo}$, $KUL01^{lo}MHCII^{hi}$ 라고 명명을 하였다. 평상 시에는 두 군집 중 $KUL01^{lo}MHCII^{hi}$ 세포들의 비율 및 수가 많음을 확인하였으며 항원 제시와 관련된 물질 (MHCII, MCHI, CD80)을 높게 발현함을 확인하였다. 반면 $KUL01^{hi}MHCII^{lo}$ 세포들은 이동능 및 대식 능력이 $KUL01^{lo}MHCII^{hi}$ 세포들에 비하여 월등했다.

단핵구/대식세포들은 염증 상황에서 중요한 역할을 하기 때문에, 두 군집의 단핵구/대식세포들이 염증 상황에서 변화를 확인하기 위하여 조류에게 LPS를 복강 투여 후 두 군집의 세포들을 확인하였다. 그 결과, 평상 시 주로 존재하던 $KUL01^{lo}MHCII^{hi}$ 세포

들의 비율 및 수가 감소하며 KUL01^{hi}MHCII^{lo} 세포들이 증가했다. 입양전달 (adoptive transfer)을 진행한 결과, 평상시에는 기증된 세포들은 초기에 KUL01^{hi}MHCII^{lo} 군집에 주로 존재하며 시간이 지남에 따라 KUL01^{lo}MHCII^{hi} 군집에서 증가함을 확인하였고, 반면 염증 상황에서는 기증된 세포들이 KUL01^{hi}MHCII^{lo} 군집에서 KUL01^{lo}MHCII^{hi} 군집으로 변하는데 걸리는 시간이 지연되어 KUL01^{hi}MHCII^{lo} 세포들이 염증 상황에서 증가함을 확인할 수 있었다. 또한 쥐에서 진행된 연구와 달리, 염증 반응에 의하여 KUL01^{hi}MHCII^{lo} 세포들의 MHCII 발현이 감소했다. 정확한 관련 기전을 밝혀지는 못하였으나 염증 상황 시 비장에서 MHCII의 발현을 억제할 수 있는 COX-2가 증가함에 따라 COX-2 잠재적 요인이라고 생각한다.

종합하면, 닭의 비장에는 두 군집의 서로 다른 표현형 및 기능을 가진 단핵구/대식세포들을 본 연구를 통해 제시하는 바이다.