Identification of Dendritic Cell Precursors in the Culture of Bone Marrow with Hematopoietic Cytokines

Hyunju In

Department of Medical Science

The Graduate School, Yonsei University

Identification of Dendritic Cell Precursors in the Culture of Bone Marrow with Hematopoietic Cytokines

Directed by Professor Jun-Young Seo

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This certifies that the Doctoral Dissertation of Hyunju In is approved.

Thesis Supervisor : Jun-Young Seo

Thesis Committee Chair : Ki Taek Nam

Thesis Committee Member : Chae Gyu Park

Thesis Committee Member : Ho-Keun Kwon

Thesis Committee Member : Tae-Gyun Kim

The Graduate School
Yonsei University

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For My Parents,

Hyung-Shin In and Hye-Kyung Ku

One thing have I desired of the LORD,
that will I seek after;
that I may dwell in the house of the LORD
all the days of my life,
to behold the beauty of the LORD,
and to enquire in his temple.

Psalm 27:4

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Sincerely,
Hyunju In

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Abbreviation

APCs : antigen presenting cells
BM : bone marrow
BM-DCs : bone marrow-derived dendritic cells
DCs : dendritic cells
cDCs : classical dendritic cells
pDCs : plasmacytoid dendritic cells
MDP : monocyte and DC progenitors
CDP : common dendritic cell progenitors
cMoP : common monocyte progenitors
pre-DCs : pre dendritic cells
Macs : macrophages
FCS : fetal calf serum
FLT3L : FMS-like tyrosine kinase 3 ligand
GM-CSF : granulocyte macrophage-colony stimulating factor
GM-DCs : granulocyte macrophage-colony stimulating factor culture dendritic cells
GM-Macs : granulocyte macrophage-colony stimulating factor culture macrophages
Mo-DCs : monocyte-derived dendritic cells
MHC : major histocompatibility complex
MLR : mixed lymphocyte reaction
TCR : T cell receptor
Tg : transgenic
TLR : Toll-like receptor

ABSTRACT

Identification of Dendritic Cell Precursors in the Culture of Bone Marrow with Hematopoietic Cytokines

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Dendritic cells (DCs), key sentinels, play crucial parts in a variety of immune responses by capturing and presenting antigens to naïve T cells and by producing the subsequent T cell activation or tolerance. *In vitro* culture systems of DCs have been widely used to study DC biology and immunology. The culture of bone marrow (BM) in the presence of a hematopoietic cytokine, Fms-like tyrosine kinase 3 ligand (FLT3L), produces plasmacytoid DCs (pDCs) and classical DCs (cDCs) showed phenotype similar to steady state DCs, whereas the culture of BM with granulocyte-macrophage colony-stimulating factor (GM-CSF) generates DCs and macrophages considered to possess inflammatory phenotypes. In the present study, the culture of BM was examined with both cytokines, i.e., FLT3L and GM-CSF in combination and 4 different subsets were identified in $CD11c^{+}MHC\ II^{+}$ cells. Meanwhile, in the culture of BM with FLT3L, $MHC\ II^{hi}$ classical DC2s (cDC2s) were identified as a

heterogeneous subset with the additional use of hematopoietic markers. CD11c^{+}MHC II^{hi} cells in FLT3L conditioned BM culture were identified as at least three different subsets and a novel population of CD11c^{+}MHC II^{hi} cells were discovered which are possess a limited capacity of antigen-presenting activity and superior capacity of taking up antigens, suggesting functionally immature DCs. This novel population from the BM culture with FLT3L was isolated and evaluated for its potential to differentiate to DCs in the BM culture containing both FLT3L and GM-CSF. As a result, the novel immature DC-like cells in CD11c^{+}MHC II^{hi} cells from BM culture with FLT3L can be activated and differentiate into mature or activated DCs, which means that the novel population is an immediate precursor cell for DCs. Furthermore, the morphology of each subset in CD11c^{+}MHC II^{hi} cells was illustrated and showed distinct characteristics. Consequently, the presence of the novel population of DC precursors in the *in vitro* culture system of DCs was demonstrated. At the transcriptional level, the newly discovered subsets in CD11c^{+}MHC II^{hi} cells were analyzed by RNA sequencing. In addition, to figure out the ontogeny of each subset, hematopoietic progenitors were cultured in the same condition that yield the MHC II^{hi} cell subsets. Collectively, in the current study, the novel populations and the novel DC precursors generated from BM culture in presence of FLT3L were identified for the first time. In summary, heterogeneity in CD11c^{+}MHC II^{+} FLT3L-derived cDCs were unveiled.

Key words : Dendritic cells, Antigen presenting cells, FLT3L, GM-CSF, Bone marrow, Bone marrow culture, Dendritic cell precursors

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I. INTRODUCTION

1. Discovery and history of dendritic cells

Dendritic cells (DCs) were first discovered by Ralph M. Steinman and Zanvil A. Cohn at 1973[1]. When closely investigating splenocytes, Steinman discovered novel cells shaped like tree which are morphologically distinct from macrophages and other cells. He named them as dendritic cells, taking after the Greek word ‘dendreon’ which translates as ‘tree’. At the time he discovered DCs, researchers had no conception regarding an immune response utilizing antigen process and presentation. Scientists noticed that lymphocytes were not enough to have the capacity to initiate immune responses following exposure to antigens and as a result, accessory cells, so called A or 3rd cells, were needed. But they did not understand the nature of these cells and rather regarded the cells were some types of macrophages[2]. However, Steinman succeeded at purifying DCs and proved DCs were the most potent accessory cells when it comes to stimulating T lymphocytes in primary mixed lymphocyte reactions[3] (MLRs). In late 1970s and 1980s, monoclonal antibodies targeting DCs

specifically had been developed and became feasible to study DC biology extensively by many immunologists[4]. With their efforts, the significance of DCs have been widely accepted.

Today, DC based therapy and DC vaccines are extremely studied in immunotherapy, which will eventually shed some lights on combating various diseases including cancer in the near future[5]. For his discovery of the DC and its role in adaptive immunity, Steinman was awarded the 2011 Novel Prize in Physiology or Medicine.

2. Characteristics of dendritic cells

Antigen presenting cells (APCs) such as DCs and macrophages are important players mediating innate and adaptive immunity. Particularly, DCs are the most potent professional APCs that stimulate and induce the proliferation of naïve T cells[3,6,7]. DCs have specialized cellular machineries to process antigens and present them to lymphocytes for subsequent immune responses.

DCs are usually located in almost all organs and tissues, from lymphoid to peripheral tissues where they can collect a variety of antigens. They are disposed in areas such as skin and gut and they are exposed to antigens there. Besides, DCs located in lymph nodes a specific manner to take lymph borne antigens and offer antigen information to the other cells. By capturing antigens, processing them and present them to lymphocytes, DCs initiate immune responses and regulate the balance between immunity and tolerance.

Hematopoietic cells have their unique morphology and DCs can also be distinguished by their shape which is non-adherent clusters of round cells having dendrites or pseudopods[8,9].

3. Dendritic cell subsets

DCs can be classified into several subsets. Generally, classical DCs (cDCs), plasmacytoid DCs (pDCs), monocyte-derived DCs (Mo-DCs), langerhans cells (LCs). They are further divided into subpopulations within tissues by the

combination of surface markers they express, functions and ontogeny (Figure 1).

cDCs are commonly defined by the expression of CD11c, MHC II and transcription factor *Zbtb46*[10] and found in various organs and tissues through the body. cDCs are the predominant subset among DCs, capable of sensing danger signals and capturing various form of antigens to induce subsequent immune responses. Functionally, cDCs prime and polarize naïve T cells and they are further divided into cDC1 and cDC2 based on their phenotype, function and location. Classical DC1s (cDC1s) are originally classified as $CD8^{+}CD11b^{-}$ DCs in lymphoid tissues, and $CD103^{+}$ DCs in non-lymphoid tissues. Recently, XCR1 was applied as a universal marker for cDC1s, since it is expressed both in lymphoid and non-lymphoid tissues of both mice and humans. *Irf8* and *Batf3* are known as global regulators of cDC1 generation[11-13]. cDC1s are specialized in cross-presentation, which means the activation of $CD8^{+}$ T cells by exogenous antigens[14]. Classical DC2s (cDC2s) exhibit $CD8^{-}CD11b^{+}CD172\alpha^{+}$ phenotype and and *Irf4* and *RelB* are implicated in cDC2 development[15]. Recent studies showed that Kruppel-like factor 4 (*Klf4*) is required in IRF4-expressing cDC2s to promote helper T cell responses *in vivo*[16].

$CD11c^{int}CD11b^{-}SiglecH^{+}B220^{+}mPDCA\text{-}1^{+}$ pDCs express MHC II, CD86, Sca-1, Ly6C and CCR9 in an activation and/or subset-specific manner[17-19]. Functionally, pDCs produce large amounts of type I interferon (IFN) and proinflammatory cytokines in response to pathogens[17,18]. Thus, pDCs are the specific DC subsets which are specialized to defend host against exogeneous pathogens like viruses.

Under inflammatory or infectious states, DCs originated from monocytes rapidly infiltrate lymphoid and non- lymphoid tissues. Monocyte-derived dendritic cells (Mo-DCs), also known as inflammatory DCs, express MHC II and CD11c, like cDCs, but also express markers for monocytes and macrophages such as CD11b, Ly6C, CD209a and CD115[20].

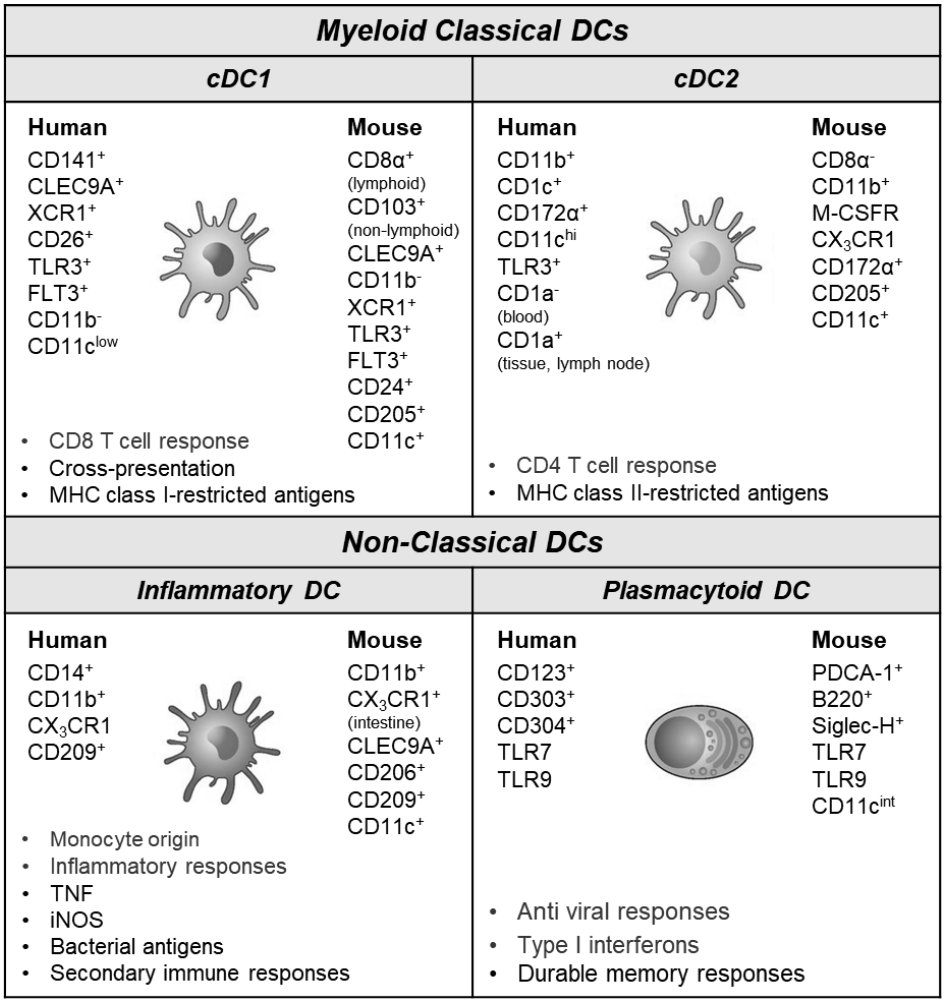


Figure 1. Characteristics of DC subsets. Representatives are common markers and functions of cDC1s, cDC2s, pDCs and Mo-DCs in summary.

4. Dendritic cell function

The key cellular player in translating innate information into adaptive immunity is DC family. DCs express a large repertoire of pattern recognition receptors (PRRs) and, in response to signals from these receptors, undergo a profound phenotypic and functional transformation, which is called maturation. DC activation, maturation, enables DC to become immunogenic APCs competent to sustain the expansion and differentiation of naïve T cells into appropriate effector cells[21-23]. DCs activated by pathogen will normally present high levels of MHC molecules bearing pathogen-derived peptides, which can engage T cell receptors on naïve T cells. This delivers the first activating signal to the T cells and is referred to as 'signal 1'. DCs activated by pathogen encounter also express various co-stimulatory molecules on cell surface, which lead ligation between co-stimulatory molecules and receptors on T cells and then give signals important for proliferation and survival of T cells (signal 2). Finally, activated DCs also produce mediators such as cytokines that act on the T cells to promote their differentiation into an effector cells (signal 3)[24].

5. Dendritic cell development

DCs are constantly being generated and replaced from precursor cells or progenitor cells which are generated from hematopoietic stem cells (HSCs) (Figure 2). Initially, it was considered obvious that DCs would be myeloid-lineage cells. DCs have many similarities to macrophages, can be generated in culture from monocytes, and are clonally associated with granulocytes and macrophages in colonies of bone-marrow cells that are grown under the influence of the 'myeloid' hormone GM-CSF[25]. It was therefore surprising when a lymphoid restricted precursor cell that was isolated from mouse thymus could generate DCs[26]. It was first assumed that this was the basis of cDC heterogeneity, with lymphoid precursors generating $CD8^+$ cDCs and myeloid precursors generating $CD8^-$ cDCs.

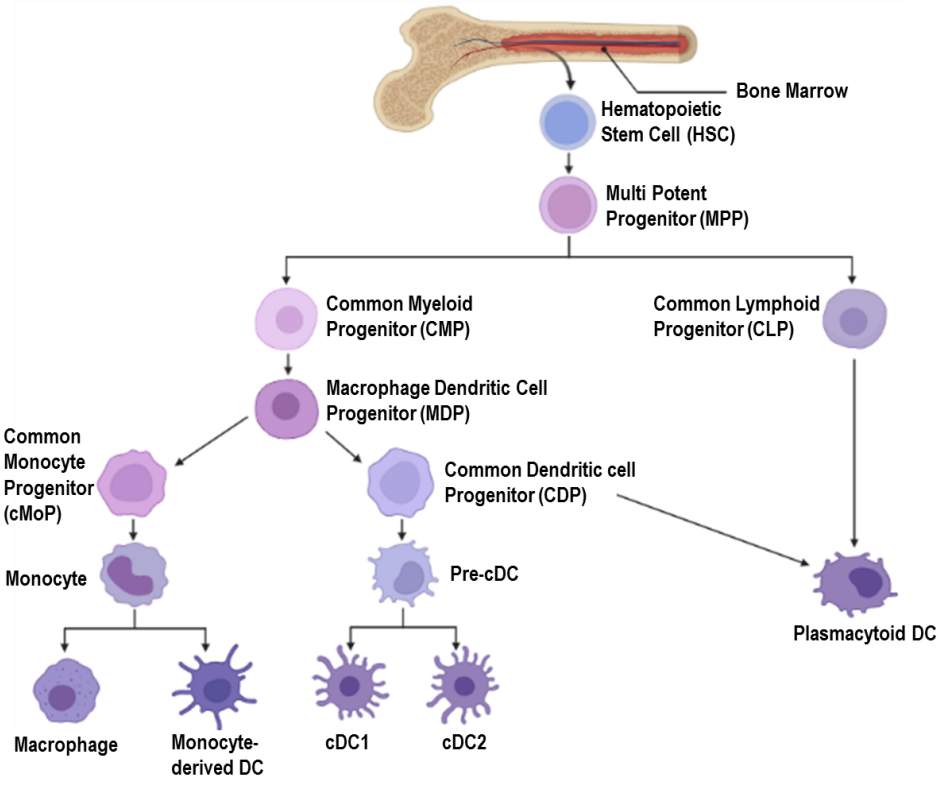


Figure 2. Schematic view of development and ontogeny of DC. DC subsets with distinct functions and markers are generated from BM progenitors and hematopoietic stem cells. The development of DCs to the respective subset counterparts arise through a multistage process.

In bone marrow (BM), developmental pathway of HSCs mainly splits into two branches, common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs)[27,28] (Figure 2). DCs are derived from common DC progenitors (CDPs) in BM, where CDPs and monocytes are derived from monocyte and DC progenitors (MDPs)[29]. CDPs are clonogenic precursor cells which have lost the potential to differentiate into macrophages or monocytes and exclusively produce pDCs and cDCs. CDPs also give rise to pre-classical DCs (pre-cDCs), which are circulating cDC-restricted progenitor cells that have lost the potential to differentiate into pDCs. Then pre-cDCs differentiate into cDCs in FLT3 ligand (FLT3L)-dependent manner in the steady state[30]. cDCs further differentiate into cDC1s via *Irf8* and cDC2s via *Irf4*. CDPs can also develop into pDCs by FLT3L. In addition, a recent study revealed that CLPs can differentiate into pDCs by the expression of *Rag1* gene. Therefore, pDCs can be generated from both myeloid and lymphoid lineages.

Monocytes, another cell originating from MDPs and common monocyte progenitors (cMoPs), also can be converted into DCs, i.e., monocyte-derived DCs (Mo-DCs), during inflammatory condition caused by microbial infection[31], as well as upon treatment of important hematopoietic cytokine for survival and proliferation of various hematopoietic cells, granulocyte macrophage-colony stimulating factor (GM-CSF)[32] and IL-4. Thus, monocyte-derived DCs arise independently of FLT3/CD135.

FLT3L, GM-CSF and M-CSF are essential cytokines which are involve in the differentiation of myeloid cells. MDPs express both FLT3 (CD135) and M-CSF receptors (M-CSFR, CD115). However, at the downstream of MDPs, when progenitors are committed to a certain lineage of cells, only specific receptors for cytokines remain. CDPs maintain the expression of FLT3 but lose M-CSFR. In contrast, cMoPs maintain the expression of M-CSFR but not FLT3. Taking into account the correlation of these expression pattern of cytokine receptors, FLT3L and M-CSF are important in normal development of DCs and

monocytic lineage cells. Mice lacking FLT or FLT3L have deficiencies in DCs, whereas M-CSF knock out mice show deficiencies in monocytic lineage cells[33].

6. Dendritic cell culture *in vitro*

DCs are efficiently generated from BM following *in vitro* culture typically with GM-CSF for a week, which has been a standard way to produce BM-derived DCs (BM-DCs) for various experiments[7,34,35], and the resultant DCs exhibit a myeloid-like phenotype. From late 1980s and early 1990s, the culture method to generate *in vitro* DCs with GM-CSF was commonly and widely used by countless researchers. But not all cells generated from BM culture through this method are DCs, whereas in the BM culture with GM-CSF, monocyte-derived macrophages resembling immature DCs also develop in addition to monocyte-derived DCs. With FLT3L, BM cells are also able to produce DCs which are similar to steady state DCs like pDCs, cDC1s and cDC2s. Recent studies reveal that cDC2s *in vivo* are heterogeneous population by transcriptional approaches. In case of DCs generated from BM culture with FLT3L, researching cDC2 heterogeneity is also needed to be performed. In this study, heterogeneous nature of cDCs generated from BM culture with FLT3L was demonstrated. Some researchers investigated DC culture with using both cytokines important to differentiation and survival for DCs, GM-CSF and FLT3L[36]. Combination of two cytokines yields novel cDC2 types *in vivo* and *in vitro* and they are shown to prevent inflammation and inflammation-caused diseases. By using DC culture *in vitro*, regulated homogeneous character of cells can be obtained and can be applied to various experiments. The importance of *in vitro* culture DCs is to investigate DC vaccines and to improve immune therapy. However, DCs existed in human peripheral blood are approximately 0.1~1% of white blood cells so that enrichment or expansion of DCs is needed[37]. In clinical approaches, it is feasible to making large amount of DCs through DC culture *in vitro*.

II. MATERIALS AND METHODS

1. Mice

C57BL/6 mice were purchased from the Orient Bio (Seongnam, Korea). C57BL/6-Tg (Tcra Tcrb) 1100Mjb/J (OT-1), B6.Cg-Tg (Tcra Tcrb) 425Cbn/J (OT-2), and B6.SJL-Ptprc^{a}Pepc^{b}/BoyJ (CD45.1) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). CD45.1^{+} OT-1 and CD45.1^{+} OT-2 mice were bred in house. Animal care and experiments were carried out according to the guidelines and protocols set and approved by the Institutional Animal Care and Use Committee of the Yonsei University College of Medicine.

2. Antibodies and reagents

The following fluorochrome-conjugated antibodies were purchased from BioLegend (San Diego, CA, USA): anti-CD3, anti-CD4, anti-CD8, anti-CD11b, anti-CD11c, anti-CD14, anti-CD19, anti-CD24, anti-CD45.1, anti-CD45.2, anti-B220/CD45R, anti-TCRβ, anti-TCR Vα2, anti-CD62L, anti-CD80, anti-CD83, anti-CD86, anti-CD103, anti-CD115, anti-CD117, anti-CD135, anti-CD172α, , anti-CD301a, anti-CD301b, anti-CCR9, anti-PD-1, anti-BTLA, anti-CCR2, anti-CCR3, anti-CCR7, anti-CXCR5, anti-CX3CR1, anti-Zbtb46, anti-Clec9a, anti-MHC I, anti-I-A/I-E (anti-MHC II), anti-NK1.1, anti-CD49b, anti-Ly6C, anti-Ly6G, anti-Gr1, anti-F4/80, anti-TER119. Anti-CD209a antibody was purchased from eBioscience (San Diego, CA, USA) and anti-CD209b was purchased from Invitrogen (Carlsbad, CA, USA). CellTrace™ CFSE or CellTrace™ violet (CTV) Cell Proliferation Kits and LIVE/DEAD™ Fixable Yellow, Blue, Far Red Dead Cell Stain Kits were purchased from Thermo Fisher Scientific Korea (Seoul, Korea) and were used according to the instructions provided by the manufacturers. FITC-conjugated ovalbumin, Rhodamine-Phalloidin (R415), GRASP65 polyclonal antibody (PA3-910) and MitoTracker™ Red CMXRos were purchased from Invitrogen. Fluoresbrite® yellow green microspheres (YGM) 1.00 μm beads (Polysciences, Warrington,

PA, USA) were purchased and sterilized by washing according to the instruction provided by the manufacturers. Ovalbumin and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse GM-CSF and FLT3L were produced and purified in house as described previously[38,39]. Anti-Calnexin antibody was purchased from Abcam (Cambridge, UK) and CpG ODN 2216 (TLRGRADE®) was purchased from Enzo Life Science (Farmingdale, NY, USA) and used according to the instructions provided by the manufacturers. Poly I:C HMW was purchased from InvivoGen (San Diego, CA, USA) and used according to the manufacturers' instructions.

3. Antibody purification and labeling

To purify mAbs 2A1 and L5 (the isotype control of 2A1 antibody)[40], the cultured supernatants of individual hybridomas were collected, filtrated before purification, the mAbs were purified with protein G (Pierce, Rockford, IL; GE Healthcare, Piscataway, NJ) column and then concentrated using Amicon® Ultra-4 Centrifugal Filter (Millipore, Burlington, MA, USA), according to the manufacturers' instructions. Quality check for purified mAb 2A1 was performed by BCA assay to quantify the amount of protein and by gel electrophoresis followed Coomassie Blue staining to check purity. Homemade mAb 2A1 was fluorescently labeled using Alexa Fluor™ 488 Antibody Labeling Kit (Invitrogen) or Alexa Fluor™ 647 Antibody Labeling Kit (Invitrogen) according to the manufacturers' instructions.

4. Bone marrow cell culture

Mice were euthanized by asphyxiation in a CO_2 chamber. Bone marrow cells were isolated from femurs and tibias of C57BL/6 mice at 8-14 weeks of age under sterile conditions as described[34,41,42], with some modifications. After lysing erythrocytes with RBC lysis buffer (Biolegend), single cell suspension of BM cells was generated by sieving the sample through cell strainers (100 μm, SPL Life Sciences, Pocheon, Korea). Then cells were counted and cultured for

8-10 days at 1×10^6 per well for GM-CSF or 2×10^6 cells per well for FLT3L culture in 24-well tissue culture plates with DMC7 containing hematopoietic cytokines as described previously[41-43]. During the culture, half of the medium in each well was carefully removed and replenished with fresh FLT3L or GM-CSF conditioned media every 2 days until harvest for use in subsequent experiments.

5. Flow cytometry

Single cell suspensions were prepared from mouse tissues or culture and were incubated in the culture supernatant of Fc receptor blocking 2.4G2 hybridoma cells for 20 minutes at 4°C followed by washing with FACS buffer (DPBS containing 2% FBS, 2mM EDTA and 0.1% sodium azide). Then, cells were incubated with appropriate cocktails of fluorochrome-conjugated mAbs and dead cell staining dye for 30 minutes at 4°C. For intracellular staining, cells were stained for surface markers as above followed by fixation, permeabilization and staining intracellular molecules with conjugated mAbs according to the manufacturers' instructions (Fixation buffer/Intracellular staining permeabilization wash buffer, Biolegend). Multiparameter analysis of each sample was performed on LSRFortessa™ flow cytometer (BD Biosciences, San Jose, CA, USA) and flow cytometric isolation of cells was performed on BD FACSAria™ II cell sorter (BD Biosciences) at the Flow Cytometry Core Facility of the Yonsei University College of Medicine. Collected data were analyzed with FlowJo software (BD Biosciences).

6. RNA sequencing analysis

Cells from BM culture were stained and sorted according to the suitable gating strategy with FACSAria™ II cell sorter as described above. For each population, total RNA was extracted by MiniBEST universal RNA extraction kit (TaKaRa Bio, Shiga, Japan) from at least 1×10^5 isolated cells. Subsequent RNA-seq procedures and analysis were performed by Macrogen (Seoul, Korea) as follows: Reverse transcription of mRNA and generation of cDNA libraries

were carried out with SMARTer Ultra low input RNA library kit and sequences with Illumina NovaSeq (Illumina, San Diego, CA, USA). The raw reads from the sequencer were preprocessed to remove low quality and adapter sequence before analysis to align the processed reads to the *Mus musculus (mm10)* using HISAT v2.1.0. After alignment, StringTie v1.3.4d was used to measure the relative abundances of genes in FPKM (Fragments Per Kilobase of exon per Million fragments mapped). Multidimensional scaling method was used to visualize the similarities among samples. The larger the dissimilarity between two samples, the further apart the points representing the experiments in the picture should be. Euclidean distance was applied as the measure of dissimilarity. Hierarchical clustering analysis was also performed using complete linkage and Euclidean distance as a measure of similarity to display the expression patterns of differentially expressed transcripts which are satisfied with |fold change|≥2. Data analysis and visualization of differentially expressed genes was conducted using GraphPad Prism 8 (GraphPad Software, La Jolla, CA) R 3.5.1 (https://www.r-project.org), Multiple Experiment Viewer software (MeV), and Morpheus software (https://software.broadinstitute.org/morpheus). Some data was analyzed using ExDega (e-biogen, Seoul, Korea). Venn diagram analysis was performed in (http://www.interactivenn.net).

7. Microscopic analysis

On day 8-9, culture of bone marrow was harvested by gentle pipetting and stained with appropriate fluorochrome-conjugated antibodies. Cells were sorted through proper gating strategies by FACS sorting with FACSAria™ II cell sorter. Obtained cells were cultured in DMC7 media supplemented with proper hematopoietic cytokines, GM-CSF and FLT3L on 96-well flat-bottom cell culture plate or 48-well culture plate overnight. Phase-contrast observations of cultures were made by means of eclipse TS100 microscope (Nikon, Tokyo, Japan) at 400 × magnification. For staining 10^5 cells were prepared on slides by cytocentrifugation (Cytospin 4, Thermo scientific) at 650rpm for 5 minutes. For

fluorescent staining, cells attached on slides were blocked with 2.4G2 for 15 minutes and 10% normal goat serum for 1 hour and then M.O.M.® (Mouse on Mouse) blocking reagent (Vector Laboratories, Inc., Burlingame, CA, USA) for 1 hour at room temperature. Primary antibody staining was performed for 2 hours at room temperature or overnight at 4°C, followed by secondary antibody staining for 1 hour at room temperature. Then the sections were stained with conjugated antibodies for 1 hour at room temperature and mounted with Dako fluorescence mounting medium (Dako, Santa Clara, CA, USA). The images were acquired immediately after staining with the Carl Zeiss LSM700 confocal microscope using 40X and 60X objectives or were taken by IX73 fluorescent inverted microscopes (Olympus, Tokyo, Japan).

8. Treatment of TLR agonists

For titration of working concentration of LPS, graded doses of LPS were added on BM culture at culture day 8; 1 pg/ml, 100 pg/ml, 10 ng/ml, 1 μg/ml. After 18 hours of incubation, cells were harvested, washed and analyzed using flow cytometry. 1 μg/ml of LPS was used for *in vitro* DC stimulation unless otherwise noted. 1 μM of CpG or 1μg/ml of poly I:C was added on BM culture at day 8. After 18h of incubation, cells were harvested, washed and stained with fluorescently labeled antibodies for flow cytometric analysis.

9. Antigen uptake

Culture of BM was treated with 100 μg/ml FITC labeled OVA or 0.000675% YGM beads for 1 hour at 37 °C or at 4 °C as control. Then, each sample was washed twice with cold DMEM and further blocked with Fc receptors and stained with the appropriate mixture of dead cell staining dye and fluorochrome-conjugated antibodies prior to being analyzed with a flow cytometer as described above.

10. Antigen presentation and T cell proliferation

To prepare APCs, cells were pulsed with 100 μg/ml of OVA on culture plates at 37°C. After 30 minutes of pulsing, suspending cells were harvested by gentle pipetting and washed thoroughly. To obtain specific populations among culture, cells were fluorescently labeled and sorted by FACS cell sorter. Splenic T cells from $CD8^+$ OT-1 and $CD4^+$ OT-2 mice were enriched after Fc block by excluding $CD19^+$, $CD49b^+$, MHC II^+, $F4/80^+$, $NK1.1^+$, $Ly6G^+$ and $CD4^+$ (for OT-1) or $CD8^+$ (for OT-2) splenocytes using appropriate biotinylated Abs and anti-biotin Dynabeads® (ThermoFisher Scientific Korea). Enriched T cells were labeled with CFSE or CTV at 37ºC for 10 minutes, washed and 2.5×10^4 T cells were cultured with APCs at various ratios in the complete media containing 57.2 μM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) for 3-5 days.

To check changes of T cell proliferating ability under LPS stimulation, APCs were sorted and co-cultured with $CD8^+$ OT-1 or $CD4^+$ OT-2 T cells in the presence of 10 μg/ml OVA and 0 μg/ml or 1 μg/ml LPS. After 3 days or 5 days of incubation, T cell proliferation was assessed by the dilution of CFSE or CTV.

11. Detection of MHC class I molecules complexed with OVA epitope peptide pOT-1

At culture day 7 or 8, FL-BM culture were incubated in the presence of 100 mg/ml of endotoxin-free OVA (EndoFit Ovalbumin, InvivoGen) or 1 mg/ml of OT-1 peptide (pOT-1, SIINFEKL) for 18 hours at 37°C. For a control, 1 mg/ml of pOT-1 was added on culture for 1 hour on ice. To determine the surface levels of H2-Kb bound to pOT-1, cells were incubated with mAb 25-D1.16 on ice for 30 min, washed, and then analyzed by flow cytometry.

12. Progenitor study

BM pre-cDCs were sorted with FACSAria™ II cell sorter as in proper gating strategy; $Lin^-CD11b^-CD11c^+$MHC II^- $CD135^+$ (Lin; CD3, CD19, CD49b,

CD115, CD117, Sca-1, Ly6C, Ly6G). 5×10^4 of sorted BM pre-DCs were cultured with FLT3L for 3 days or 6 days. 2×10^6 of whole BM cells without RBCs from CD45.1 mice were co-cultured as filler cells. At culture day 2 or day 5, LPS was added on culture to stimulate cells. After 18 hours of incubation, cells were harvested, washed, analyzed by flow cytometry. Filler cells and cells derived from sorted pre-cDCs were distinguished by the expression of CD45.1 or CD45.2 on cell surface. BM monocytes were sorted according to the gates as in followed; $Lin^-CD115^+CD11b^+Ly6G^-Ly6C^{hi}$ (Lin; CD3, CD19, CD49b, CD117, Sca-1, NK1.1, CD11c, MHC II, CD135). BM cMoPs were sorted according to the gates as followed; $Lin^-CD117^+CD115^+CD16/23^+CD34^+CD135^-Ly6C^+$ (Lin; CD3, CD11b, CD11c, CD19, CD49b, Sca-1, NK1.1, B220, TER119, MHC II, Ly6G). CDPs were sorted as in gates followed; $Lin^-CD117^{int}CD115^+CD135^+$ (Lin; CD3, CD11b, CD11c, CD19, CD49b, Sca-1, Ly6C, Ly6G, MHC II, B220, IL-7Rα). All sorted progenitor cells were cultured with the aids of whole BM filler cells from $CD45.1^+$ mice.

13. Transmission electron microscopy

Cells were fixed with 2% Glutaraldehyde and 2% Paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 12 hours; washed with 0.1M phosphate buffer; post-fixed with 1% OsO4 in 0.1M phosphate buffer for 2 hours; dehydrated with a series of ascending ethanol (50, 60, 70, 80, 90, 95, 100, and 100%) for 10 minutes each; infiltrated with propylene oxide for 10 minutes. Then, specimens were embedded with a Poly/Bed 812 kit (Polysciences) and polymerized in an electron microscope oven (TD-700, DOSAKA, Japan) at 65°C for 12 hours. The block was cut with a diamond knife in the ultramicrotome into 200 nm semi–thin section and stained toluidine blue for observation of optical microscope. The region of interest was then cut into 80 nm thin sections using the ultramicrotome, placed on copper grids, double stained with 3% uranyl acetate for 30 minutes and 3% lead citrate for 7 minutes, and imaged with a

transmission electron microscopy (JEM-1011, JEOL, Tokyo, Japan) at the acceleration voltage of 80 kV equipped with a Megaview III CCD camera (Soft Imaging System, Germany).

14. Statistical analysis

Experiments with multiplicate samples were analyzed for statistical comparisons between different groups using with ANOVA and unpaired Student's t-test using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Statistical significance is denoted by the p values equal or below 0.05 (*), 0.01 (**), and 0.001 (***). Data were plotted for graphs with GraphPad Prism 5 and GraphPad Prism 8.

III. RESULTS

1. A specific marker for DCs, 2A1

2A1 is one of the mAbs generated by early efforts[35] to produce new reagents that specifically detect DCs in the culture of BM with GM-CSF as well as in lymphoid tissues in situ. However, its targets express inner cell space so that more efforts are needed to access intracellular regions. Moreover, molecules that 2A1 binds is unknown so far, albeit I've studied extensively to figure out what 2A1 targets by numerous ways. Although 2A1 has weakness to study cells alive, there are also some advantages that 2A1 can mark highly mature DCs or activated DCs which are expressing MHC II abundantly. In this study, 2A1 was purified by chromatographic methods from 2A1 secreting hybridoma cells, labeled with fluorescence and utilized to various experiments to unravel the complexity of DC biology.

2. 2A1 Antigen is highly expressed in DCs generated from the culture of BM with GM-CSF

Monoclonal antibody (mAb) 2A1 was demonstrated to detect DCs expressing high levels of MHC class II molecules (MHC II^{hi} DCs) in the culture of BM with GM-CSF[35,44]. However, the use of mAb 2A1 in research has been rare because the antigen (from now on also named as "2A1") recognized by mAb 2A1 is localized intracellularly[35,44,45] and requiring the permeabilization of cell membranes prior to the flow cytometric detection (Figure 3). Consequently, the expression of 2A1 has not been fully investigated in DCs from various subsets and tissues. To examine the expression of 2A1 accurately, highly pure mAb 2A1 was prepared from the culture of 2A1 hybridoma in the media containing fetal bovine serum with ultra-low IgG content (Figure 4) and then mAb 2A1 was conjugated with fluorescent dyes in house. With this newly produced reagent, cells in the culture of BM with GM-CSF for their expression of 2A1 were first examined (Figures 5 and 6). Although 2A1 was hardly detected from

BM cells in the beginning, about 15% of the cells expressed high levels of 2A1 after 8 days of culture with GM-CSF (Figure 5A). Virtually all the cells with high levels of 2A1 expression ($2A1^{hi}$ cells) were identified as GM-DCs (DCs derived from BM culture with GM-CSF[46]) and nearly all the GM-DCs were $2A1^{hi}$ cells (Figures 5B and 5C). Accordingly, GM-Macs (macrophages derived from BM culture with GM-CSF[46]) hardly contained any $2A1^{hi}$ cells. More abundant expression of 2A1 in GM-DCs was similarly observed in the expression of co-stimulatory molecules such as CD80, CD83, and CD86, while CD11b, CD115, and CX_3CR1 were more abundantly.

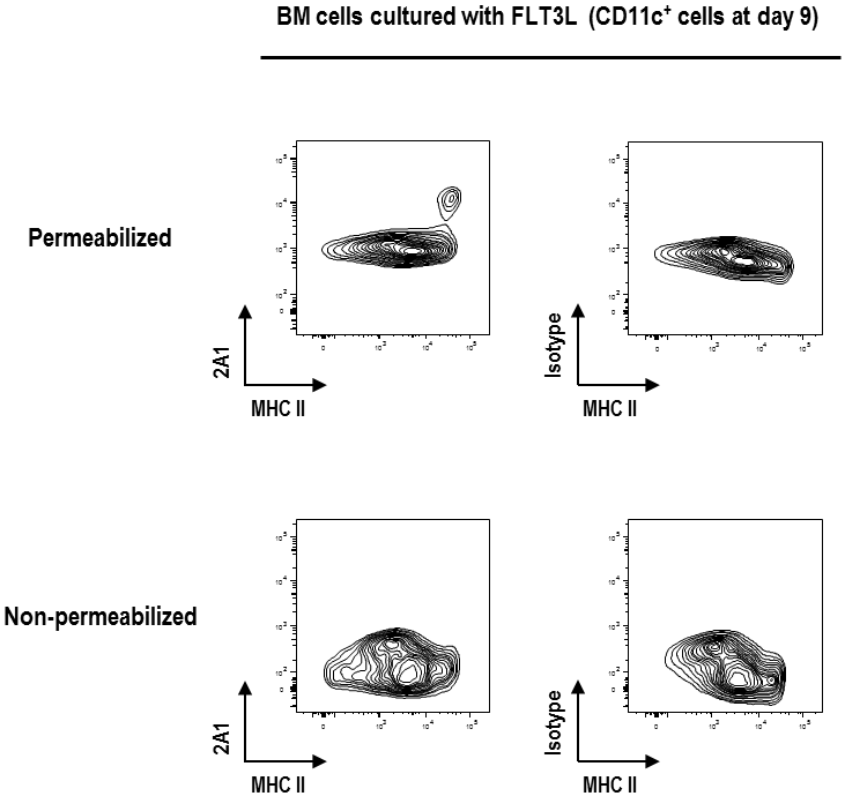


Figure 3. 2A1 express intracellular region of DCs in BM culture. $CD11c^+$ cells were analyzed at FLT3L culture day 9. Top, cells were stained with 2A1 after fixing and permeabilizing cells, bottom, 2A1 staining was performed without fixation and permeabilization.

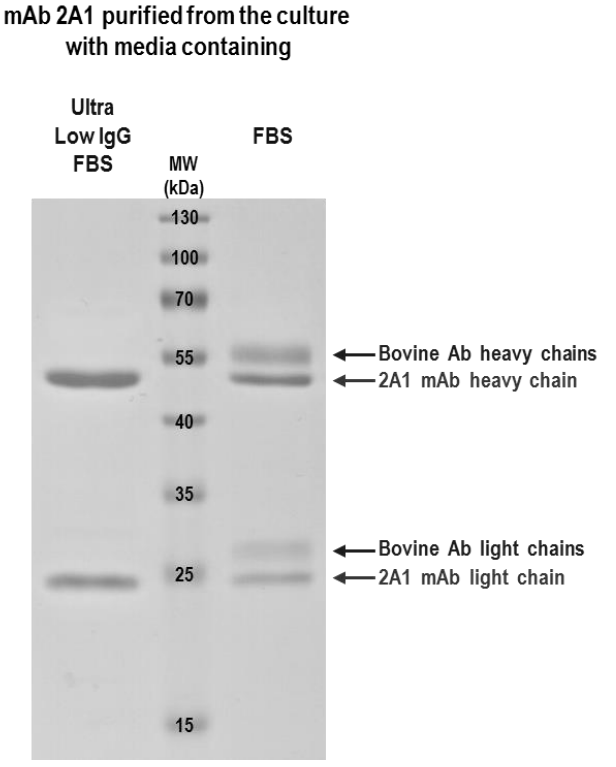
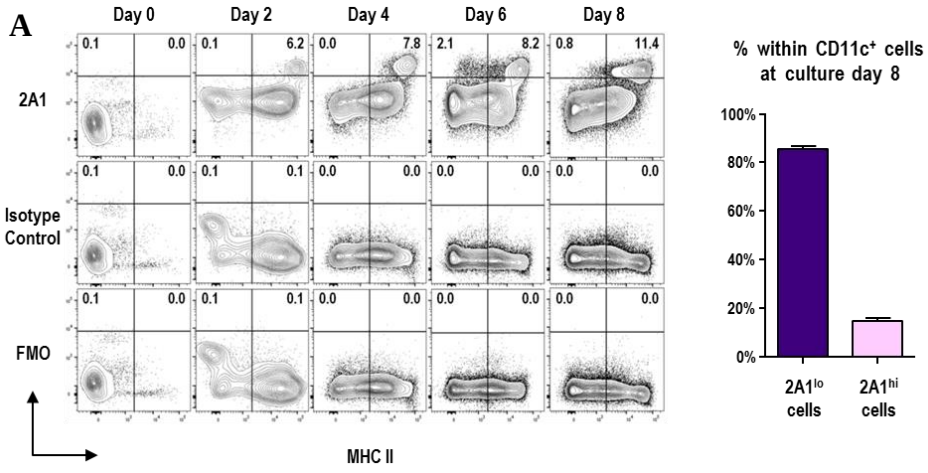
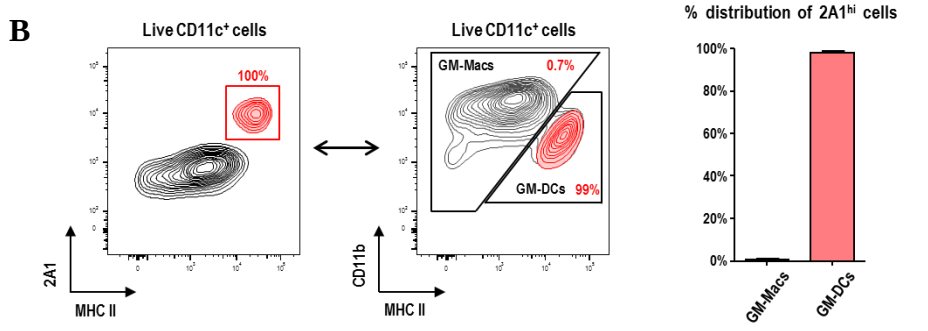


Figure 4. Quality check for home-made mAb 2A1. Hybridoma cells secreting mAb 2A1 were culture in the presence of normal FBS or Ultra low IgG FBS. 2A1 antibody was purified by chromatographic way using protein G beads from culture supernatant containing mAb 2A1. Same amount of purified 2A1 from both conditions were stained with Coomassie Blue to check the quality. In the normal FBS condition, nearly half of the proteins are bovine antibodies originated from FBS.





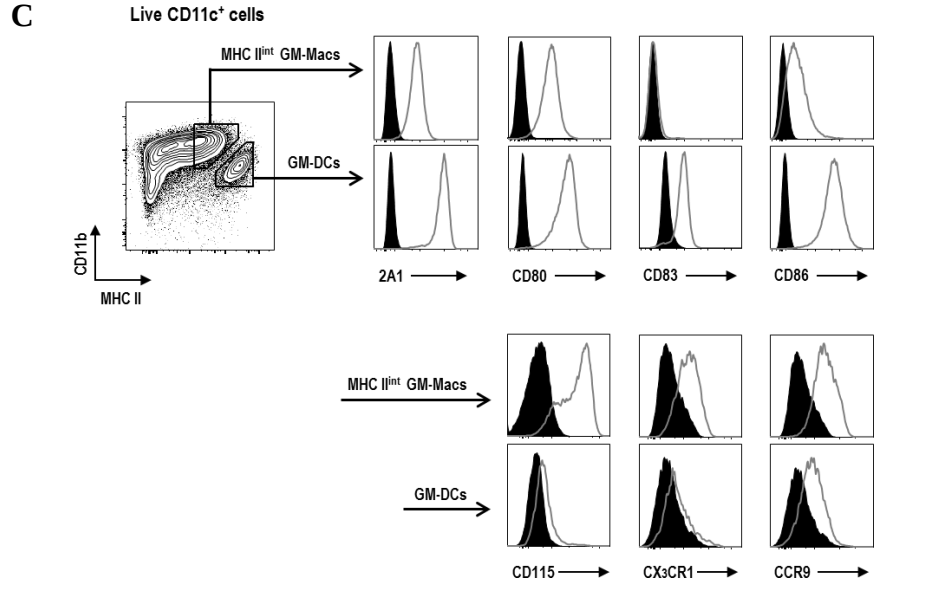


Figure 5. 2A1 expression on cells from BM culture with GM-CSF. (A) Kinetics of $CD11c^{+}2A1^{hi}$ cell development from GM-CSF-supplemented BM culture. Day 0 data were generated from freshly isolated BM cells. (B) Distribution of $2A1^{hi}$ cells in GM-CSF cultures at day 8. Shown are the results of a representative experiment out of ten independent experiments performed. (C) Phenotype of GM-CSF cultures at day 8. GM-DCs, GM-Macs are gated. Filled black histograms mean isotype controls of each marker. Representative flow cytograms are from at least 4 independent experiments.

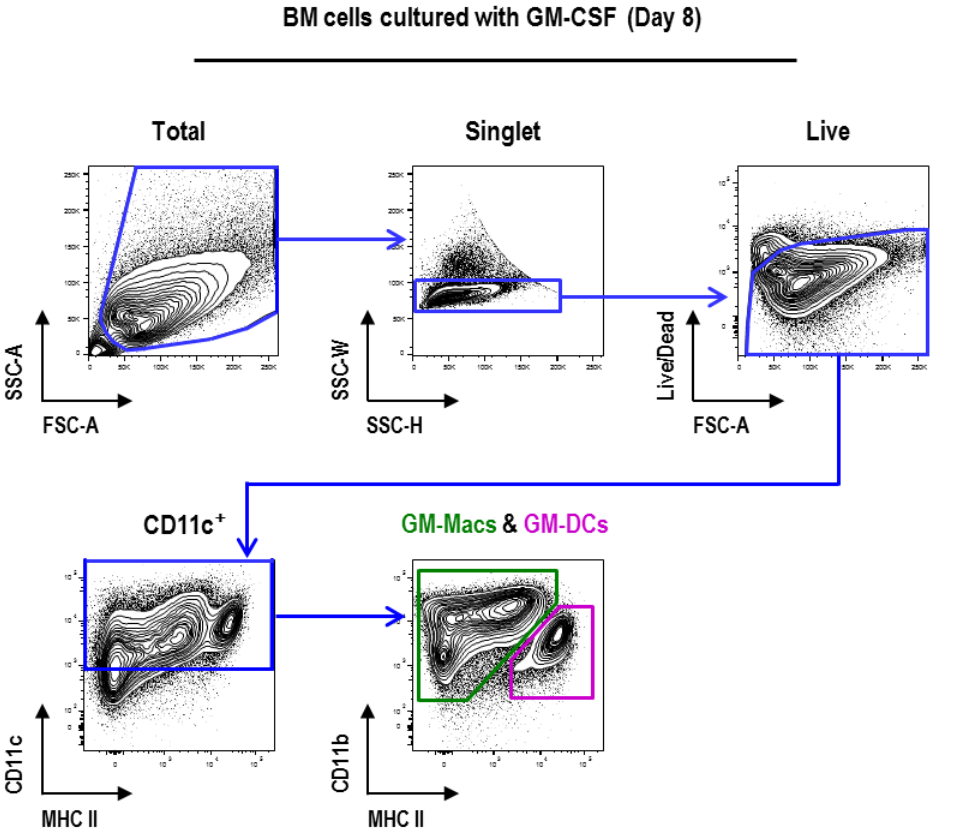


Figure 6. Gating strategy for BM culture with GM-CSF. BM cells are cultured with GM-CSF, harvested, stained, and analyzed on culture day 8. $CD11c^+$ cells in green pentagon indicate GM-Macs and $CD11c^+$ cells in purple pentagon are GM-DCs. Representative flow cytograms are from 5 independent experiments.

3. 2A1^{hi} cells are found in MHC II^{hi} DC subsets in the culture of BM with FLT3L

When BM cells were cultured with FLT3L for more than a week, most of cells in the culture become CD11c^{+} DCs (Figure 9). Cells in the culture of BM with FLT3L were also examined for their 2A1 expression and 2A1^{hi} cells were composed approximately 10% of the cells after 9 days (Figure 8A). All those 2A1^{hi} cells in the culture of BM with FLT3L were found in MHC II^{+} cDCs and no 2A1^{hi} cells were in MHC II^{-} pDCs (Figure 8B). Interestingly, a vast majority (about 90%) of 2A1^{hi} cells was identified as cDC2s and the other small fraction (about 10%) was cDC1s (Figures 8B and 11A), although about 20% of cells in the cDC2 subset were 2A1^{hi} and less than 10% in the cDC1 subset were 2A1^{hi} (Figure 4B). To further understand the distribution of 2A1^{hi} cells among the cDC subsets, cDCs were examined based on their levels of MHC II expression. In the culture of BM with FLT3L, about 20% of cDCs belonged to the cDC1 subset and the other 80% of cDCs belonged to the cDC2 subset. However, almost 95% of MHC II^{hi} cDCs were cDC2s whereas less than 50% of MHC II^{lo} cDCs were cDC2s (Figure 11A). Meanwhile, almost entirely all 2A1^{hi} cells were found to be MHC II^{hi} cDCs (Figure 11A) and MHC II^{hi} cDCs were composed of about 30% of 2A1^{hi} cells and around 70% of 2A1^{lo} cells (Figure 11A). These observations suggest that the high expression of MHC II is necessary but not sufficient for cDCs to become 2A1^{hi} regardless of cDC subset.

Although the antigen that 2A1 targets were unknown so far, there are some early researches using 2A1 mAb to stain DCs and lymphoid tissues such as LNs and spleen. 2A1 specifically labels MHC II DCs arise from BM culture with GM-CSF[25,44] and stains T cell zones or DC-rich regions in LNs and spleen[45]. As a result, the localization within culture DCs is to be elucidated. To illustrate the subcellular location of 2A1, various markers that label cellular organelles were used with 2A1 (Figure 10). In the culture of BM with FLT3L, cells that highly express 2A1 have relatively small amount of cytoplasmic contents. 2A1 locates

through space near the nucleus to the plasma membrane, showing granule-like shapes (Figure 10, left 2nd and left 3rd). However, the intracellular localization of 2A1 seemed quite ambiguous because none of organelle markers overlapped with 2A1 stain (Figure 10). Ribosome and lysosome can be potent candidates for subcellular regions that 2A1 exists so that further study would be needed to delineate the accurate cellular localization of 2A1.

The findings above indicate evidently that, within MHC II^{hi} cDCs in the culture of BM with FLT3L, there are at least 2 distinct populations, i.e., $2A1^{hi}$ cDCs and $2A1^{lo}$ cDCs (Figure 7). To further elucidate those distinct cDC populations, the expression of surface molecules with relationship to the intracellular level of 2A1 were extensively examined. (Figure 12) Then the following was found that, among MHC II^{hi} cDCs, the high expression of CD83 was strongly correlated with the high levels of 2A1 (Figure 11B), and virtually all the cDCs expressing CD115 and/or CX_3CR1 were $2A1^{lo}$ (Figure 11C). Accordingly, MHC II^{hi} cDCs were classified into three different populations: $CD115^+CX_3CR1^+CD83^-$ $2A1^{lo}$ cDCs, $CD115^-CX_3CR1^-CD83^-$ $2A1^{lo}$ cDCs, and $CD115^-CX_3CR1^-CD83^+$ $2A1^{hi}$ cDCs (Figure 13). Interestingly, all those 3 populations were present only in MHC II^{hi} cDC2s but not in the other cDCs (Figure 13A and 14B). With decreasing levels of MHC II, $CD115^-CX_3CR1^-CD83^+$ $2A1^{hi}$ cDCs disappeared from the cDC2 subset. Meanwhile, MHC II^{hi} cDC1s were composed of $CD115^-CX_3CR1^-CD83^-$ $2A1^{lo}$ and $CD115^-CX_3CR1^-CD83^+$ $2A1^{hi}$ cDCs but deficient in $CD115^+CX_3CR1^+CD83^-$ $2A1^{lo}$ cDCs (Figure 13). Also, $CD115^-CX_3CR1^-CD83^+$ $2A1^{hi}$ cDC1s became undetectable with lowering levels of MHC II (Figure 14A and 14B).

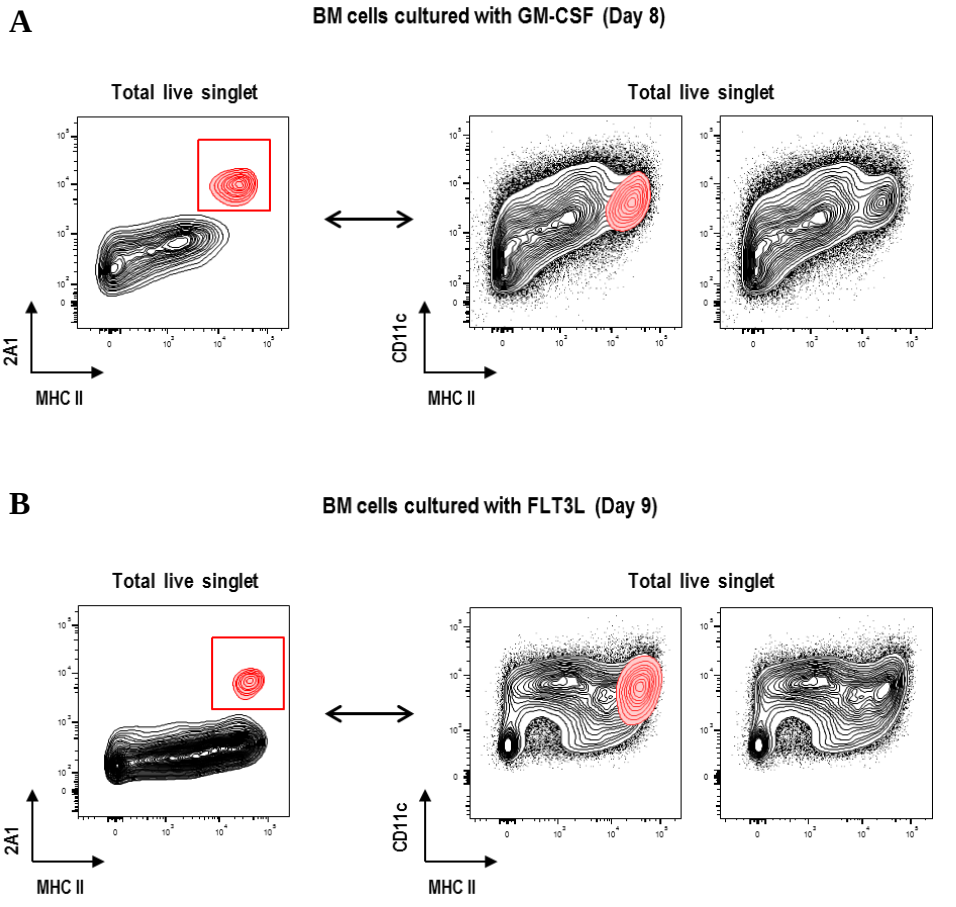


Figure 7. 2A1 were expressed specifically MHC II^{hi} DCs both GM-CSF and FLT3L culture. (A) Cells were analyzed at culture day 8 in GM-CSF condition. 2A1^{hi} MHC II^{hi} cells colored with red in the box were marked at CD11c and MHC II plot. (B) Representatives are cells from BM culture day 9 in FLT3L condition. Gates are same as figure 7A.

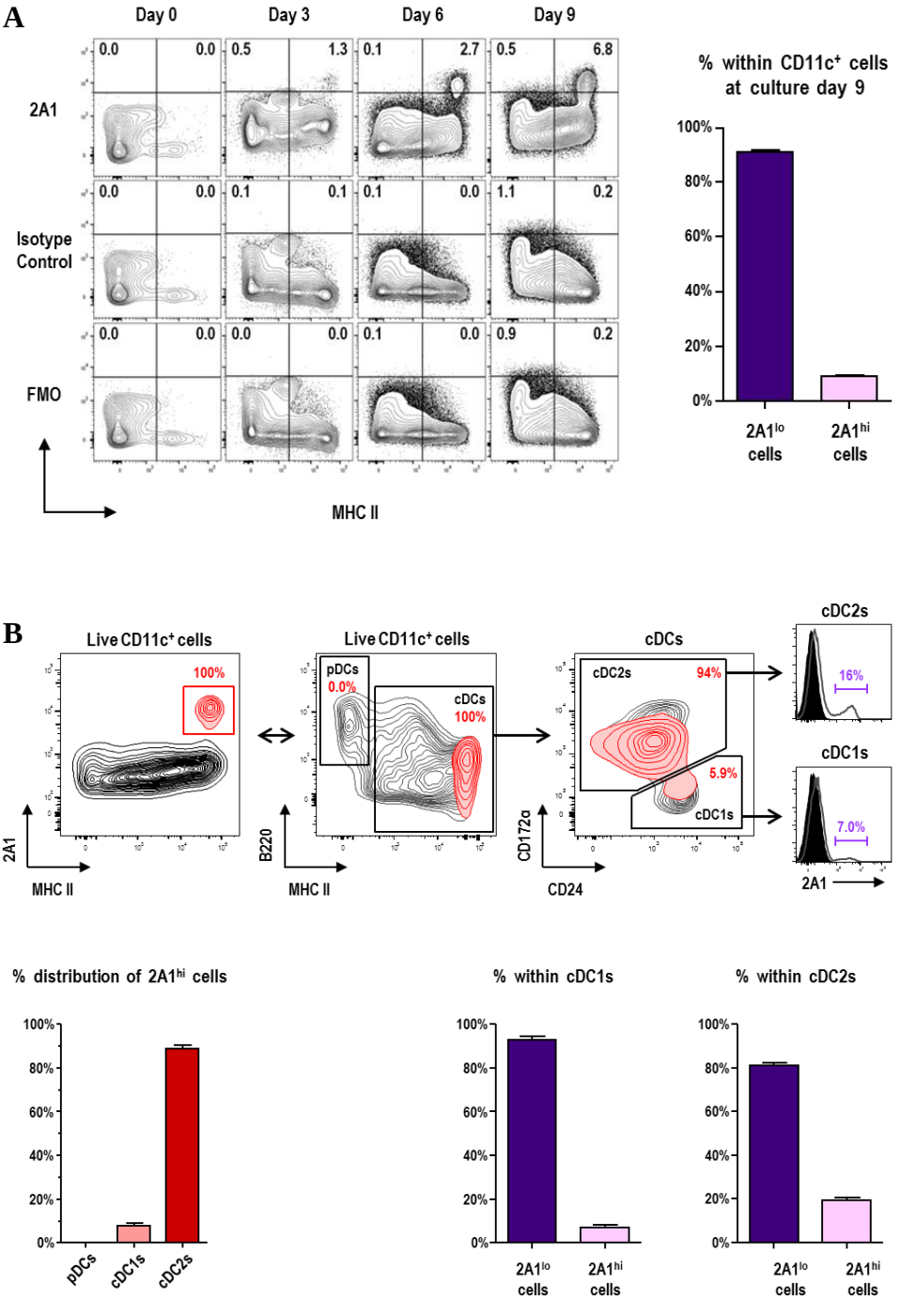


Figure 8. 2A1 expression on cells from BM culture with FLT3L. (A) Kinetics of $CD11c^{+}$ $2A1^{hi}$ cell development from FLT3L-supplemented BM cultures. Left, representative plots, right, statistical graph. (B) Distribution of $2A1^{hi}$ cells in FLT3L cultures at day 9. Shown are the results of a representative experiment out of ten independent experiments performed.

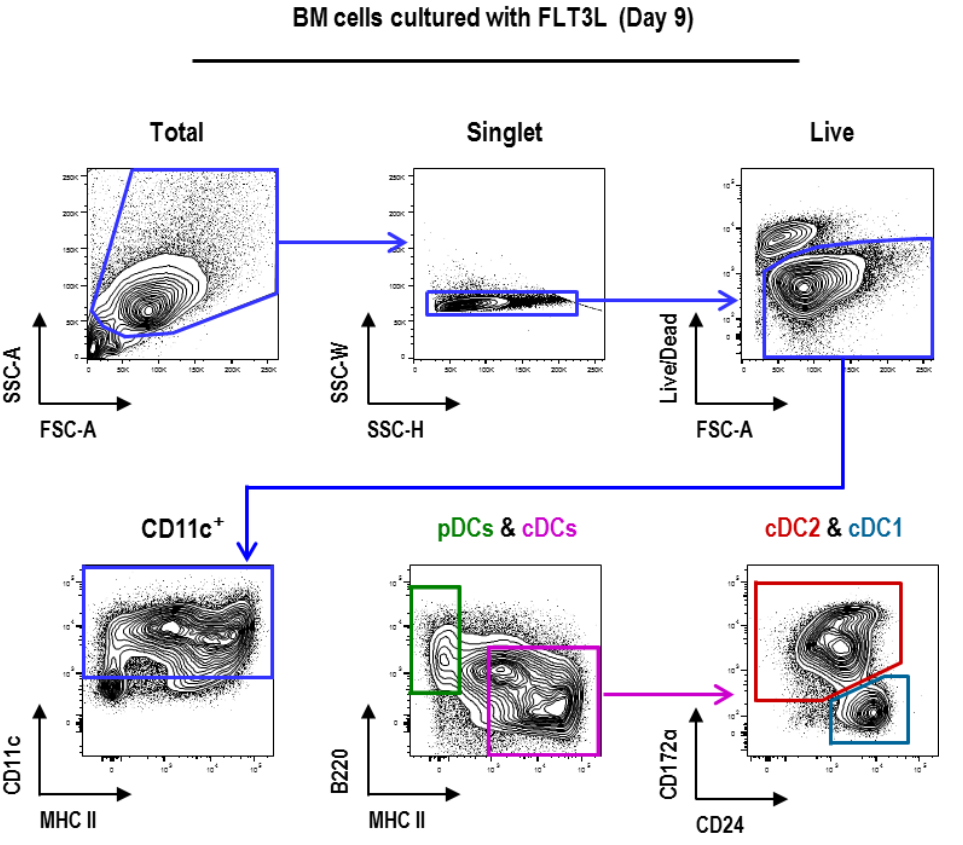


Figure 9. Gating strategy for BM culture with FLT3L. BM cells were cultured with FLT3L, harvested, stained and analyzed on day 9. Green, $B220^{+}MHC\ II^{-}$ pDCs; fuchsia, $B220^{-}MHC\ II^{+}$ cDCs; red, $B220^{-}MHC\ II^{+}CD172a^{+}CD24^{-}$ cDC2s; blue, $B220^{-}MHC\ II^{+}CD172a^{-}CD24^{+}$ cDC1s.

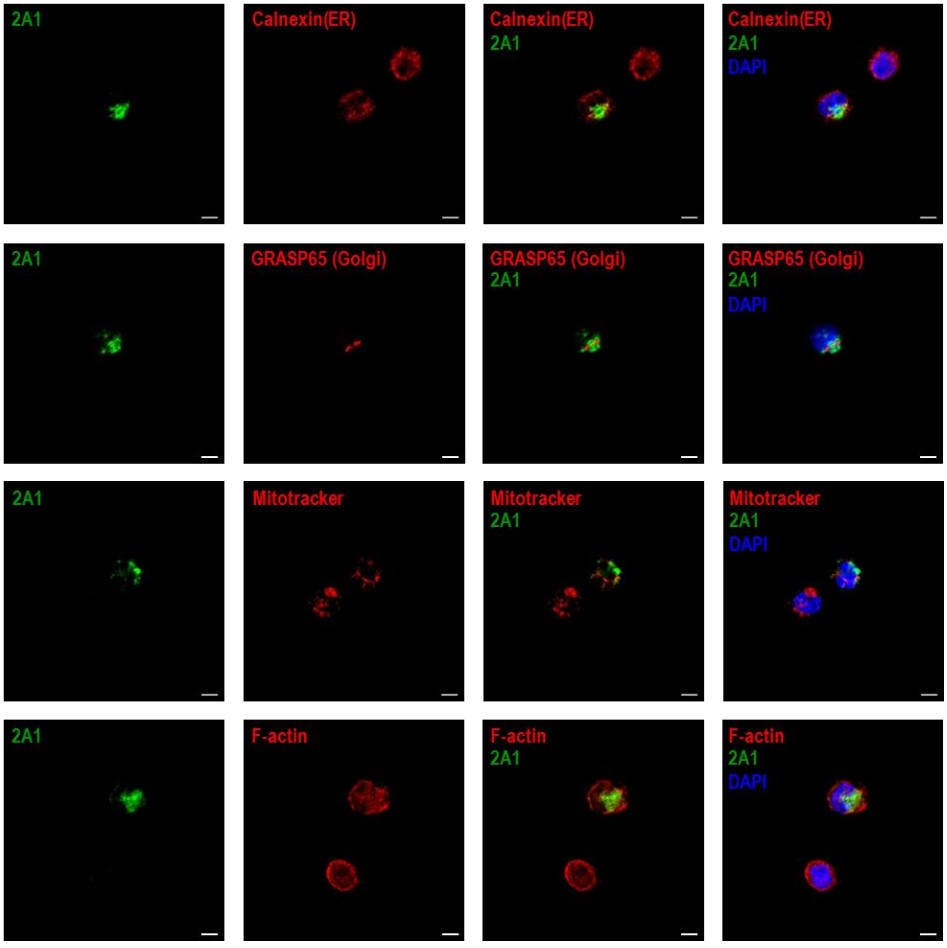
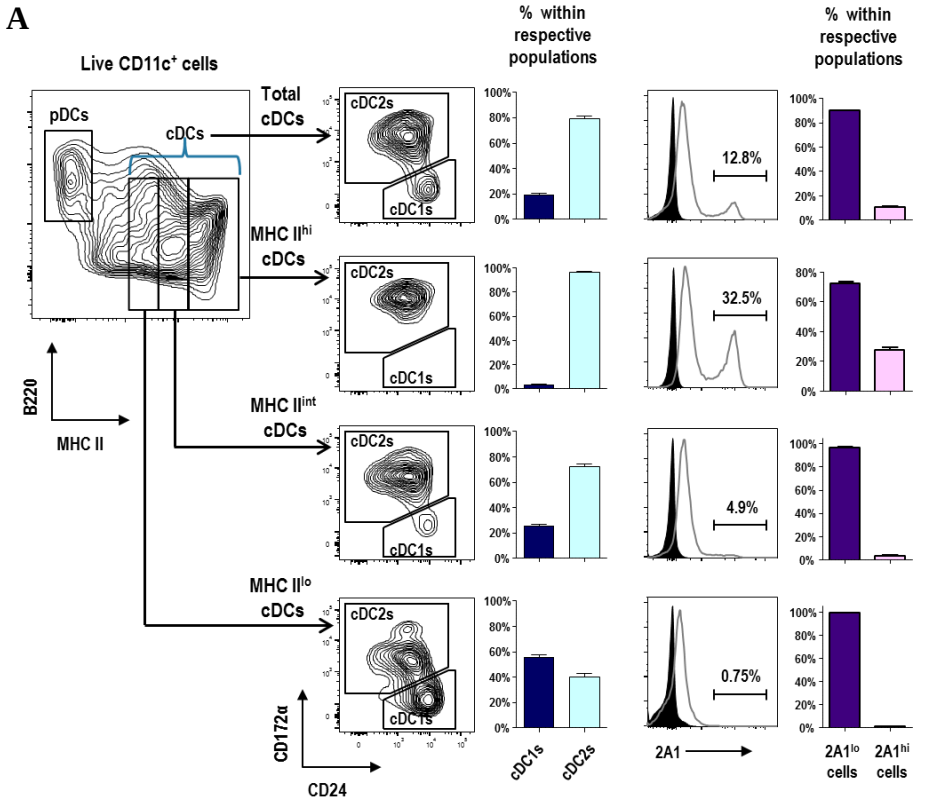


Figure 10. Cellular localization of 2A1 in the cells from BM culture with FLT3L. Cells were harvested, cytospined and stained with indicated markers for cellular organelles at culture day 8. Confocal immunofluorescent analysis of cells from BM culture with FLT3L using indicated markers for cellular organelles (red) and 2A1 (green). Mitochondria were stained with MitoTracker™ Red CMXRos (red). ER and Golgi apparatus were marked with anti-Calnexin and GRASP65 antibodies respectively. Actin filaments (F-actin) were labeled with Rhodamine-Phalloidin (red). Blue pseudocolor represents DAPI which is a fluorescent DNA dye. Scale bar = 5μm



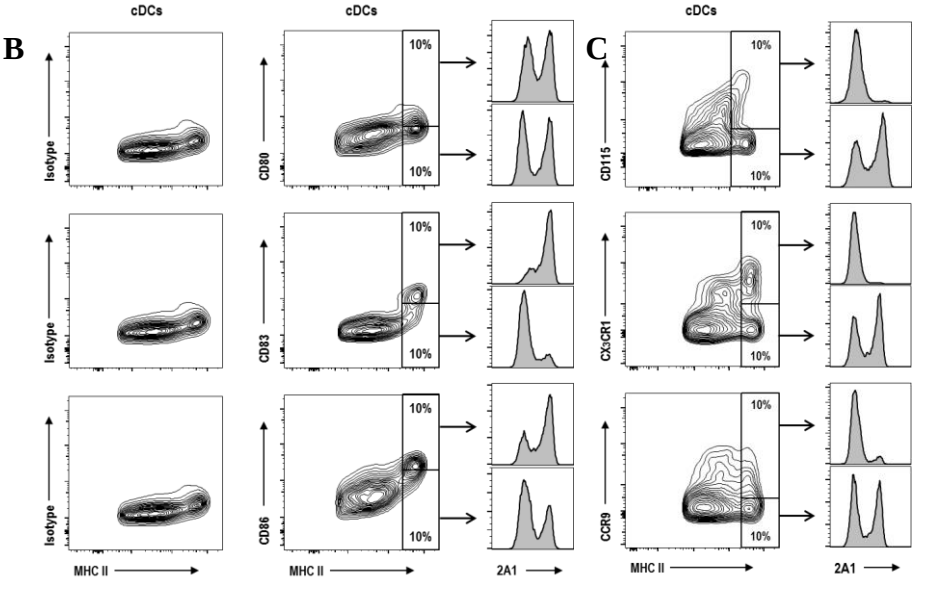


Figure 11. cDC2s can be subdivided with additional hematopoietic markers. **(**A) Distribution of cDC1, cDC2 or 2A1^{hi} cells within MHC II^{hi}, MHC II^{mid}, MHC II^{lo} cDCs in FLT3L cultures at day 9. Shown are the results of a representative experiment out of ten independent experiments performed. (B) Positive correlation between T cell co-stimulatory molecules and 2A1 within MHC II^{hi} cDCs in FLT3L cultures at day 9. Left column represents isotype controls of each marker. (C) Negative correlation between hematopoietic markers and 2A1 within MHC II^{hi} cDCs in FLT3L cultures at day 9.

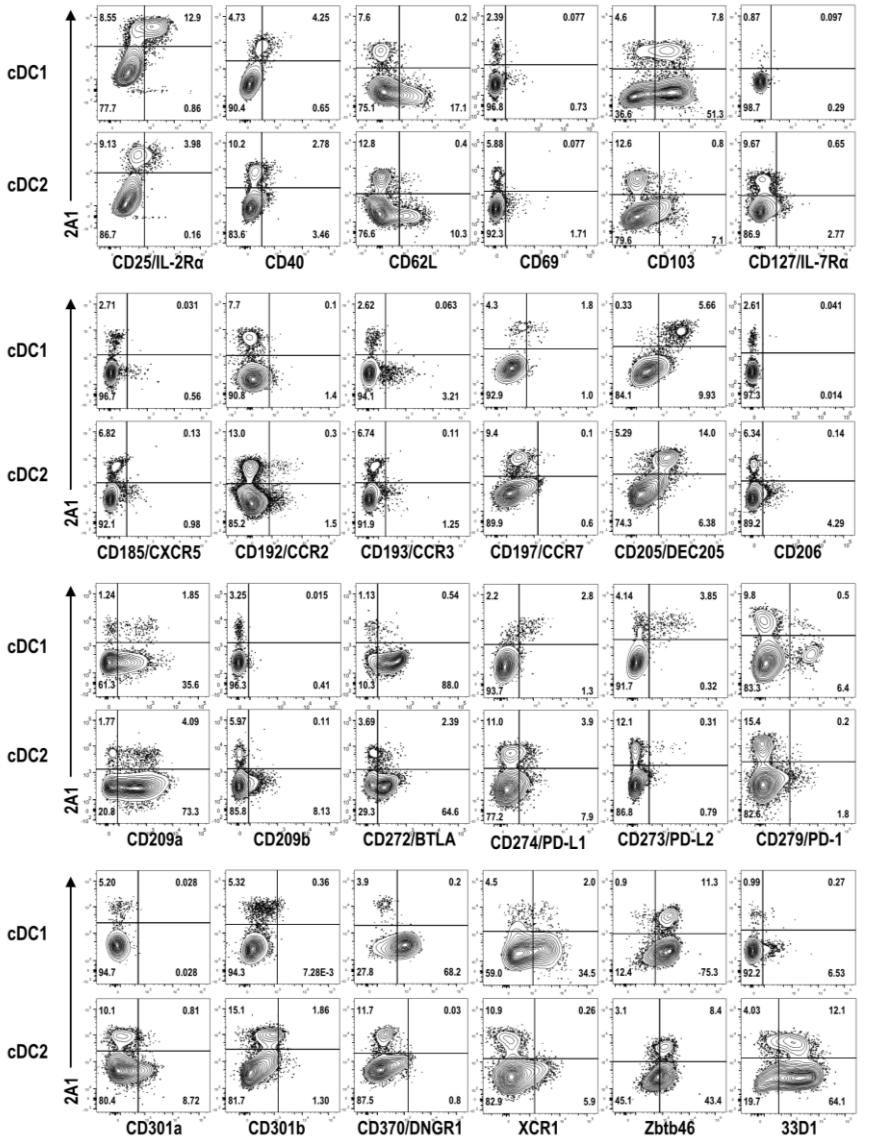


Figure 12. Expression of 2A1 and various molecules in cDC1s and cDC2s from FL-BM culture. Expression of 2A1 and various molecules are analyzed in cDC1s and cDC2s from FL-BM culture at culture day 8 or 9. For Zbtb46 staining, nuclear permeabilization was performed. The frequencies of individual quadrants based on FMO or isotype control are shown. Representative flow cytograms shown are from 3 independent experiments.

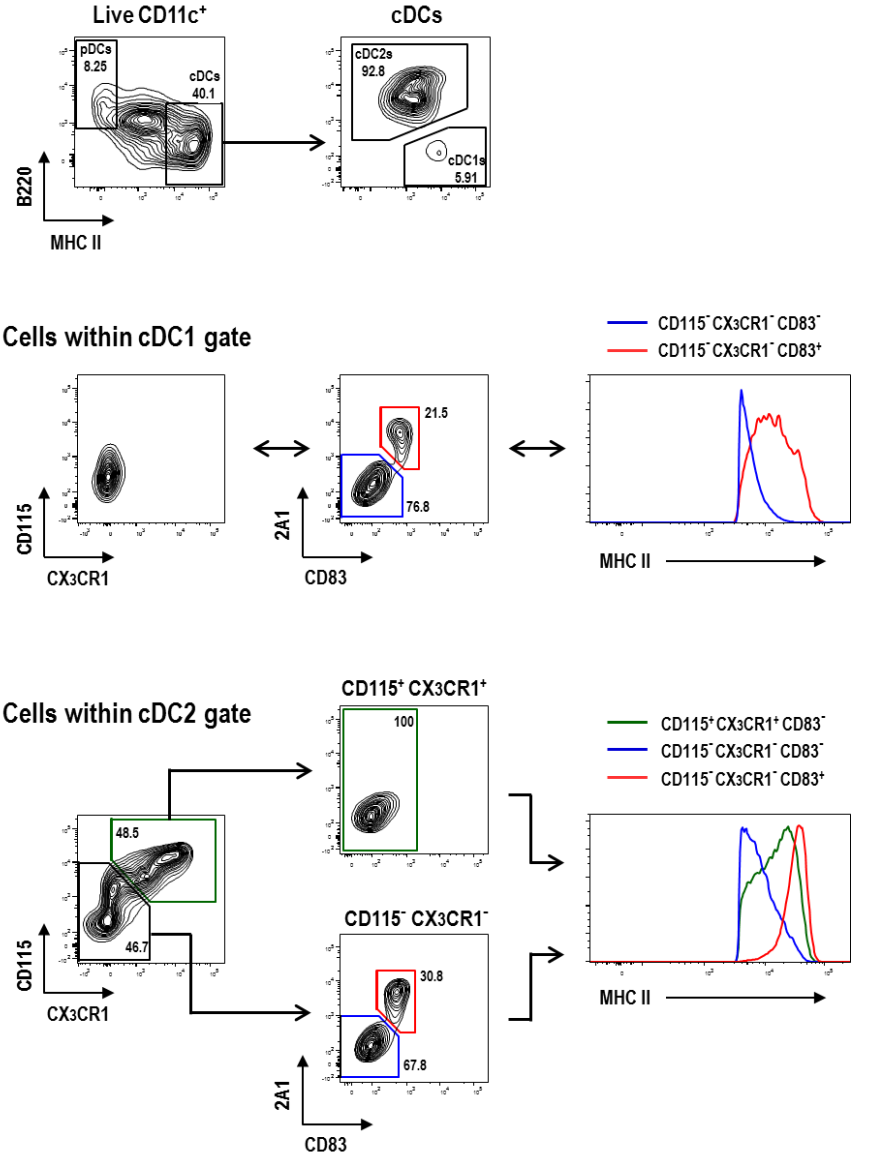
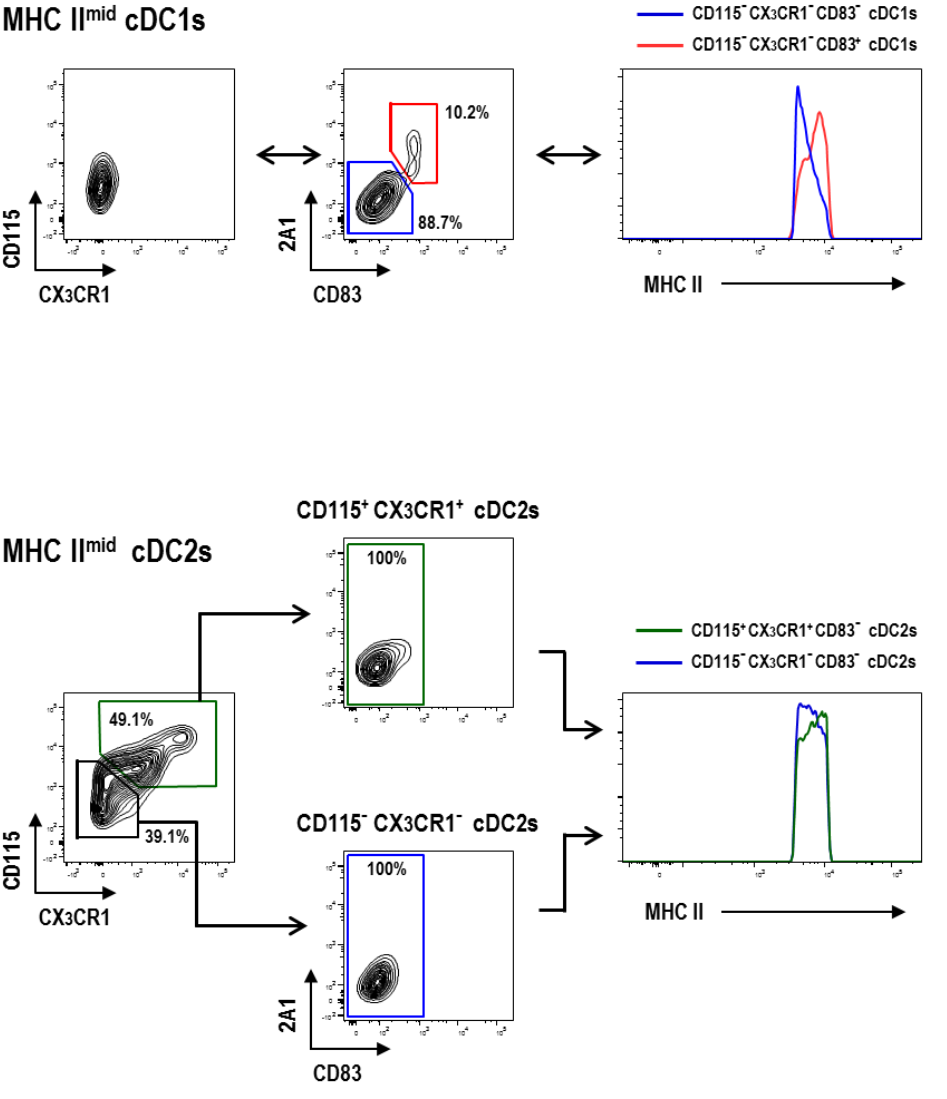


Figure 13. Heterogeneity of MHC II^{hi} cell subsets from BM culture with FLT3L. Cells were harvested by gentle pipetting and analyzed at culture day 9. Among CD11c^{+}MHC II^{+}CD172a^{+}CD24^{-} cDC2s, three different subsets were identified; CD115^{-}CX$_3$CR1^{-}CD83^{-} cDC2s (blue), CD115^{-}CX$_3$CR1^{-}CD83^{+} cDC2s (red), CD115^{+}CX$_3$CR1^{+}CD83^{-} cDC2s (green).

A



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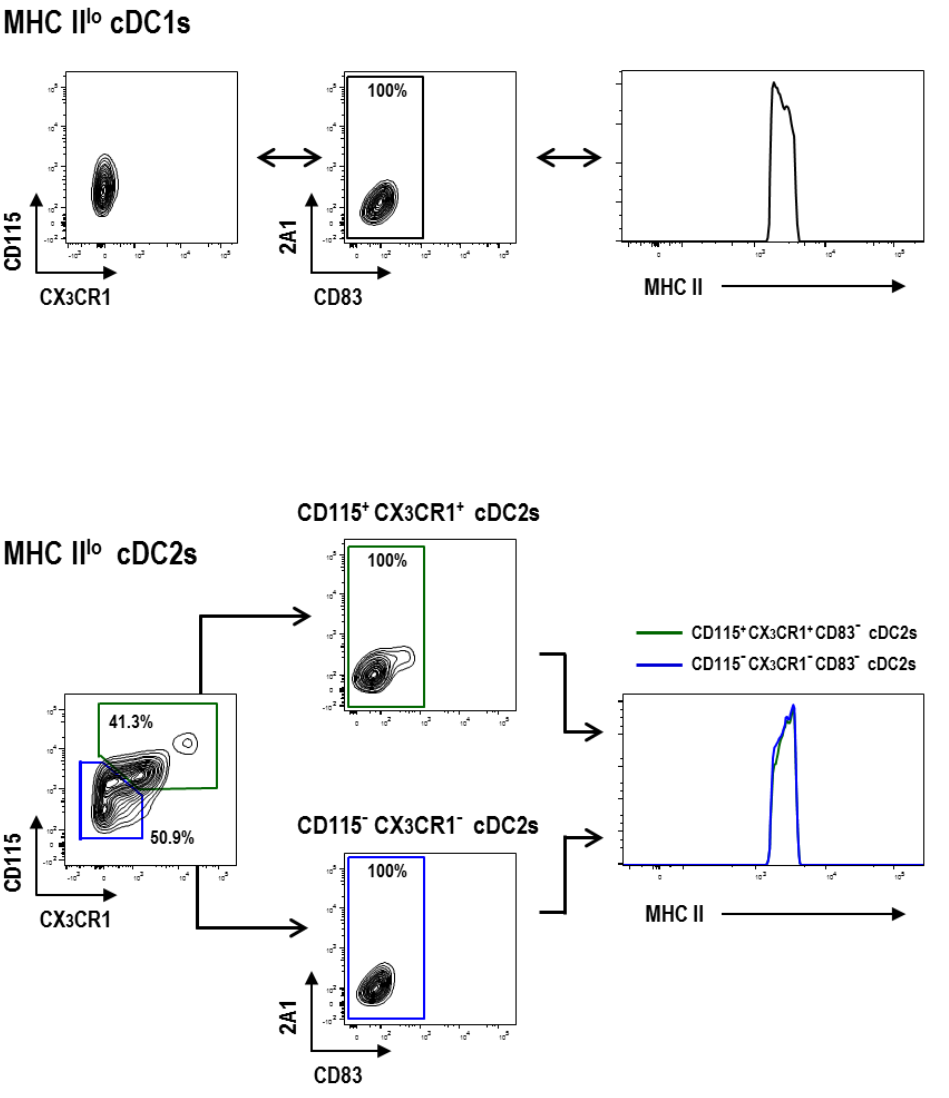


Figure 14. Heterogeneity of cDCs from BM culture with FLT3L. Representatives are MHC II^{mid} cDCs (A) or MHC II^{lo} cDCs (B) within CD11c^{+} cells in BM culture with FLT3L at day 9. Cells were harvested, stained and analyzed by flow cytometry.

4. Heterogeneity in morphology of MHC II^{hi} cell subsets from BM culture with FLT3L

Morphology is one of the criteria to discern different cell types of hematopoietic cells[1]. DCs have unique morphology with dendrites and form cell clusters on culture plate[1,47]. The three populations of MHC II^{hi} cells can be discriminated by their morphological characters. Cells from BM culture with FLT3L were harvested on day 8 and sorted into three subsets according to the gates as in Figure 11 (lower panels) using flow cytometry followed by the additional culture in FLT3L supplemented condition. Notably, both CD115$^-$ CX$_3$CR1$^-$CD83$^-$ cells and CD115$^-$CX$_3$CR1$^-$CD83$^+$ cells exhibited dendrites and clusters of non-adherent cells, albeit different in size of clusters. (Figure 15A and 15B). Whereas CD115$^+$CX$_3$CR1$^+$ cells showed slightly adherent morphology with larger in size compared to CD115$^-$CX$_3$CR1$^-$ cells, especially the large cytoplasm and had few dendrites or pseudopods, which is similar to BM derived macrophages[48] (Figure 15A and 15B). Besides, CD115$^-$CX$_3$CR1$^-$ CD83$^+$ cDC2s exhibited the typical electron microscopic features of DCs[44,49-51] that the irregularly shaped nuclei possessed a dense rim of heterochromatin and small nucleoli; the cytoplasm comprised many scattered mitochondria, short slips of rough endoplasmic reticulum, few electron-dense membrane-bound lysosomes but many electron-lucent vesicles including multivesicular bodies of varying size in the Golgi region (Figure 16), while CD115$^+$CX$_3$CR1$^+$ cells displayed more frequent electron-dense membrane-bound lysosomes (Figure 17). Thus, the three subsets of MHC II^{hi} cells are different cell types discriminated by their phenotype and morphology.

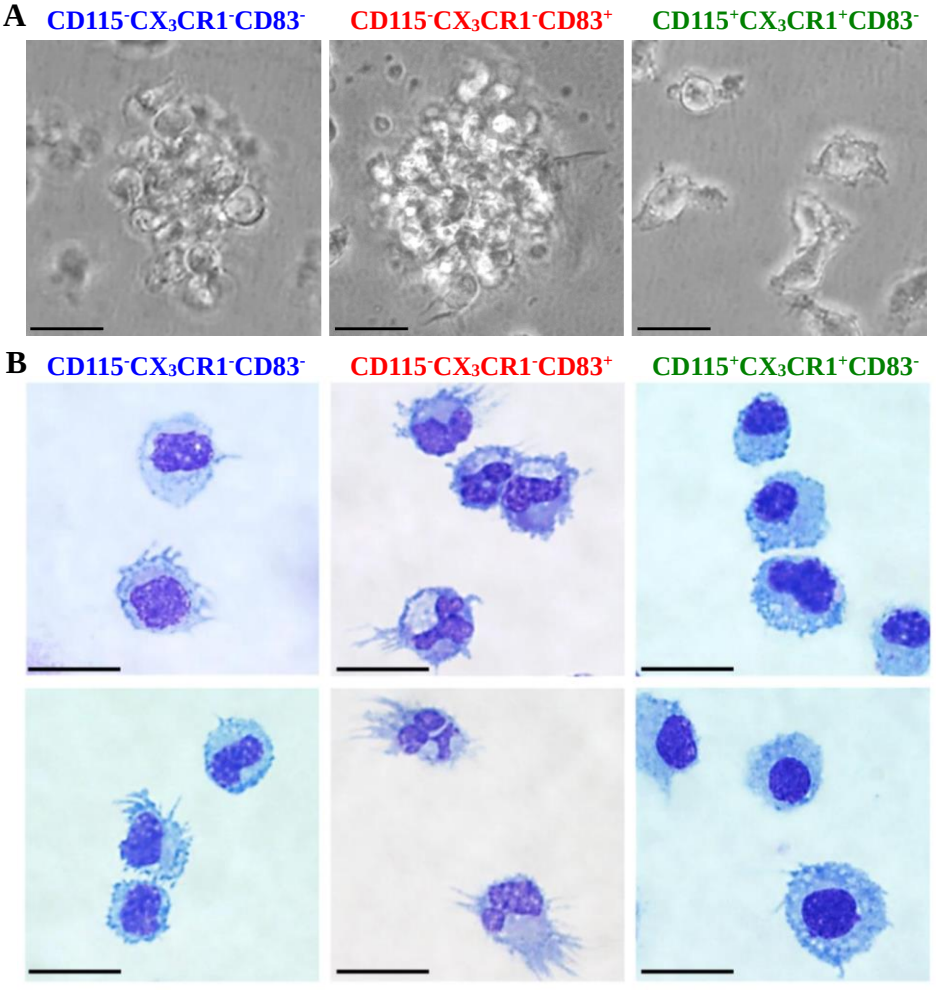


Figure 15. Morphology of MHC II^{hi} cell subsets from BM culture with FLT3L. (A) Morphological characteristics of MHC II^{hi} cell subsets from FLT3L culture. At culture day 8, MHC II^{hi} cells were sorted by flow cytometry according to the gates as in Figure 9 (lower panels). Isolated cells in each subset were cultured overnight before taking pictures. Scale bar, 20 μm. (B) Diff-Quik staining images of sorted MHC II^{hi} cell subsets. Cells were sorted as in Figure 9 (lower panels) and attached on slides by cytospin. Then, cells were stained with Diff-Quik staining solutions and images were obtained. Magnification, 600X; scale bar, 20 μm.

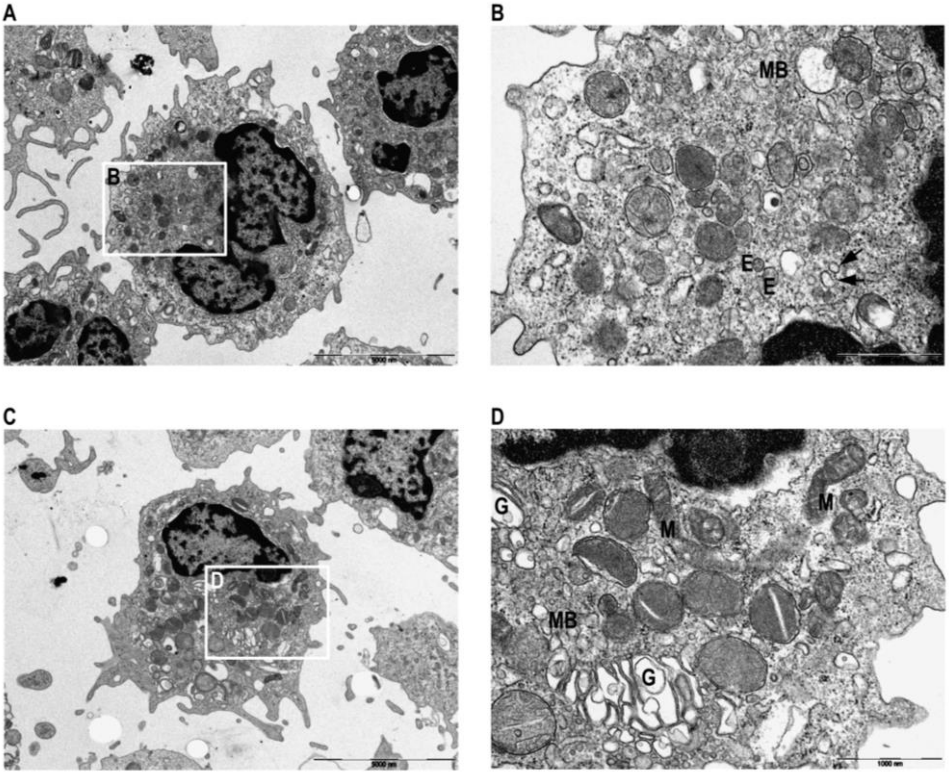


Figure 16. Electron micrographs of CD115^{-}CX$_3$CR1^{-}CD83^{+} cDC2s from FL-BM culture. MHCII^{hi}CD115^{-}CX$_3$CR1^{-}CD83^{+} cells isolated from FL-BM culture are stained for electron micrographic analysis. (A, C) CD115^{-}CX$_3$CR1^{-}CD83^{+} cDC2s exhibit dendrites or short protrusions in a lower magnification. (B, D) Close-ups of the perinuclear region show profiles of ER, mitochondria, and vacuoles. Multivesicular bodies are scattered through cytosol. Arrow, ER; G, Golgi; E, endosome; M, mitochondria; MB, multivesicular bodies. (A, C) Bar = 5000 nm; (B, D) Bar = 1000 nm.

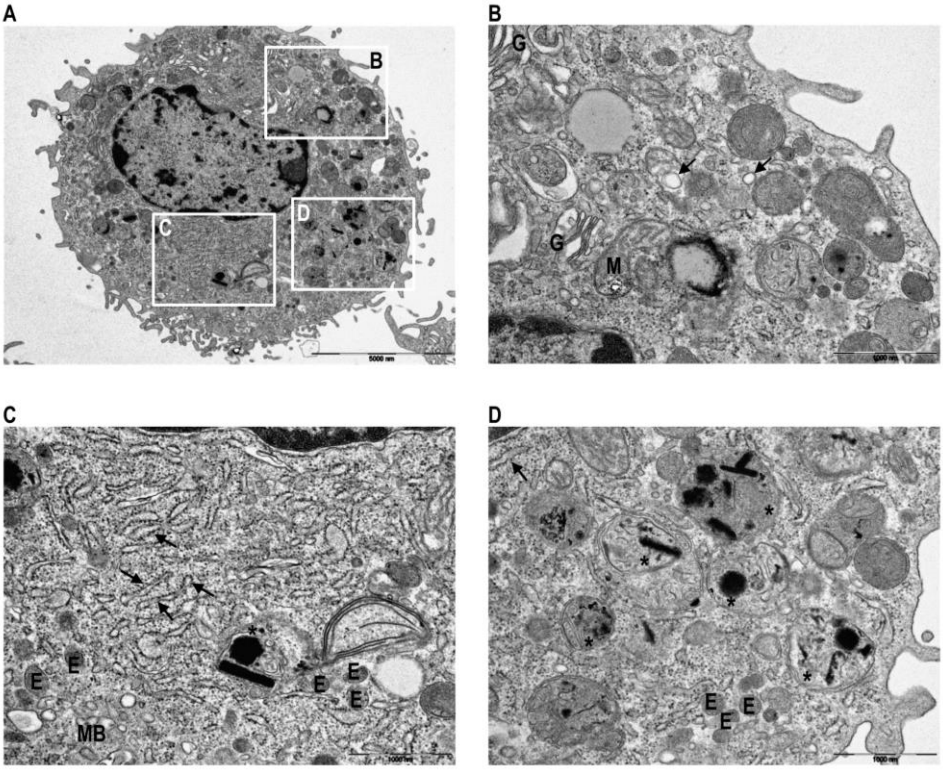


Figure 17. Electron micrographs of $CD115^{+}CX_3CR1^{+}$ cells from FL-BM culture. $MHCII^{hi}CD115^{+}CX_3CR1^{+}$ cells isolated from FL-BM culture are stained for electron microscopic analysis. (A) $CD115^{+}CX_3CR1^{+}$ cells exhibit larger cytosol compared to $CD115^{-}CX_3CR1^{-}CD83^{+}$ cDC2s (Figure 16). (B-D) Inner cell spaces are shown in a higher magnification. Abundant ER and Golgi apparatus are shown in perinuclear regions. Some lysosomal or phagocytic structures are in cytoplasmic region near plasma membrane. Arrow, ER; G, Golgi; E, endosome; M, mitochondria; black asterisk, lysosome or phagolysosome; MB, multivesicular bodies. (A) Bar = 5000 nm; (B-D) Bar = 1000 nm.

5. Heterogeneity of cDC2 subsets from FLT3L conditioned culture in phenotype and morphology

cDCs, commonly defined by the expression of CD11c and MHC II, comprise mainly two subsets, cDC1s and cDC2s[52]. This initial division reflects developmental and functional heterogeneity among DCs[12,53,54]. Especially cDC2s in various tissues and organs, specified by the expression of CD11b and CD172α/SIRPα, comprise a heterogeneous population of cells with differential surface expression of ESAM, Mgl2 (CD301b), etc[54,55]. These cDC2 subsets exhibit variable dependence on *IRF4* and Notch signaling[55,56] and appear to have distinct functional roles[57,58], suggesting further diversification within cDC2s. Recent efforts revealed the existence of distinct cDC2 lineages, their transcriptional identity and developmental origins[59]. Of note, it is important to describe the heterogeneity of cDC2s and their functions. At this point of view, the importance of studying cDC2 heterogeneity *in vitro* is also rising.

cDC2s in the culture of BM with FLT3L, previously known as one population, are newly described into three subtypes through their heterogeneous nature of phenotype and morphology as shown above; $CD115^{-}CX_3CR1^{-}CD83^{-}$, $CD115^{-}CX_3CR1^{-}CD83^{+}$, $CD115^{+}CX_3CR1^{+}CD83^{-}$ cells (Figure 13, 14A, 15-17).

6. Gene expression profiles reveal heterogeneity of MHC II^{hi} cell subsets in BM culture with FLT3L

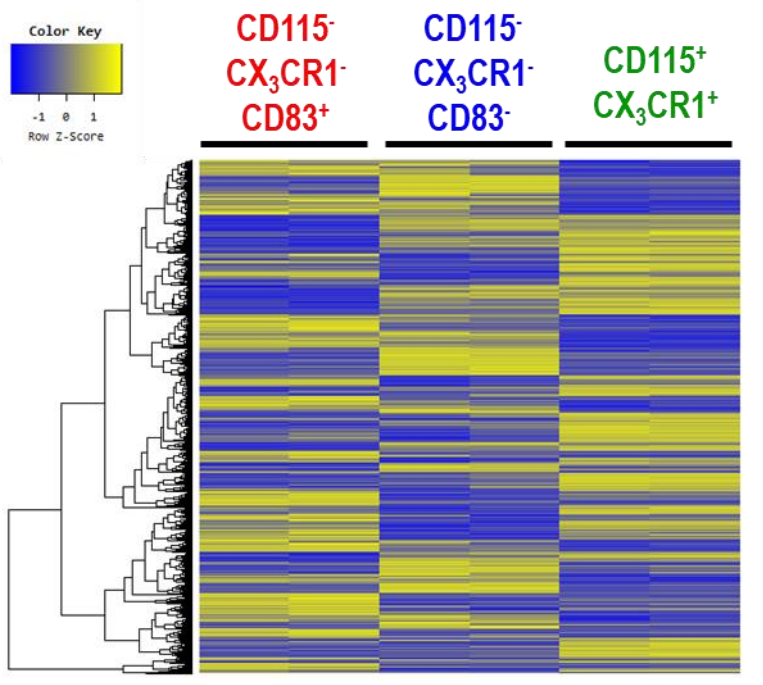
At a transcriptional level, subsets in MHC II^{hi} cells have distinct characteristics of transcriptional profiles (Figure 18A). The global gene expression pattern of three MHC II^{hi} cell subsets shows different property of each subset. cDC1s and cDC2s from BM culture with FLT3L can be categorized with the surface expression of CD83 molecules. In other words, CD83^{+} cDC1s and CD83^{+} cDC2s are similar in gene expression and CD83^{-} cDC1s and CD83^{-} cDC2s have analogous translational characters by PCA assay (Figure 18B). More interestingly, CD115^{+}CX$_3$CR1^{+} cells from FLT3L condition are far from the other cells from FLT3L condition. Rather, they exhibit similar characteristics with GM-Macs in gene expression by PCA assay (Figure 18B).

To be more specific, CD115^{-}CX$_3$CR1^{-}CD83^{+} cells express activated DC genes such as *CCR7* and *CCL22* (Figure 19A). CD115^{-}CX$_3$CR1^{-}CD83^{-} cells express cDC core genes including *Kmo, Flt3* and DC associated genes such as *CD209a* (encoding DC-SIGN) (Figure 19B and 19C). In contrast, CD115^{+}CX$_3$CR1^{+} cells express macrophage signature genes including *Lyz2, Apoe, Csf1r, Clec4e, Mmp12* (Figure 19A).

To further understand the nature of MHC II^{hi} cell subsets, expression of genes related to cDCs[15,60] were analyzed and shown by heatmaps and hierarchical clustering (Figure 20). Genes related to cDCs are upregulated in both CD115^{-}CX$_3$CR1^{-}CD83^{-} and CD115^{-}CX$_3$CR1^{-}CD83^{+} cells, whereas in CD115^{+}CX$_3$CR1^{+} cells, most of cDC signature genes were downregulated compared to the other two MHC II^{hi} cell subsets except *Anpep,* which encodes the aminopeptidase CD13 involved in the metabolism of regulatory peptides and the cleavage of peptides bound to MHC II molecules of APCs[61] (Figure 20A and 20B). In contrast, genes related to macrophages showed substantial upregulation in CD115^{+}CX$_3$CR1^{+} cells relative to their expression in CD115^{-}CX$_3$CR1^{-}CD83^{-} and CD115^{-}CX$_3$CR1^{-}CD83^{+} cells except several genes including

1810011H11Rik which is also known as *tmem273* and its function unknown; *Dok3* which plays a critical role in TLR3 signaling and IFN-β production[62]; and *Pecr* which encodes peroxisomal trans-2-enoyl-CoA reductase participated in chain elongation of fatty acids[63] (Figure 21A). However, in case of expression of monocyte-associated genes, some are relatively enriched in $CD115^{+}CX_3CR1^{+}$ cells and the others are upregulated in $CD115^{-}CX_3CR1^{-}CD83^{-}$ and $CD115^{-}CX_3CR1^{-}CD83^{+}$ cells (Figure 21B). Plus, the lists of highly expressed genes in each subset show their distinct characteristics in a transcriptional level (Figure 22-24). In conclusion, the gene expression profiles reveal heterogeneity of MHC II^{hi} cell subsets in BM culture with FLT3L.

A



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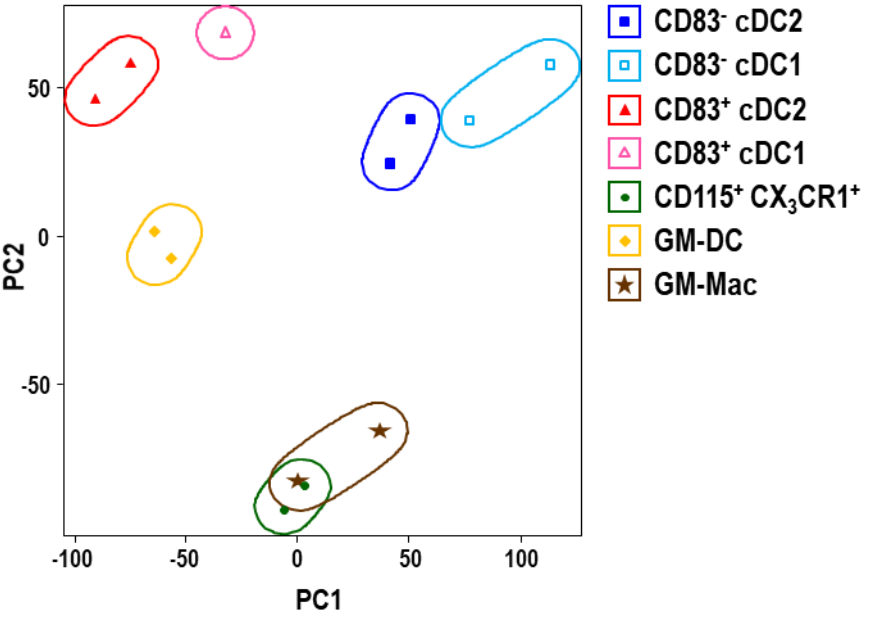
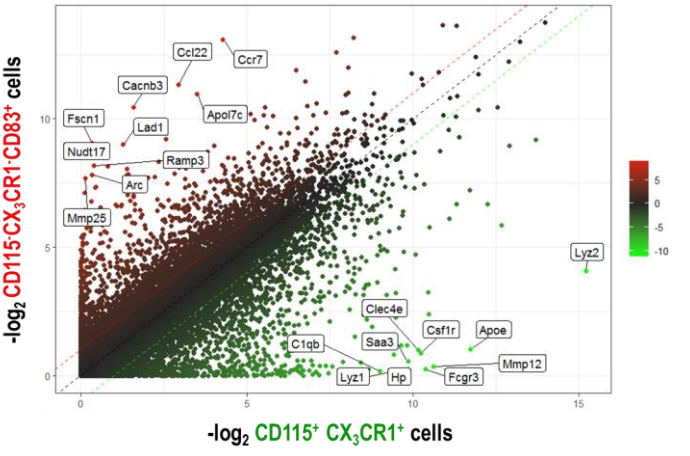


Figure 18. Transcriptional profiles reveal heterogeneity of MHC II^{hi} cell subsets in BM culture with FLT3L. At culture day 8, cells from BM culture with FLT3L or GM-CSF were sorted by FACS sorting according to the gates as in Figure 9 (for FLT3L culture) or Figure 4 (for GM-CSF culture). For each population, total RNA was extracted from at least 1×10^5 cells and RNA sequencing was performed. (A) Heatmap displays the one-way hierarchical clustering using z score for normalized value of randomly selected genes that are differentially expressed in MHC II^{hi} cell subsets in BM culture with FLT3L. (B) Principal component analysis of each subset was performed and shown.

A



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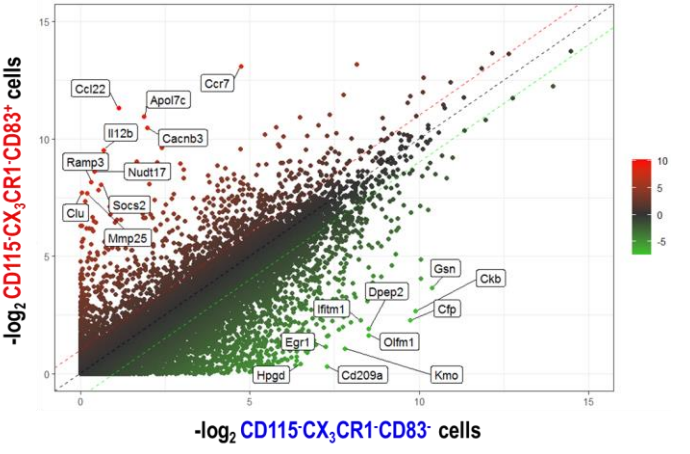

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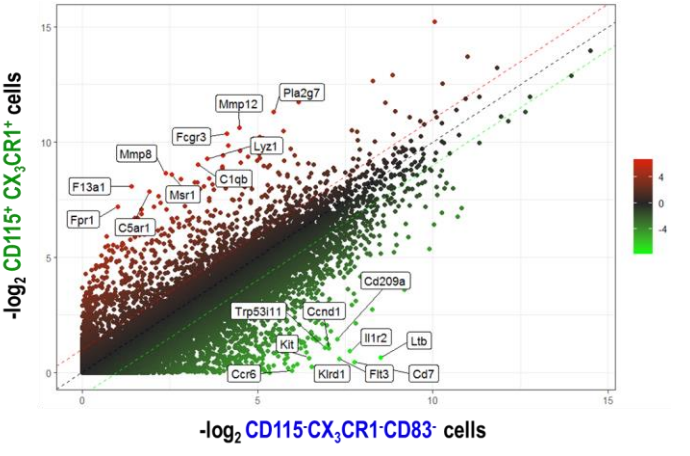


Figure 19. MHC II^{hi} cell subsets showed distinct characteristics in gene expression. (A) Scatter plot highlighting genes differentially expressed in CD115^{-}CX$_3$CR1^{-}CD83^{+} cells over CD115^{+}CX$_3$CR1^{+} cells. (B) Scatter plot highlighting genes differentially expressed in CD115^{-}CX$_3$CR1^{-}CD83^{+} cells over CD115^{-}CX$_3$CR1^{-}CD83^{-} cells. (C) Scatter plot highlighting genes differentially expressed in CD115^{+}CX$_3$CR1^{+} cells over CD115^{-}CX$_3$CR1^{-}CD83^{-} cells. Unexpressed genes were excluded when analyzed. Dashed lines mean fold change = 2.

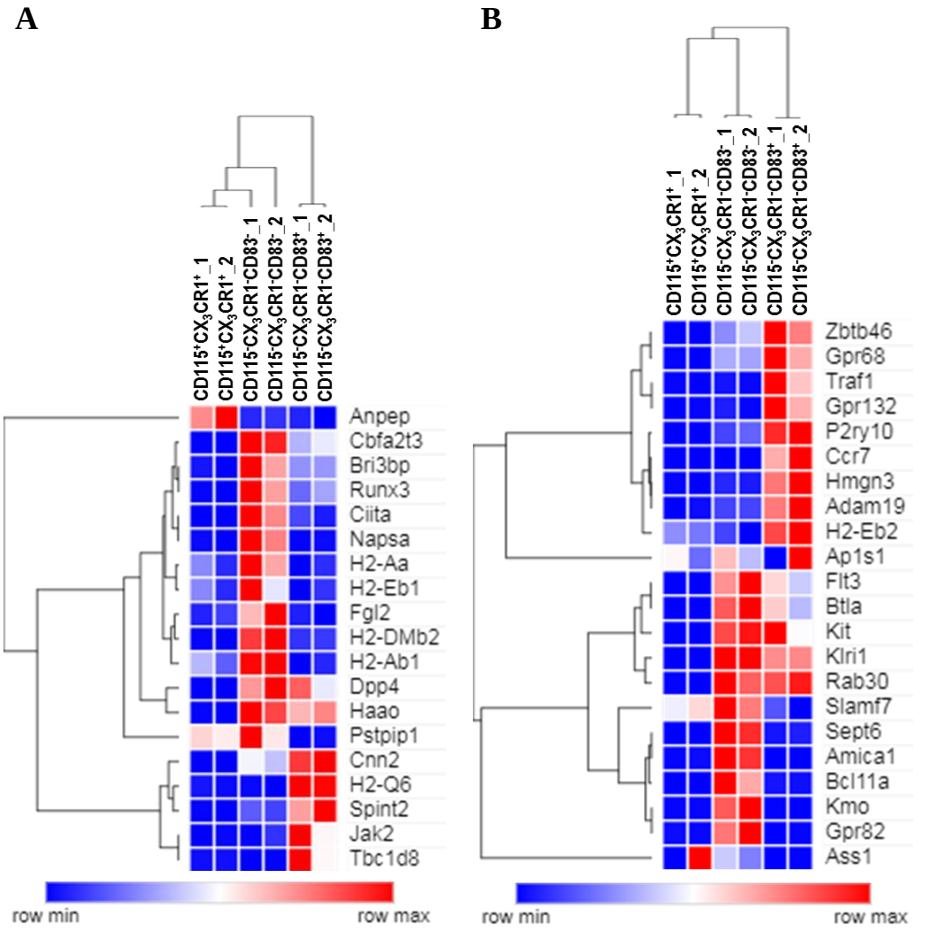


Figure 20. Differentially expressed genes related to cDCs in MHC II^{hi} cell subsets in BM culture with FLT3L. Heatmaps showing hierarchical clustering by one minus Pearson correlations of MHC II^{hi} cell subsets using DC signature genes (A) and core cDC signature genes (B)[15,60].

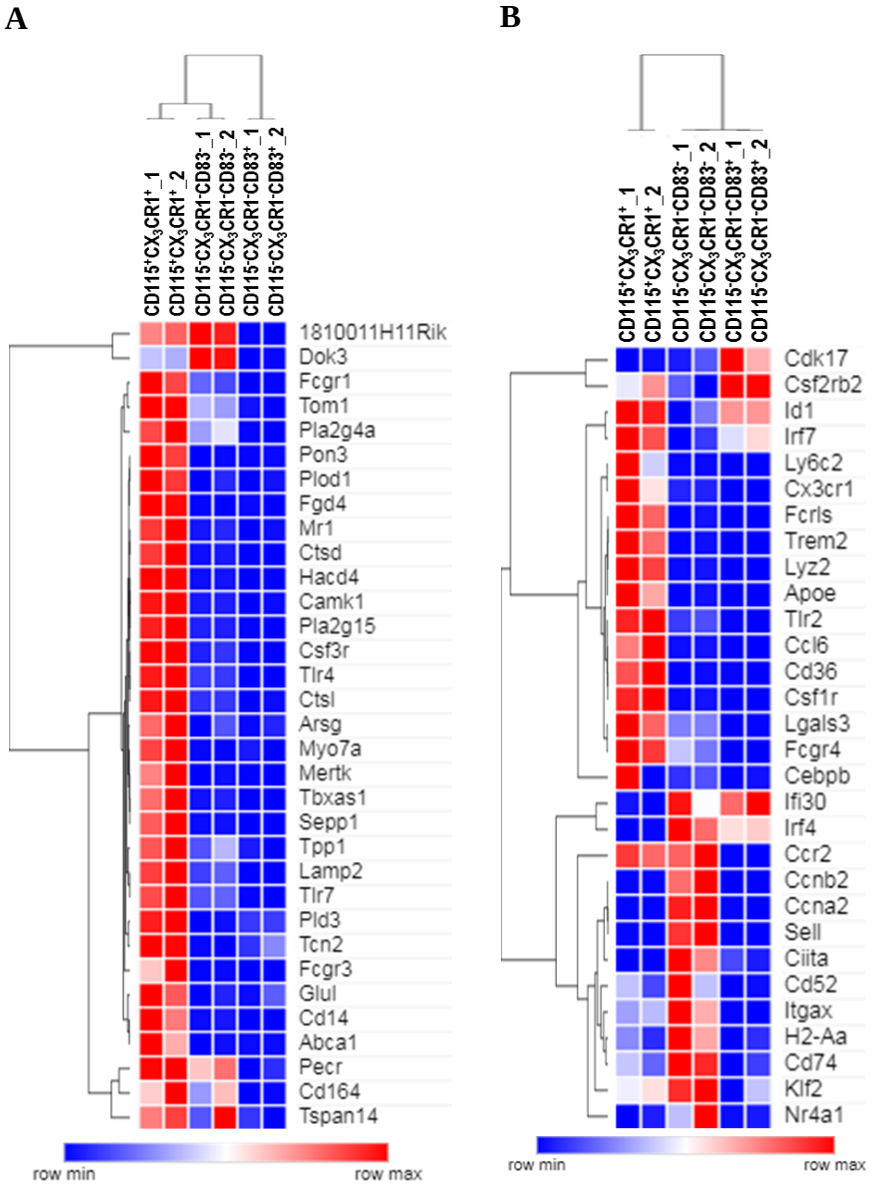


Figure 21. Hierarchical clustering and differentially expressed genes related to monocytes and tissue macrophages in MHC II^{hi} cell subsets in BM culture with FLT3L. Shown are heatmap and hierarchical clustering by one minus Pearson correlations of MHC II^{hi} cell subsets using tissue macrophage signature genes (A) and monocytes signature genes (B)[64,65].

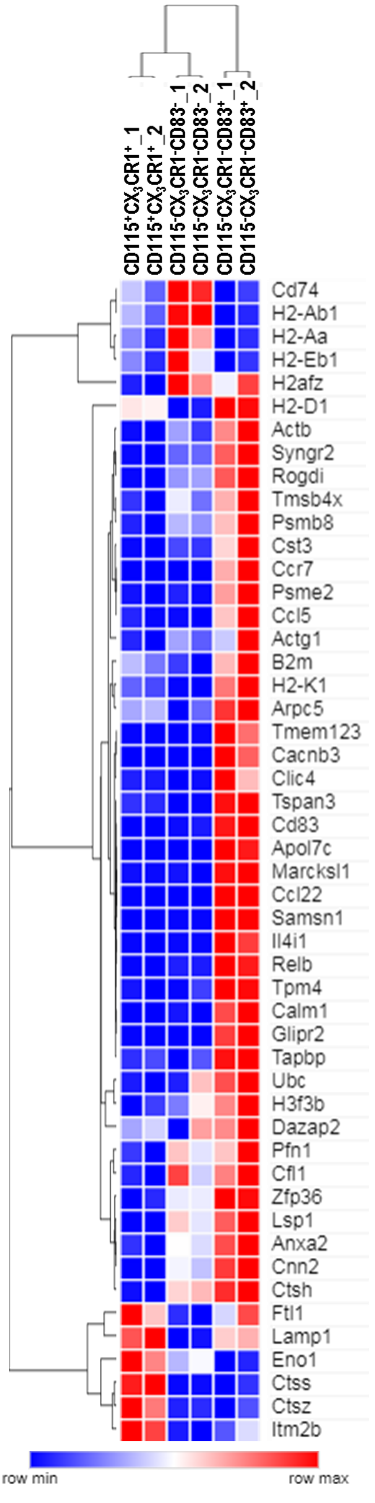


Figure 22. Heatmap of upregulated genes in MHC II^{hi} CD115^{-} CX$_3$CR1^{-} CD83^{+} cells in BM culture with FLT3L. Shown are the heatmap and hierarchical clustering of the top 50 genes that were highly expressed in MHC II^{hi} CD115^{-} CX$_3$CR1^{-} CD83^{+} cells.

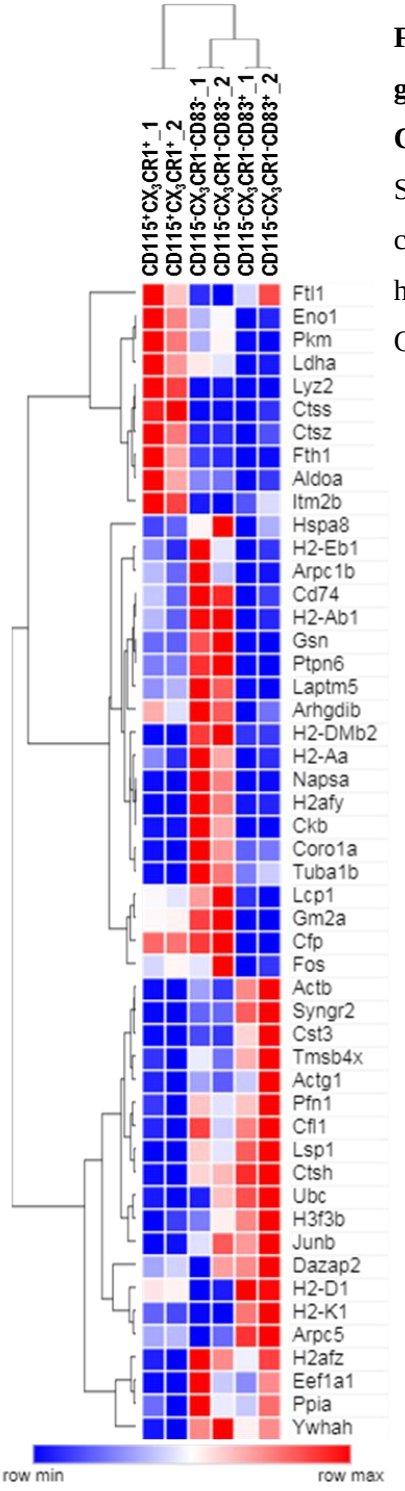


Figure 23. Heatmap of upregulated genes in MHC II^{hi} CD115^{-} CX$_3$CR1^{-} CD83^{-} cells in BM culture with FLT3L. Shown are the heatmap and hierarchical clustering of the top 50 genes that were highly expressed in MHC II^{hi} CD115^{-} CX$_3$CR1^{-} CD83^{-} cells.

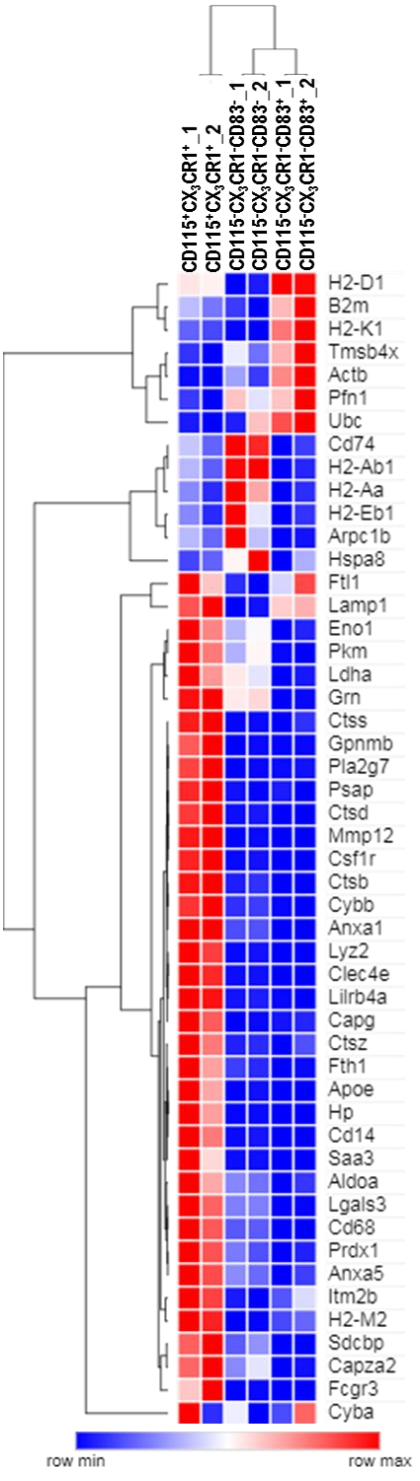


Figure 24. Heatmap of upregulated genes in MHC II^{hi}CD115^{+}CX$_3$CR1^{+} cells in BM culture with FLT3L. Shown are heatmap and hierarchical clustering of the top 50 genes that were highly expressed in MHC II^{hi} CD115^{+}CX$_3$CR1^{+} cells.

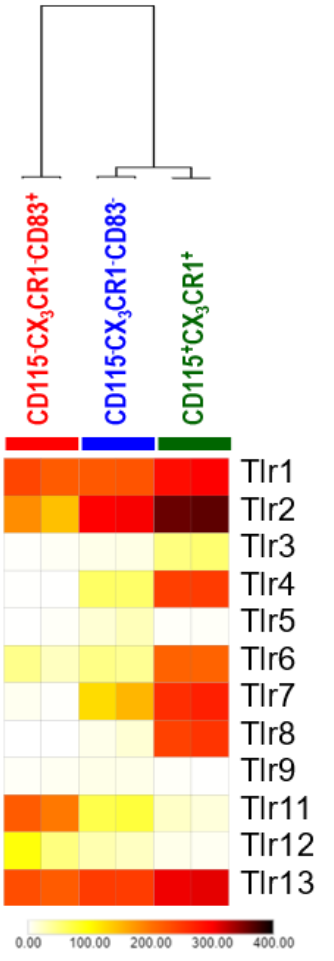


Figure 25. Gene expression of Toll-like receptors in BM cultures. Heatmap shows hierarchical clustering on normalized FPKM read counts of TLR gene expression in cDC2-gated cell subsets from FL-BM culture.

7. $CD115^{+}CX_3CR1^{+}$ cells in the culture of BM with FLT3L exhibit stronger endocytic ability than $CD115^{-}CX_3CR1^{-}$ cells

DCs can also take up various antigens to act as sentinels[41,42,66]. Immature DCs mainly take up antigens whereas mature DCs engulf smaller amount of antigens compared to immature DCs and present them to the other cells as professional APCs. To check the endocytic and phagocytic ability of MHC II ^{hi} cell subsets, either soluble (FITC-labeled ovalbumin, FITC-OVA) or particulate (Yellow-green microsphere, YGM) forms of fluorescently labeled antigens were treated on culture. An hour after the incubation, endocytosis of FITC-OVA or phagocytosis of YGM by MHC II ^{hi} cell subsets were assessed using flow cytometric approach. When incubated with soluble protein antigens (FITC-OVA), $CD115^{+}CX_3CR1^{+}$ cells take up antigens more efficiently than $CD115^{-}CX_3CR1^{-}$ cells (Figure 26A). Similar results were observed with particulate antigens (YGM) but fluorescently labeled beads were phagocytosed slightly more efficient way by $CD115^{-}CX_3CR1^{-}$ cells compared to soluble antigens (Figure 26B). Therefore, in the culture of BM with FLT3L, $CD115^{+}CX_3CR1^{+}$ cells showed to have greater antigen-uptake capacity like macrophages or immature DCs whereas $CD115^{-}CX_3CR1^{-}$ cells have poorer endocytic and phagocytic activities, which is the typical feature of DCs.

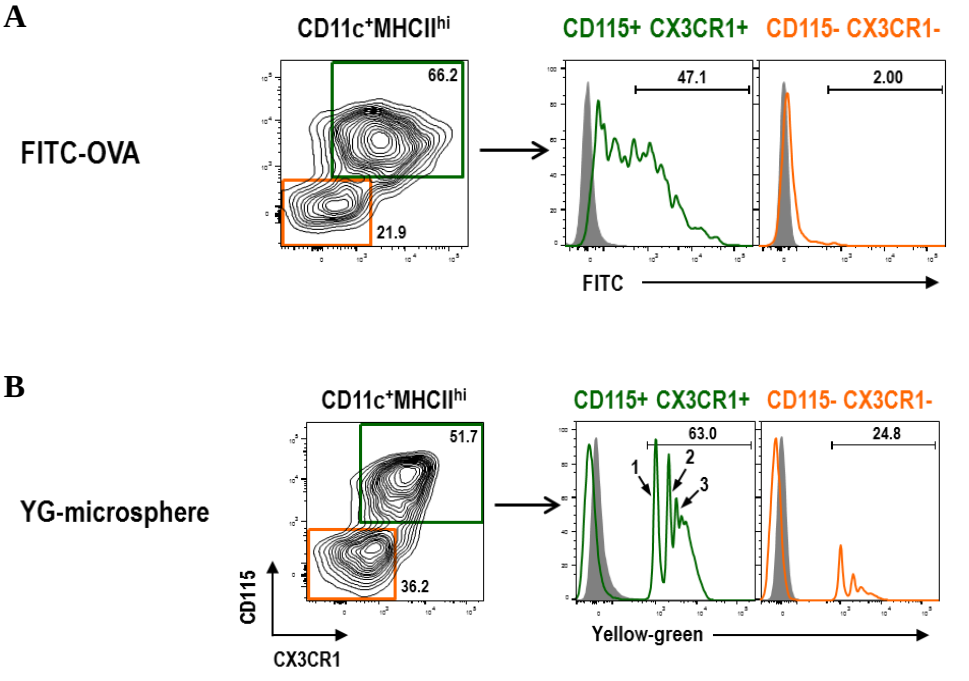
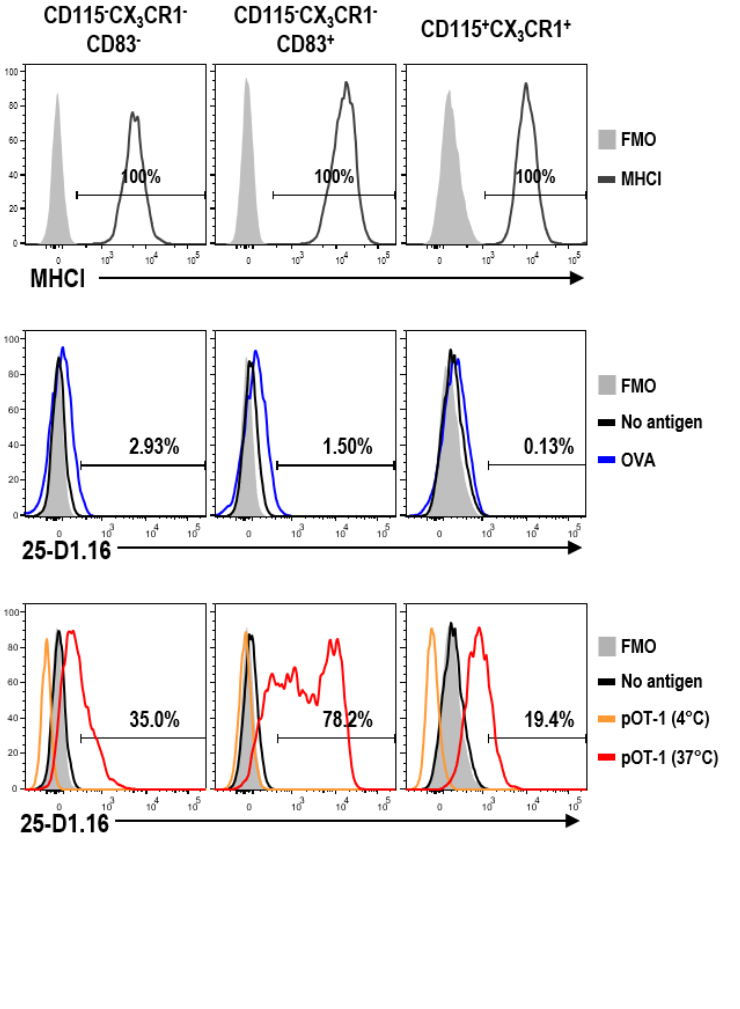


Figure 26. Phagocytosis and pinocytosis of MHC II^{hi} cells in the culture of BM with FLT3L. (A) On FLT3L culture day 9, cells were incubated with 100 μg/ml of FITC-conjugated OVA protein for 30 minutes to test pinocytosis of MHC II^{hi} cells. Cells were harvested after incubation, washed, stained and analyzed. Gray histograms are each subset from non-treated control. (B) On FLT3L culture day 9, cells were incubated with 0.000675% YGM for 1 hour to check phagocytic ability of each subset. Cells were harvested after incubation, washed, stained and analyzed. Gray histograms are non-treated control of each subset.

8. $CD115^{-}CX_3CR1^{-}CD83^{+}$ cells in FLT3L culture stimulate $CD4^{+}$ T cells in an effective manner than $CD115^{+}CX_3CR1^{+}$ cells *in vitro*

DCs are efficient in processing and presenting antigens to T cells while macrophages can not[46,66,67]. Because $CD115^{-}CX_3CR1^{-}$ cells exhibit quite incompetent capacity in taking up antigens, antigen presenting ability of each subset was examined. To evaluate the ability to proliferate naïve T cells of cDC2 subsets as professional APCs, each subset was pulsed with appropriate concentration of model antigen, OVA, followed by co-culture with OT-I $CD8^{+}$ or OT-II $CD4^{+}$ T cells which have transgenic TCR specific to OVA peptide and protein in various ratios. In the co-cultures of graded doses of each $MHCII^{hi}$ subpopulation with naive OVA-specific T cells, $CD115^{-}CX_3CR1^{-}CD83^{+}$ cells strongly inducing the proliferation of $CD8^{+}$ OT-I T cells (Figure 28A). In case of OT-II $CD4^{+}$ T cell proliferation, $CD115^{-}CX_3CR1^{-}$ cells showed greater ability to expand T cells than $CD115^{+}CX_3CR1^{+}$ cells regardless of the expression of CD83 (Figure 28B). Overall, $CD115^{+}CX_3CR1^{+}$ cells are poor at presenting antigens to both $CD4^{+}$ T and $CD8^{+}$ T cells despite of their MHC II expression at a high level.

Although the $MHCII^{hi}CD83^{-}CD115^{+}CX_3CR1^{+}$ cDC2-gated cells expressed high levels of MHC class I molecules (MHCI) on surface (Figure 27, upper panels and graph) and took up antigens efficiently (Figures 26A and 26B), the $CD115^{+}CX_3CR1^{+}$ cells were poor at processing OVA antigen and presenting OT-1 peptide epitope on MHCI (Figure 27, middle panels, lower panels and their graphs). DCs are considered as professional APCs with low endocytic and phagocytic capacities, as compared to other mononuclear phagocytes (7, 26, 42–44). Therefore, $MHCII^{hi}$ cDC2-gated cells in the FL-BM culture likely consist of $CD115^{-}CX_3CR1^{-}CD83^{-}$ immature cDC2s, $CD115^{-}CX_3CR1^{-}CD83^{+}$ mature cDC2s, and $CD115^{+}CX_3CR1^{+}$ $CD83^{-}$ non-DCs.



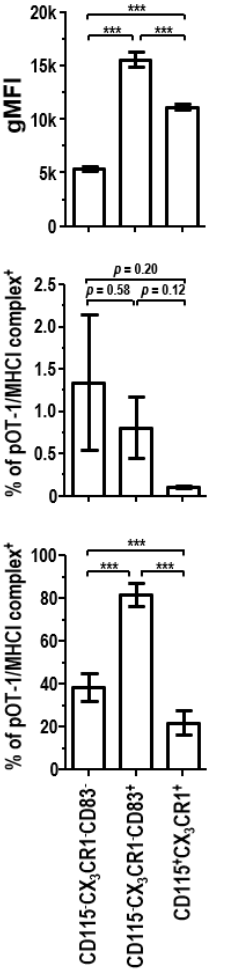
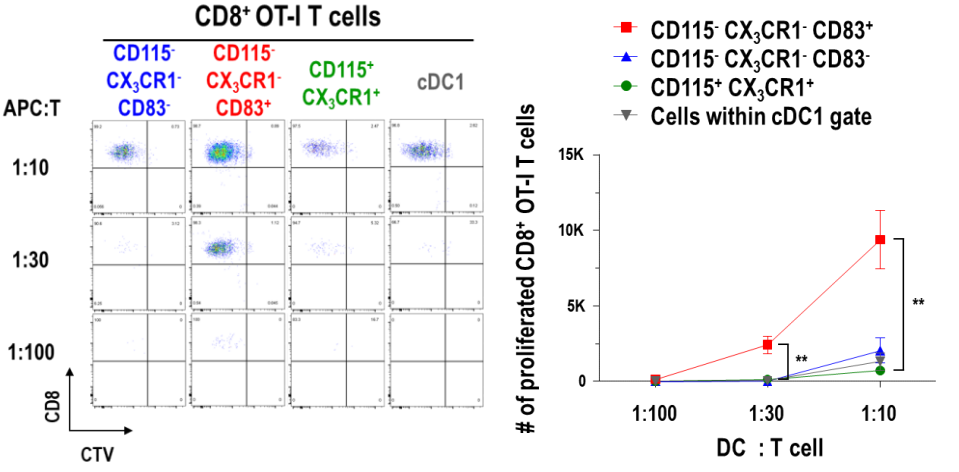


Figure 27. Expression of MHCI and antigen presentation by MHCI on MHCII^{hi} cDC2-gated cell subsets in FL-BM culture. At day 9 of FL-BM culture, MHCII^{hi} cell subsets are analyzed to show their expression of surface MHCI with gMFI value (upper panels and graph). In parallel, FL-BM cultures are treated with 100 μg/ml of endotoxin-free OVA protein (middle panels and graph) or 1 μg/ml of OT-1 peptide (pOT-1, SIINFEKL) (lower panels and graph) for 18 hours before being analyzed for the surface levels of pOT-1/MHCI complex (H-2K^{b} bound to pOT-1) by staining with mAb 25-D1.16. For the 4°C controls, FL-BM culture is incubated with pOT1 on ice for 1 hour. Representatives are from 3 independent experiments and graphs are shown in mean ± SEM (n = 3).

A OT-I day 3



B OT-II day 5

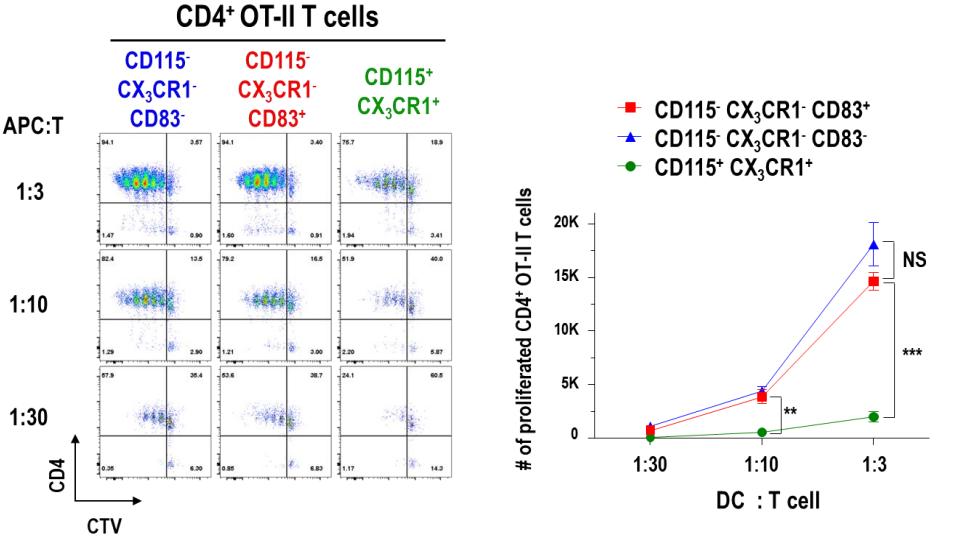


Figure 28. Proliferation of antigen-specific T cells. (A) APCs were pulsed with 100μg/ml ovalbumin for 30 minutes before sorting. 2.5×10^4 splenic $CD8^+$ T cells isolated from OT-I mice were co-cultured with indicated ratio of APCs for 3 days. (B) APCs were pulsed with 100 μg/ml ovalbumin for 3 hours before sorting. 2.5×10^4 splenic $CD4^+$ T cells isolated from OT-II mice were incubated with indicated ratio of APCs for 4 days. Proliferation of CTV-labeled naive splenic $CD8^+$ OT-1 (A) and $CD4^+$ OT-2 (B) T cells are assessed following co-culture with graded doses of the respective APCs. Representative data are shown from more than 3 independent experiments. Error bars indicate mean ± SEM across multiplicate sample. NS, not significant. *$p\leq0.05$; **$p\leq0.01$; ***$p\leq0.001$; ****$p\leq0.0001$.

9. Stimulation of TLR activates MHC II^{hi} cells in the BM culture with FLT3L

Toll-like receptors (TLRs) sense various ligands derived from microbial components such as carbohydrates, nucleic acids and lipoproteins[21,22,31,68]. Phagocytes including dendritic cells have TLRs on cell surface or inner cell spaces to detect foreign danger signals and activate defense mechanisms. Ligation of TLR agonist is commonly employed to induce maturation of DCs. To investigate whether MHC II^{hi} cells from FLT3L culture were activated by TLR signaling, graded doses of lipopolysaccharide (LPS) were treated on BM culture for 18 hours. 1 pg/ml of LPS had no effect on cells in BM culture with FLT3L (Figure 29). When more than 10 ng/ml of LPS was treated, $CD83^+$ $2A1^+$ cells were augmented among MHC $II^-CD115^-CX_3CR1^-$ cells (Figure 29). Interestingly, under LPS stimuli, $CD115^+CX_3CR1^+$ cells were decreased dramatically and $CD115^-CX_3CR1^-CD83^+2A1^+$ cDC2s were increased in number (Figure 29 and 30).

To clarify this phenomenon, MHC $II^{hi}CD115^+CX_3CR1^+$ cells were purified by FACS sorting (Figure 31A), were cultured again for 3 days to stabilize cells (Figure 31B), and then LPS were added to stimulate cells (Figure 32A and 32B). About 20% of cells were activated spontaneously from $CD115^+CX_3CR1^+$ to $CD115^-CX_3CR1^-$ phenotype without the additional stimulus (Figure 32A, control). After 18 hours of LPS incubation, half of $CD115^+CX_3CR1^+$ cells became $CD115^-CX_3CR1^-$ cells which possess properties of activated DCs (Figure 32A), although the decrease was not that dramatic as in the cells among $CD45.1^+$ filler cells (Figure 32B).

Next, to test whether $CD115^+CX_3CR1^+$ cells gain the ability to proliferate T cells when stimulated, OVA-specific T cell proliferation was conducted with LPS. When given LPS, $CD115^-CX_3CR1^-$ cells displayed greater capability to proliferate both $CD8^+$ OT-I and $CD4^+$ OT-II T cells. In contrast, the stimulatory ability was not prominently changed by stimulation of the cells with LPS, which

commonly used to induce DC maturation (Figure 33).

When TLR9 was ligated by TLR agonists, CpG and polyI:C, $CD115^+$ cells were evidently disappeared, albeit the expression of CX_3CR1 remained. CpG treatment increased $CD115^-CX_3CR1^-CD83^+2A1^+$ cells in a moderate manner whereas polyI:C markedly amplified $CD115^-CX_3CR1^-CD83^+2A1^+$ cells and reduced $CD115^-CX_3CR1^-CD83^-$ cells. (Figure 30).

Overall, MHC $II^{hi}CD115^+CX_3CR1^+$ cells have potentials to differentiate into $MHCII^{hi}CD115^-CX_3CR1^-CD83^+$ DCs in the culture of BM with FLT3L after LPS treatment in a dose dependent manner. Therefore, $CD115^+CX_3CR1^+$ cells are the immediate precursor cells for DCs in the culture of BM with FLT3L.

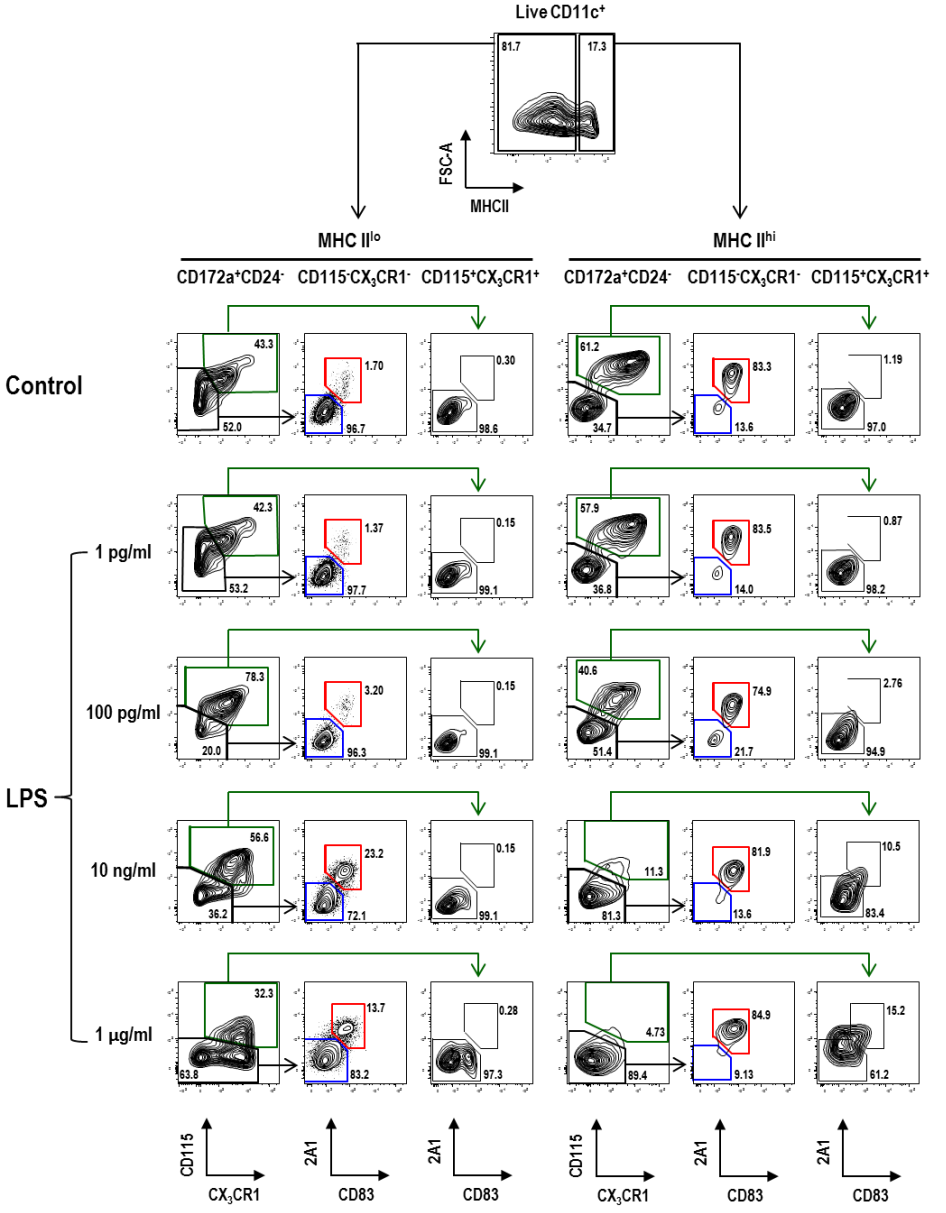


Figure 29. LPS treatment on BM culture with FLT3L. Graded doses of LPS were treated on BM culture with FLT3L at culture day 8; 1 pg/ml, 100 pg/ml, 10 ng/ml, 1 μg/ml. After 18 hours of incubation, cells were harvested and analyzed using flow cytometry. $CD11c^+$ cells were divided into 2 populations by expression level of MHC II molecules.

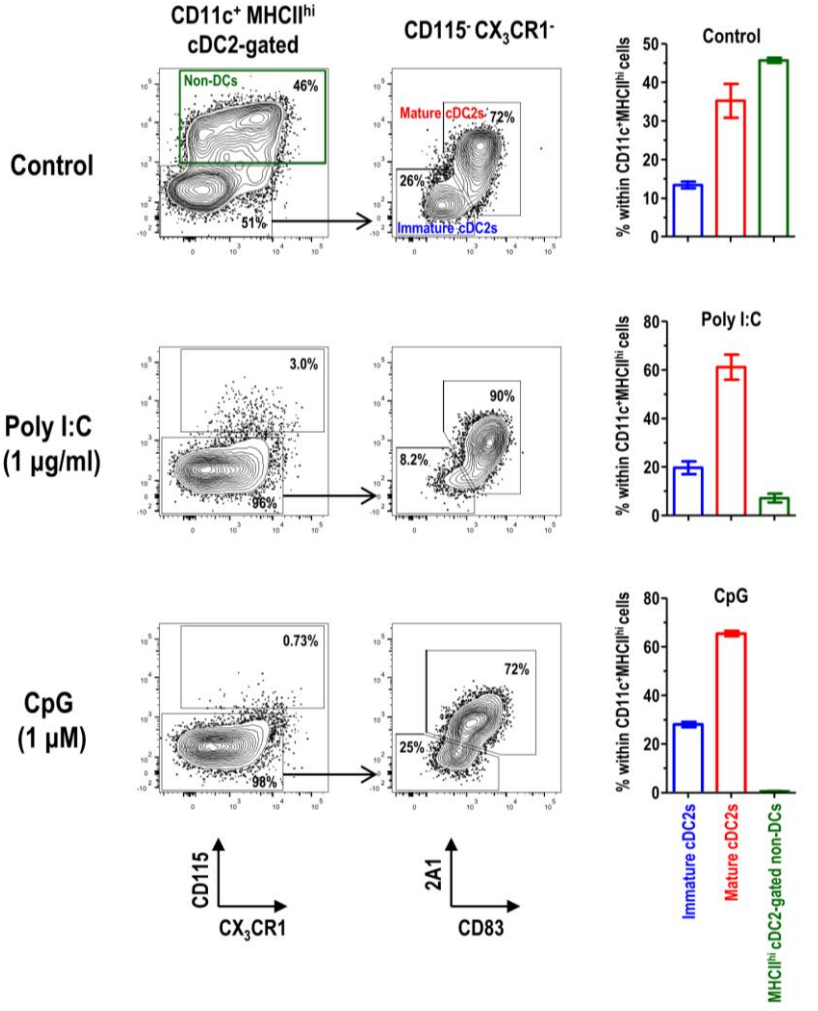


Figure 30. MHCII^{hi} cDC2-gated non-DCs diminish by treatment with TLR agonists. FL-BM culture is treated with poly I:C (1μg/ml) or CpG (1 μM) at culture day 8. After 18 hours of treatment, cells are harvested, washed, and stained with fluorescently labeled antibodies for flow cytometric analysis. CD11c^{+}MHCII^{hi} cDC2-gated cells are shown with two different gating strategies (A and B). Representatives shown are from 4 independent experiments. Graphs are shown in mean ± SEM (n = 4).

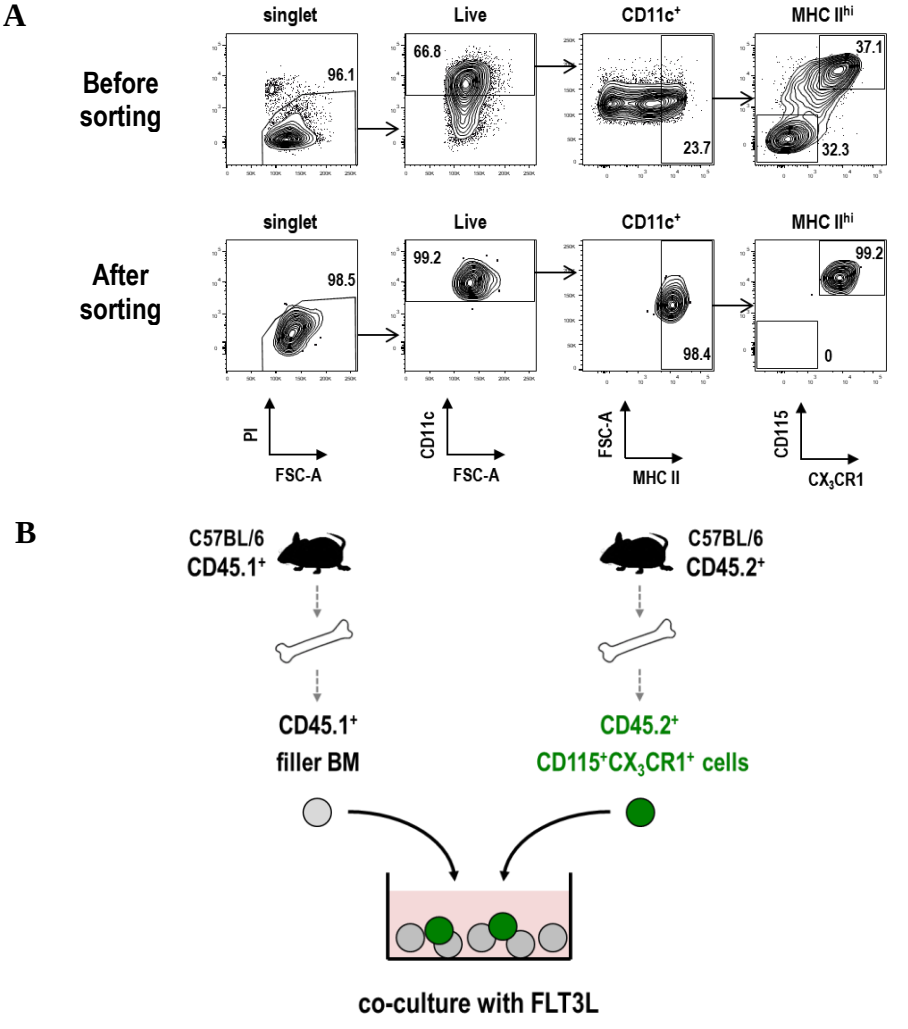


Figure 31. Experimental and sorting scheme of MHC II^{hi}CD115^{+}CX$_3$CR1^{+} cells from BM culture with FLT3L. (A) Shown are the gating strategy for CD11c^{+}MHC II^{hi}CD115^{+}CX$_3$CR1^{+} cells (upper panels, before sorting) and sorting purity of CD11c^{+}MHC II^{hi}CD115^{+}CX$_3$CR1^{+} cells (lower panels, after sorting) from BM culture with FLT3L at culture day 8. About 99% purity of CD11c^{+}MHC II^{hi} CD115^{+}CX$_3$CR1^{+} cells were obtained by FACS sorting. (B) Experimental scheme to evaluate the developmental potential of MHC II^{hi} CD115^{+}CX$_3$CR1^{+} cells.

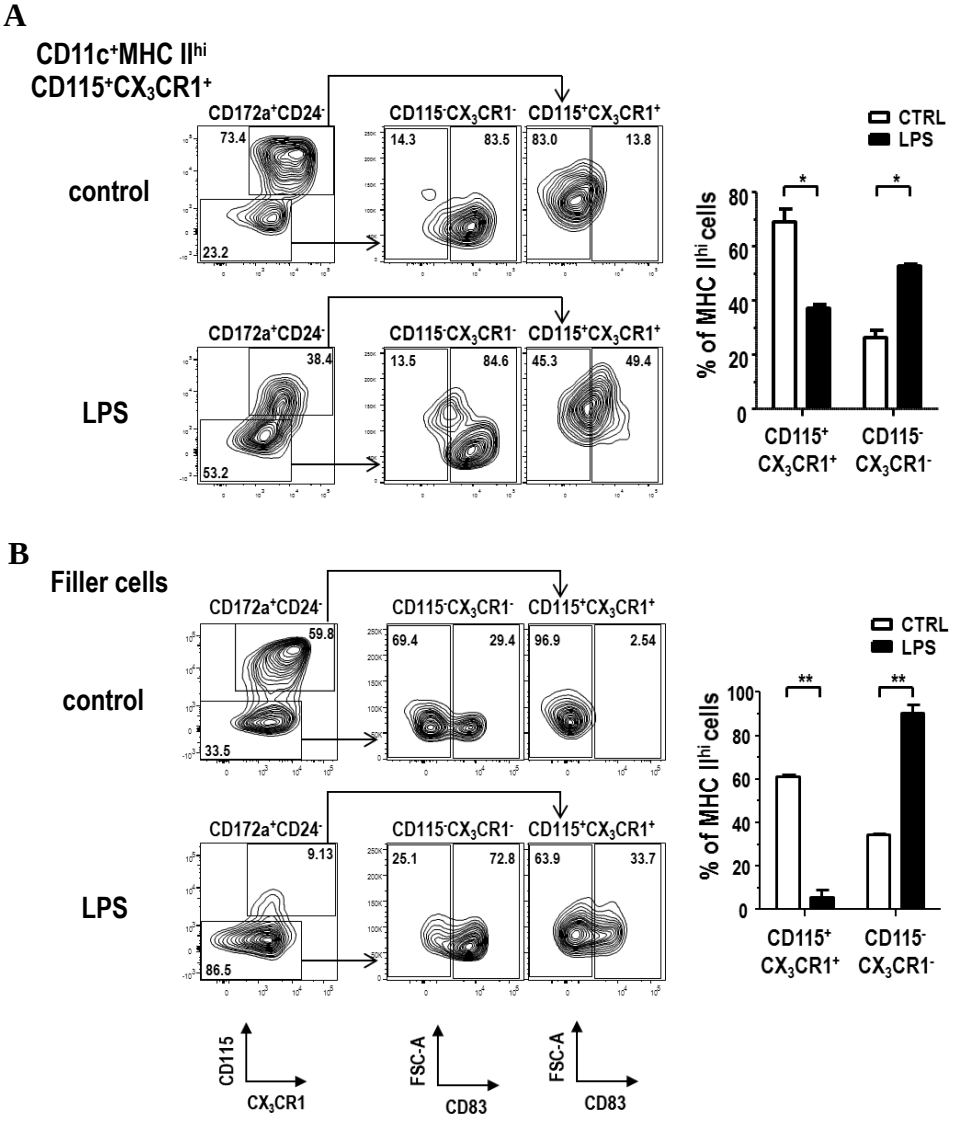


Figure 32. LPS treatment on MHC II^{hi}CD115^{+}CX$_3$CR1^{+} cells from BM culture with FLT3L. 7×10^4 of sorted CD11c^{+}MHC II^{hi}CD115^{+}CX$_3$CR1^{+} cells were cultured per well with 2×10^6 of freshly isolated BM cells from CD45.1^{+} mice at 24-well culture plates in FLT3L conditioned media. After three additional days of culture, 1 μg/ml of LPS were treated on culture for 18 hours. At culture day 4, cells were harvested, washed and analyzed by flow cytometry. Controls mean LPS untreated cells. (A, left) CD45.2^{+}CD11c^{+}MHC II^{hi} CD172a^{+}CD24^{-} cells were gated. (B, left) CD45.1^{+}CD11c^{+}MHC II^{hi} CD172a^{+}CD24^{-} filler cells were gated. (A, right) Shown are the changes of proportion between CD115^{+}CX$_3$CR1^{+} and CD115^{-}CX$_3$CR1^{-} cells over MHC II^{hi} cells. (B, right) Shown are the changes of proportion between CD115^{+}CX$_3$CR1^{+} and CD115^{-}CX$_3$CR1^{-} cells over MHC II^{hi} cells within filler cell gates. Error bars indicate mean ± SEM across multiplicate sample. Representative data are shown from more than two independent experiments.

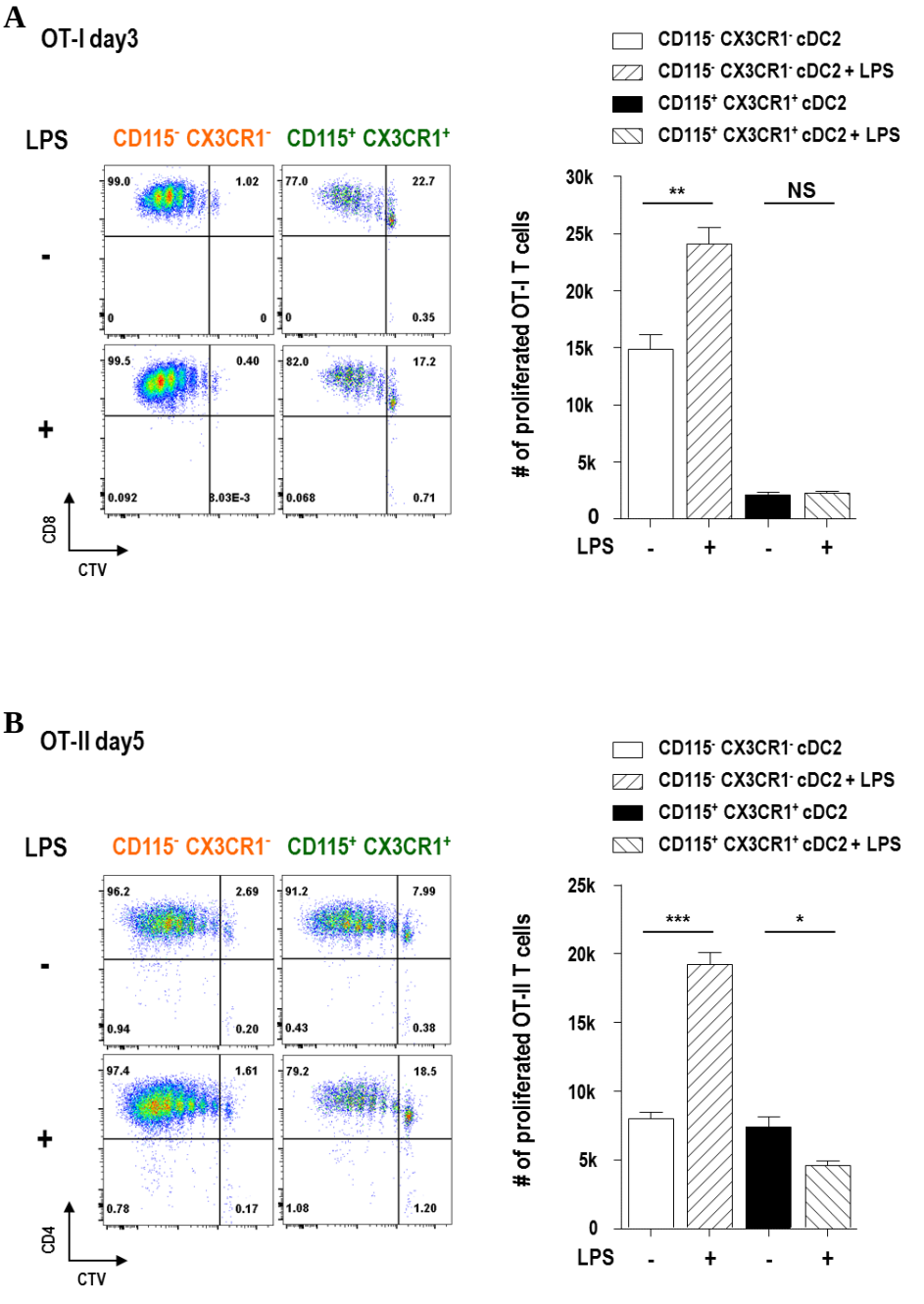


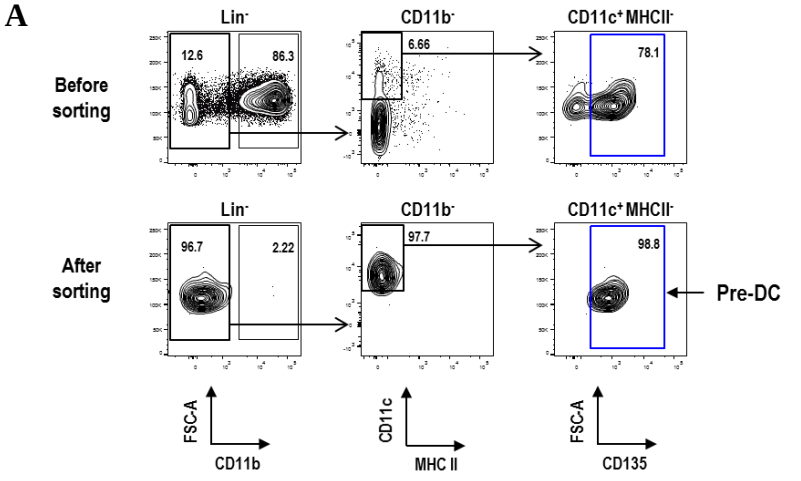
Figure 33. Evaluation of T cell proliferating ability of MHC II^{hi} cell subsets upon LPS stimulation. At culture day 8, slightly adhered cells in BM culture were harvested and sorted according to the gates as in Figure 9 (lower panels). Purified APCs were cultured with 2.5×10^4 of CTV labeled splenic CD8$^+$ OT-I T cells (A) or CD4$^+$ OT-II T cells (B) in the presence of 10 μg/ml OVA and 0 μg/ml or 1 μg/ml LPS. To assess proliferation of CD8$^+$ OT-I and CD4$^+$ OT-II T cells, CTV^{lo} proliferated OT-I and OT-II cells were analyzed on day 3 and day 5, respectively. Representative flow cytometric plots of CD8$^+$ OT-I (A) and CD4$^+$ OT-II (B) T cell proliferation are shown. Representative data are shown from more than 2 independent experiments of quadruplicate. Error bars indicate mean ± SEM across multiplicate sample. NS, not significant; *p≤0.05; **p≤0.01; ***p≤0.001.

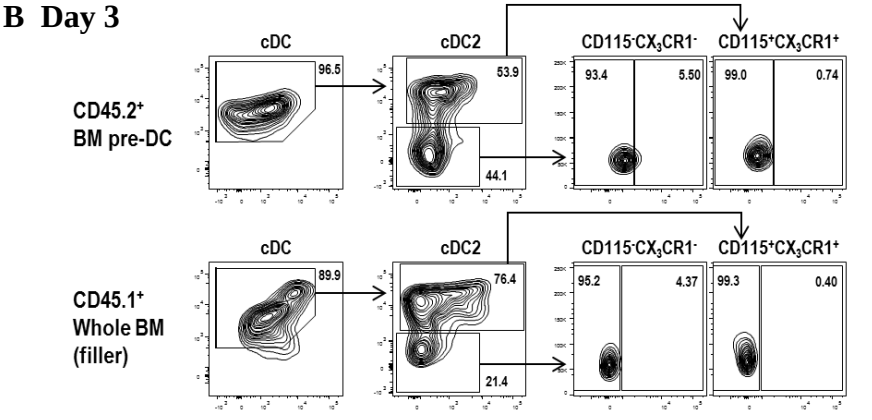
10. MHC II^{hi} cells are generated from pre-cDCs, not monocytes in BM culture with FLT3L

Developmental process of DCs progresses from MDPs to CDPs that give rise to pDCs and cDCs, but not monocytes, and finally to committed precursors of cDCs which termed pre-cDCs[30]. Pre-cDCs enter LNs through and migrate along special vessels, high endothelial venules (HEVs), and then disseminate and incorporate into the DC network. Further cDC development involves cell division, which is controlled in part by T cells and by the receptors, FLT3[30].

With this immediate precursors for cDCs at steady state condition, pre-cDCs[69], the origin of MHC II^{hi} cell subsets *in vitro* was identified and demonstrated. Pre-DCs were isolated using flow cytometry and then were cultured in FLT3L condition (Figure 34A). Due to the limitation in gaining enough cells, whole BM cells isolated from CD45.1 mice were used as filler cells to nourish the small sorted cell fraction. With the aids of filler cells, 2.5×10^4 pre-cDCs differentiated into cDCs even for a short period *in vitro* with FLT3L (Figure 34B). After 6 days of culture, all the MHC II^{hi} cell subsets including activated DCs which express CD83 were produced from pre-cDCs (Figure 34C). Next, to test whether the MHC II^{hi} cells arise from pre-cDCs are reactive to immune stimuli, LPS was added on culture at day 2 and 5 for 18 hours respectively. At both 3 days and 6 days, MHC II^{hi}CD115$^+$CX$_3$CR1$^+$ cells disappeared in pre-cDC gates and filler cell gates, albeit approximately 25% of cells from fillers remained CD115 molecules at intermediate level at day 3 for (Figure 35A and B). Most of the cells within MHC II^{hi} cDC gates were CD115$^-$ CX$_3$CR1$^-$ cells composed of CD83$^-$ and CD83$^+$ cells in 3:7 ratio, indicating that MHC II^{hi} cells originate from pre-cDCs and the cells are able to be activated or maturated when immune stimuli given (Figure 35B).

Collectively, pre-cDCs are the immediate precursors for MHC II^{hi} cell subsets, especially DC precursors exhibit CD115$^+$CX$_3$CR1$^+$ phenotype in the culture of BM with FLT3L.





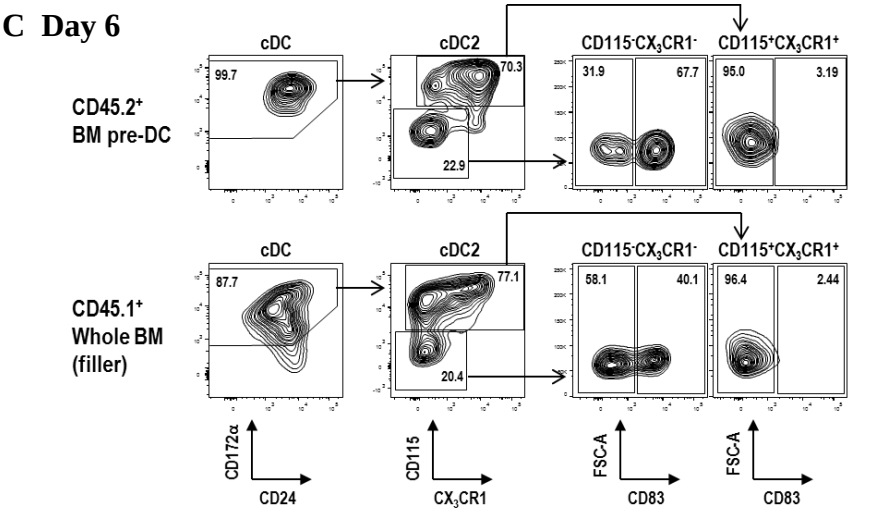


Figure 34. MHC II^{hi} cells are generated from pre-cDCs in the culture of BM with FLT3L. (A) Gating (upper panels) and sorting purity (lower panels) of BM pre-cDCs. (B, C) 2.5×10^4 of sorted Lin$^-$CD11b$^-$CD11c$^+$MHC II$^-$ CD135$^+$ pre-DCs were cultured with FLT3L for 3 days (B) or 6 days (C). 2×10^6 of whole BM cells from CD45.1 mice were co-cultured as filler cells. Lin; CD3, CD19, CD49b, CD115, CD117, Sca-1, Ly6C, Ly6G. cDC gates mean live CD11c$^+$MHC II^{hi} cells. Representative data are shown from more than 2 independent experiments.

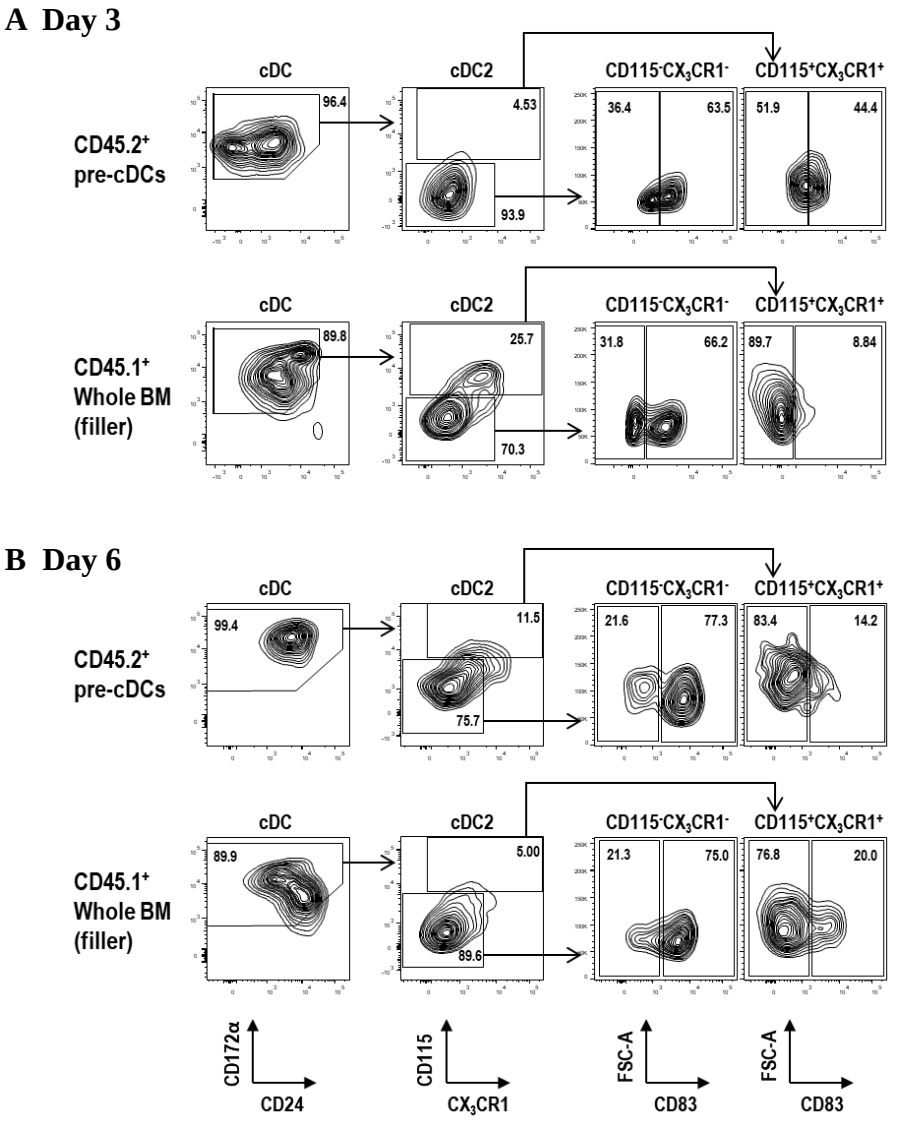


Figure 35. MHC II^{hi} cells generated from pre-cDCs in FLT3L condition are activated upon LPS treatment. Cells were sorted and cultured same as in Figure 22. At culture day 2 (A) or day 5 (B), 1 μg/ml of LPS was added on culture to stimulate cells. After 18 hours of incubation, cells were harvested, washed, stained and analyzed by flow cytometry. Representatives are shown from more than 2 independent experiments.

11. Monocytes have no potential to differentiate into MHC II^{hi} cells in the BM culture with FLT3L

Steady state DCs require FLT3L[70], whereas monocytes are dependent upon macrophage colony-stimulating factor (M-CSF)[71]. FLT3L$^{-/-}$ mice show severe deficiency in populating DCs[72,73], whereas in mice lacking M-CSF receptors (CD115), monocytes are missing[74,75]. As a result, DCs are generally independent of monocytes in the steady state. However, in case of infection or inflammation, monocytes can rapidly differentiate into DCs, which term Mo-DCs[31,76]. In addition to *in vivo* experiments, countless of studies have revealed that Mo-DCs are derived from monocytes *in vitro* in the presence of hematopoietic cytokine, GM-CSF, which is important for differentiation and survival of Mo-DCs[76,77]. Monocytes, upon culture for a week with GM-CSF and/or IL-4, acquire a typical dendritic morphology, lose the capacity to phagocytose which is the characteristics specific for macrophages, and strong ability to initiate adaptive immunity.

Monocytes are much more abundant than DCs in blood and bone marrow, so utilization of the monocytes as a reservoir for DCs needs to be elucidated not only with GM-CSF which is the already established method but also with FLT3L. To test whether monocytes have potentials to differentiate into MHC II^{hi} cells in case of FLT3L condition, BM monocytes were sorted and cultured in FLT3L conditioned media for 6 days with CD45.1^{+} filler BM cells. Lin^{-} CD115^{+}CD11b^{+}Ly6G^{-}Ly6C^{hi} fraction of BM was gated and sorted as BM monocytes over 98% purity (Figure 36A). Cells from filler gates developed in a normal manner in FLT3L condition (Figure 36B, lower panels), whereas monocytes only give rise to MHC II^{int} cells when cultured with FLT3L (Figure 36B, upper panels). Nevertheless, monocytes have no potential to differentiate into all the three MHC II^{hi} cell subsets in FLT3L condition, especially CD115^{+}CX$_3$CR1^{+} cells which exhibit the hematopoietic molecules related to monocyte lineage, CD115 and CX$_3$CR1 (Figure 36B, upper panels). And even

18 hours of LPS stimulation was fail to generate MHC II^{hi} cells from BM monocytes. Taken together, monocytes are not the immediate precursor cells for MHC II^{hi} cell subsets in the culture of BM with FLT3L.

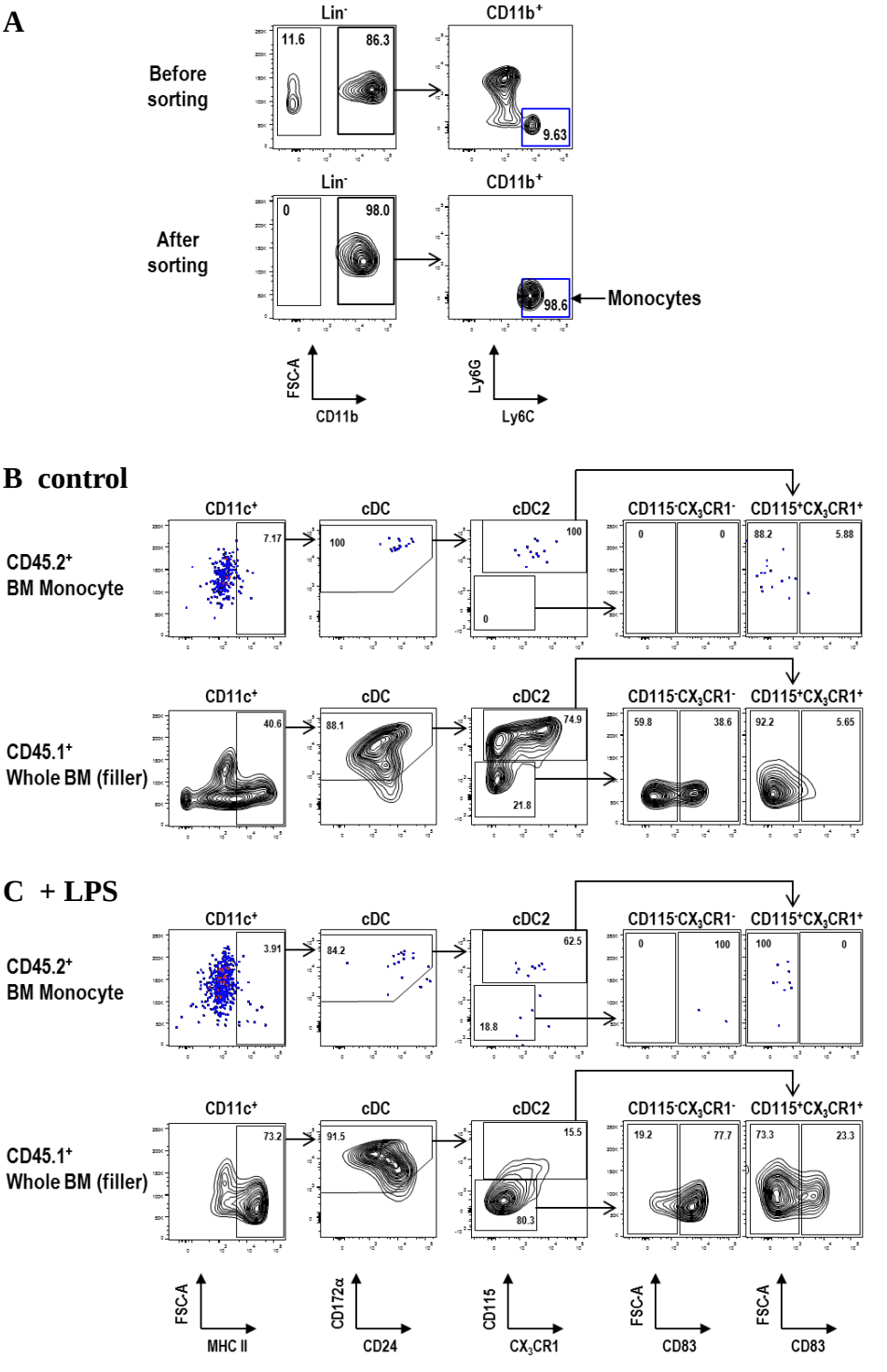


Figure 36. MHC II^{hi} cells are not generated from BM monocytes in the culture of BM with FLT3L. (A) Gating (upper panels) and sorting purity (lower panels) of BM monocytes. (B, C) 2.5×10^4 of sorted Lin$^-$ CD115$^+$CD11b$^+$Ly6G$^-$Ly6C^{hi} monocytes were cultured with FLT3L for 6 days. 2×10^6 of whole BM cells from CD45.1 mice were co-cultured as filler cells. (B) Cells were normally cultured in FLT3L conditioned media as unstimulated controls. (C) At culture day 5, 1 μg/ml of LPS were added on culture for 18 hours. After incubation, cells were harvested by gentle pipetting, washed, stained and analyzed. Sorted cells and filler cells were discriminated by surface expression of CD45.1 or CD45.2 molecules. Lin; CD3, CD11c, CD19, CD49b, CD115, CD117, Sca-1, MHC II. cDC gates mean live CD11c$^+$MHC II^{hi} cells. Representative data are shown from more than 2 independent experiments.

12. cMoPs are not the progenitors for MHC II^{hi} cells in the culture of BM with FLT3L

cMoPs are clonogenic, monocyte- and macrophage-restricted progenitor cells derived from the MDP. cMoPs are Ly6C^{+} proliferating cells present in the BM and spleen that produced the major monocyte subsets and macrophages, but not DCs[48]. To confirm that monocytes cannot generate MHC II^{hi} cells including CD115^{+}CX$_3$CR1^{+} cells in the culture with FLT3L, the progenitors for monocytes, cMoPs were isolated and cultured with CD45.1 filler BM cells in FLT3L condition (Figure 37A). 1.25×10^{4} of sorted Lin^{-}CD117^{+}CD115^{+} CD16/32^{+}CD34^{+}CD135^{-}Ly6C^{hi} cMoPs were cultured for 8 days and only MHC II$^{lo\text{-}int}$ cells were generated, not MHC II^{hi} cells (Figure 37B, upper panels). Simultaneously, cells within filler gates generate normally including all the MHC II^{hi} cells (Figure 37B, lower panels). The expression level of CD135/FLT3, receptors for FLT3L, on cell surface of cMoPs is low-to-negative so that cMoPs are merely reactive to FLT3L[48] (Figure 37A). Also, LPS stimulation was fail to generate MHC II^{hi} cells from cMoPs (Figure 37C, upper panels), similar to the monocytes. As a result, monocytes and monocyte-restricted progenitors, cMoPs, are not the progenitors for MHC II^{hi} cells in the culture of BM with FLT3L.

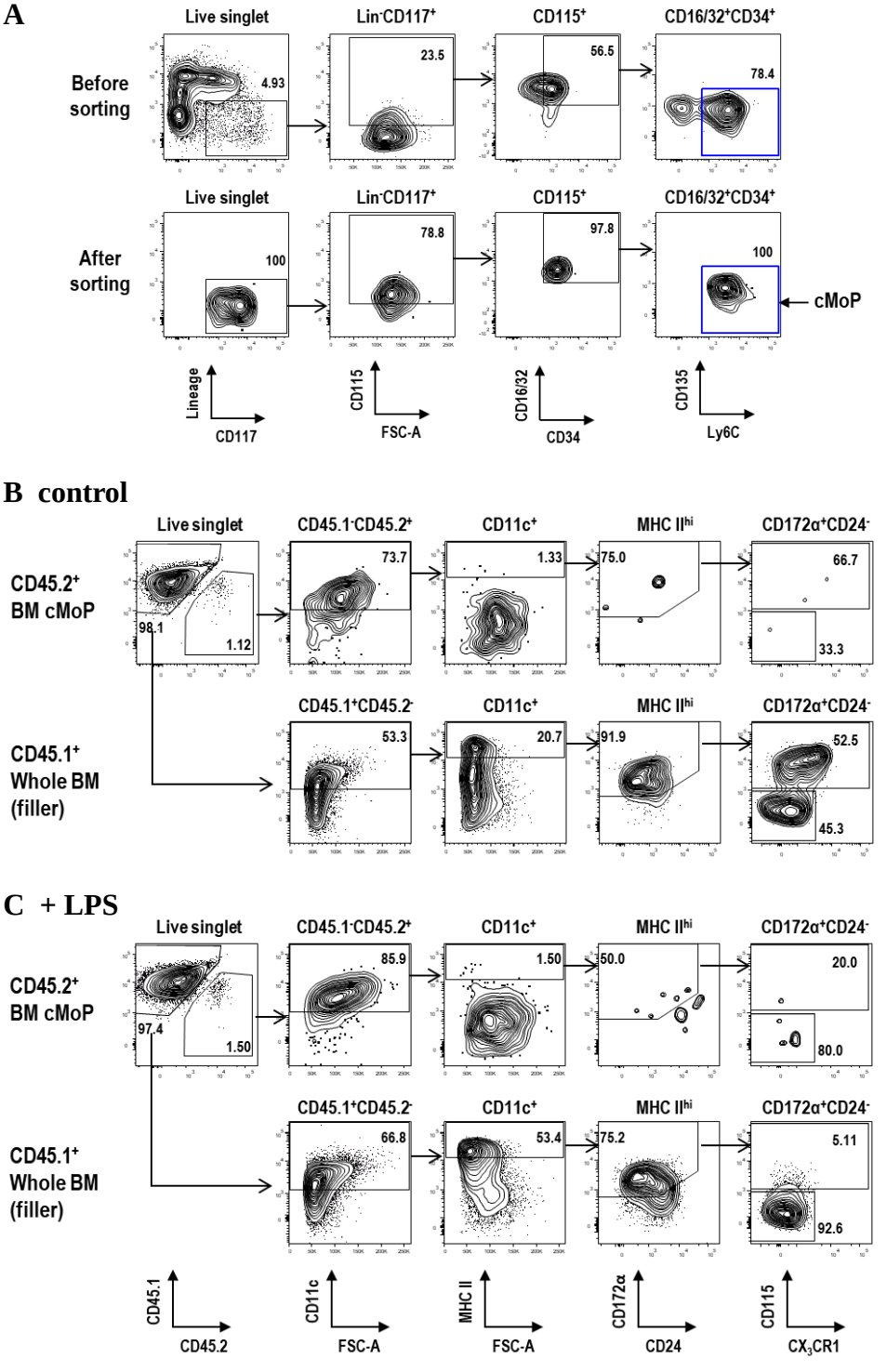
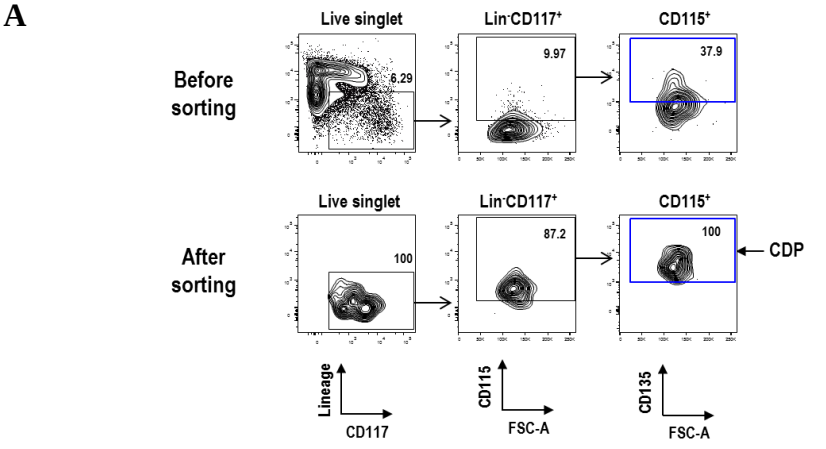
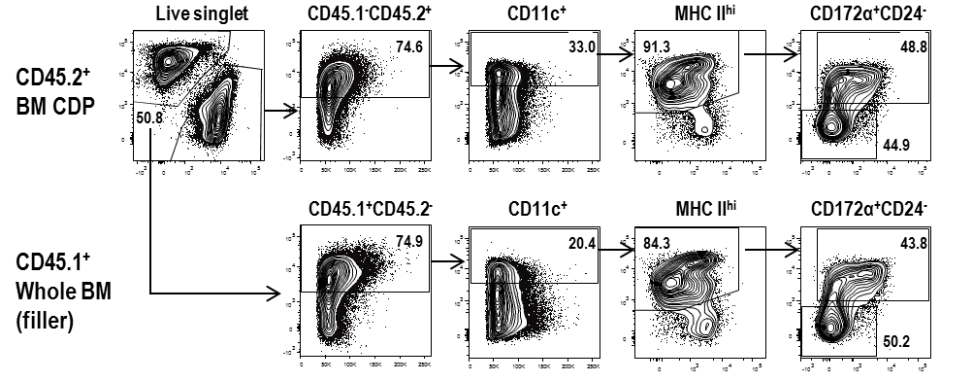


Figure 37. cMoPs are not the progenitors for MHC II^{hi} cells in the culture of BM with FLT3L. (A) Sorting scheme and purity check for BM cMoPs. Whole BM cells were stained with appropriate cocktail of antibodies without Fc block and were purified by FACS sorting as in the shown gates. (B, C) 1.25×10^4 of sorted Lin$^-$CD117$^+$CD115$^+$CD16/32$^+$CD34$^+$CD135$^-$Ly6C^{hi} cMoPs were cultured with FLT3L for 8 days. 2×10^6 of whole BM cells from CD45.1 mice were co-cultured as filler cells. (B) Cells were cultured in FLT3L conditioned media as unstimulated controls. (C) At culture day 7, 1 μg/ml of LPS were added on culture for 18 hours. After LPS incubation, cells were harvested by gentle pipetting, washed, stained and analyzed. Sorted cells and filler cells were discriminated by surface expression of CD45.1 or CD45.2 molecules. Lin; CD3, CD11b, CD11c, CD19, CD49b, NK1.1, B220, Ly6G, TER119, Sca-1, MHC II. Representative data are shown from more than 2 independent experiments.

13. CDPs give rise to MHC II^{hi} cells through pre-cDC pathway in FLT3L condition.

CDPs are common DC-restricted progenitors in BM[78]. To confirm that pre-cDCs are the precursors for MHC II^{hi} cell subsets in FLT3L condition, CDPs, the progenitors for pre-cDCs, were isolated and cultured with filler BM cells from CD45.1 mice (Figure 38A). Lin^{-}CD117^{+}CD115^{+}CD135^{+} cells were sorted as CDPs (Lineage markers are written in figure legend of figure 38). After 8 days of culture, all the MHC II^{hi} cells were shown to be generated from CDPs, even the input number of cells was one-fifth of the input cell number in cMoP culture (Figure 38B). Upon LPS stimulation, CD115^{+}CX$_3$CR1^{+} cells generated from CDPs were activated and gained CD115^{-}CX$_3$CR1^{-} phenotype as in cells from CD45.1 filler gates (Figure 38C). In summary, CDPs give rise to MHC II^{hi} cells through pre-cDC pathway in FLT3L condition.





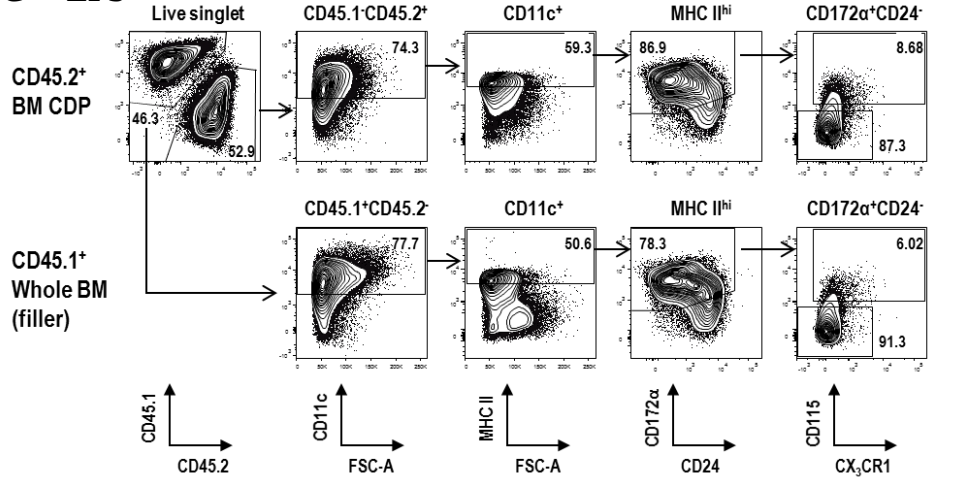


Figure 38. MHC II^{hi} cells are generated from CDP lineage. (A) Sorting strategy and purity check for CDPs (BM Lin^{-}CD117^{+}CD115^{+} CD135^{+} cells). Whole BM cells were stained and purified by FACS sorting as in the shown gates. Lin; CD3, CD11b, CD11c, CD19, CD49b, Gr-1, Ly6G, NK1.1, CD127, Sca-1, MHC II, TER119. (B, C) 2.5×10^{3} CDPs and whole BM from 45.1 mice as filler cells were cultured in FLT3L conditioned media for 8 days. 2×10^{6} of whole BM cells from CD45.1 mice were co-cultured as filler cells. (C) At culture day 7, 1 μg/ml of LPS were added on culture for 18 hours. After LPS incubation, cells were harvested by gentle pipetting, washed, stained and analyzed. Sorted cells and filler cells were discriminated by surface expression of CD45.1 or CD45.2 molecules. Representative data are shown from more than 2 independent experiments.

IV. DISCUSSION

There are numerous studies performed with GM-CSF derived BMDCs, also known as Mo-DCs and much of their biology was discriminated in various ways. Although many parts were demonstrated, DCs produced from BM culture with FLT3L were relatively unknown compared to GM-CSF culture. FLT3 is a receptor tyrosine kinase with homology to the kinases c-Kit and c-Fms. FLT3L is sufficient to induce the differentiation of both pDCs and cDCs from progenitors *in vitro*, and it supports the maintenance of DCs in steady-state lymphoid organs *in vivo*[79]. So far, there are no studies specifically examining subpopulation of cDC2s from BM culture in FLT3L condition. In the current study, heterogeneous nature of cDC2s or MHC II^{hi} cells generated from BM culture with FLT3L was identified at first time. The cDC2s can be divided into several subsets with the additional use of hematopoietic markers CD115, CX_3CR1 and CD83. With the combination of these three markers plus previously known markers, the three distinct MHC II^{hi} cell populations found in the BM culture with FLT3L are (1) $CD115^-CX_3CR1^-CD83^-$ cDC2s, (2) $CD115^-CX_3CR1^-CD83^+$ cDC2s and (3) $CD115^+CX_3CR1^+CD83^-$ cells.

Not only by the expression pattern of cell surface markers but also by the morphology, these subsets can be distinguished. $CD115^+CX_3CR1^+CD83^-$ cells exhibit larger in size and have lesser dendrites or pseudopods than the other MHC II^{hi} cells whereas $CD115^-CX_3CR1^-$ cDC2s, both $CD83^-$ and $CD83^+$, show typical dendritic cell morphology, which forms clusters with a lot of dendrites and shows slightly adhered or non-adhered morphology. And Giemsa staining illustrate that $CD115^-CX_3CR1^-CD83^-$ cells display dendrites on cell surface, $CD115^-CX_3CR1^-CD83^+$ cells have long protrusions or dendrites and shrunk nucleus, and that $CD115^+CX_3CR1^+$ cells show large cytoplasm and short pseudopods similar to macrophages.

Moreover, at the functional point of view, they showed difference in endocytic and phagocytic capacity. $CD115^+CX_3CR1^+$ cells are superior at taking

up various antigens such as fluorescence conjugated protein or fluorescently labeled particles. On the other hand, $CD115^{-}CX_3CR1^{-}$ cDC2s are poor at both endocytosis and phagocytosis compared to $CD115^{+}CX_3CR1^{+}$ cells. One of the typical functional criteria of DCs that are commonly used is to test APC function, i.e., MLRs and antigen-specific T cell proliferation. Using model antigen, ovalbumin, antigen-specific T cell proliferation was evaluated. When OVA-specific T cells were co-cultured with the three MHC II^{hi} cells respectively, $CD115^{+}CX_3CR1^{+}$ cells definitively exhibit incompetent ability to proliferate both $CD4^{+}$ and $CD8^{+}$ T cells compared to $CD115^{-}CX_3CR1^{-}$ cDC2s. For $CD4^{+}$ T cell proliferation, $CD115^{-}CX_3CR1^{-}$ cells are superior at antigen presentation regardless of the expression of CD83 on the cell surface. However, in case of cross presentation, only $CD115^{-}CX_3CR1^{-}CD83^{+}$ cDC2s can successfully proliferate $CD8^{+}$ T cells, indicating that $CD115^{-}CX_3CR1^{-}CD83^{+}$ cDC2s have some special machinery to process exogenous antigens compared to the other MHC II^{hi} cells from BM culture with FLT3L. Collectively, $CD115^{+}CX_3CR1^{+}$ cells showed immature DC phenotype which is identified as good phagocytes and poor antigen presenting cells. In this regard, it is important to assess in further studies whether the immediate DC precursors and activated DCs from FLT3L culture might stimulate and differentiate T cells to certain types in a specific condition, such as T_H1 (type 1 T helper cell), T_H2, T_H17 and T_{reg} cells (regulatory T cells).

Sensing and defeating microbial infections is essential for survival and vitality of organisms. Many immune cells including DCs have receptors against foreign molecules such as PRRs and TLRs. LPS, ligand for TLR4, which is commonly employed to induce DC maturation was used. To test whether the three MHC II^{hi} cells can be activated of changed to sudden ways, TLR agonists including LPS were used. Consequently, LPS treatment can stimulate or activate MHC II^{hi} $CD115^{+}CX_3CR1^{+}$ cells into $CD115^{-}CX_3CR1^{-}CD83^{+}$ cDC2s, which exhibit activated phenotype and morphology among MHC II^{hi} cells. However, T

cell proliferation of $CD115^{+}CX_3CR1^{+}$ cells was not prominently changed by LPS stimulation because the reactivity to LPS might decrease during isolation processes for some reasons. The reactivity to LPS recovered when sorted cells were cultured with fresh BM filler cells for the additional several days (total 4 days post-sort). Gathering this information, $CD115^{+}CX_3CR1^{+}$ cells are considered as the immediate precursors for DCs in BM culture with FLT3L.

Ontogeny is one of the important features to identify cellular characters[52]. To figure out the origin of MHC II^{hi} cells generated from BM culture in FLT3L condition, culture of hematopoietic progenitors and precursors for DCs in BM were assessed. The immediate precursors for cDCs, pre-cDCs were cultured with FLT3L for 6 days and all the MHC II^{hi} cells were generated from pre-cDCs. Also, the known progenitors for pre-cDCs, CDPs were isolated, cultured in FLT3L condition and cDCs including all the MHC II^{hi} cells were produced from CDPs, indicating that MHC II^{hi} cell subsets are CDP to pre-cDC-lineage. Next, to check the possibility whether monocytes and their committed progenitors give rise to MHC II^{hi} cell subsets in response to FLT3L or not, the cells were sorted and cultured. From monocytes and cMoPs, no MHC II^{hi} cell was generated and even LPS treatment was fail to activate or stimulate cells to MHC II^{hi} phenotype. In conclusion, in the BM culture with FLT3L, MHC II^{hi} cells including all the three of $CD115^{-}CX_3CR1^{-}CD83^{-}$, $CD115^{-}CX_3CR1^{-}CD83^{+}$ and $CD115^{+}CX_3CR1^{+}$ cells are CDP lineage.

Beyond the ontogeny, *in vivo* relevance of the MHC II^{hi} cell subsets remains to be clarified. So far, numerous efforts to discover the *in vivo* corresponding cell types for the MHC II^{hi} cell subsets have been, however, the exact correspondence has yet to be found. Indeed, the MHC II^{hi} cell subsets in the BM culture, especially $CD115^{+}CX_3CR1^{+}$ cells, might be generated in the specific *in vitro* condition. Nevertheless, when taking account of the characteristics of chemokine receptor CX_3CR1, the researches on $CD115^{+}CX_3CR1^{+}$ cells are still important. CX_3CR1 and its ligand CX_3CL1/fractalkine is known as a leukocyte

trafficking regulator, which performs both chemotactic and adhesive functions[80]. And in some inflammatory diseases such as rheumatoid arthritis and atopic dermatitis, CX_3CR1-CX_3CL1 axis plays an important role in the trafficking of leukocytes during the inflammation[81,82]. In this regard, multifaceted approaches are needed to demonstrate the *in vivo* phenotype of MHC II^{hi} cell subsets.

Immunizations using antigen-loaded DCs may represent powerful methods of inducing immunity, especially antitumor immunity when using tumor antigens[6]. For the development of conventional DC vaccines so far, hematopoietic cytokine GM-CSF has been used to generate DCs from human blood monocytes in large numbers[83]. However, GM-CSF can only produce DCs possessed inflammatory phenotype, not steady state DCs. Therefore, FLT3L, which involves in differentiation and survival for steady state DCs, has potential to be utilized for future DC vaccines when steady state DCs are required. At the point of vaccine development, $CD115^+CX_3CR1^+$ cells, the immediate precursors for DCs, can be potent tools for DC vaccine. To improve autologous cell vaccine therapy which utilizes one's own progenitor or precursor cells, understanding the biology of in vitro DCs is to be enhanced. Finally, characterization of the novel precursors and DC subsets in BM culture with FLT3L might help to understand and guide the utilization of the DCs in therapeutic clinical settings.

V. CONCLUSION

In current study, the novel precursor cells for DCs and DC subsets *in vitro* were identified and characterized in various ways. The MHC II^{hi} cDC2s can be further divided into several subsets with the hematopoietic molecules CD115, CX_3CR1 and CD83. With the combination of these three markers plus previously known cDC markers, the three distinct MHC II^{hi} cell subsets were identified and demonstrated in the culture of BM with FLT3L; (1) $CD115^-CX_3CR1^-CD83^-$ cDC2s, (2) $CD115^-CX_3CR1^-CD83^+$ cDC2s and (3) $CD115^+CX_3CR1^+CD83^-$ cells.

Not only by the phenotype but also by the morphology, these subsets can be distinguished. $CD115^-CX_3CR1^-$ cDC2s, both $CD83^-$ and $CD83^+$, showed typical dendritic cell morphology, which forms non-adherent clusters with dendrites, whereas $CD115^+CX_3CR1^+CD83^-$ cells exhibited large cytoplasm and short dendrites or pseudopods compared to the other MHC II^{hi} cells. Wright-Giemsa stain illustrates that $CD115^-CX_3CR1^-$ $CD83^-$ cells display dendrites on cell surface, $CD115^-CX_3CR1^-CD83^+$ cells have long protrusions or dendrites and shrunk nucleus, and that $CD115^+CX_3CR1^+$ cells show large cytoplasm and short pseudopods similar to macrophages.

Also, gene expression profiles reveal that the heterogeneity of the three MHC II^{hi} cell subsets. LPS stimulation which is commonly used to 'DC maturation' changed the phenotype of MHC II^{hi} cells, especially $CD115^+CX_3CR1^+$ cells. When LPS given, $CD115^+CX_3CR1^+$ cells undergo maturation or activation and are to possess $CD115^-CX_3CR1^-CD83^+$ phenotype. As a result, $CD115^+CX_3CR1^+$ cells are the immediate precursors for DCs in BM culture with FLT3L.

Ontogeny of MHC II^{hi} cell subsets was also delineated. All the three MHC II^{hi} cell subsets were generated from CDPs through pre-cDCs, not cMoPs and monocyte. In conclusion, MHC II^{hi} cell subsets produced in the BM culture with FLT3L were identified and characterized.

It is well known that FLT3L-induced BM-DCs do not fully recapitulate the phenotype of cDCs in vivo. It is also recognized that developing better phenotypes of FLT3L-induced BM-DCs in vitro requires the addition of still many unidentified factors into FL-BM culture[84]. Our discovery of a large number of MHCII^{hi} pre-cDC2s in FL-BM culture may reflect less than perfect in-vitro culture conditions for FLT3L-induced BM-DCs. If so, it is not likely that the in-vivo counterpart cells of the MHCII^{hi} pre-cDC2s exist in large numbers. Nonetheless, in the future studies, it will be important to determine and characterize an in-vivo counterpart of the MHCII^{hi} pre-cDC2s identified in FL-BM culture.

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ABSTRACT(IN KOREAN)

골수 배양 속에 존재하는 수지상세포의 새로운 전구세포 규명

<지도교수 서준영>

연세대학교 대학원 의과학과

인현주

수지상세포는 다양한 면역 작용에 있어 중요한 역할을 하는 면역세포로서, 체내에서 항원을 수집하고 처리하여 T 세포에 전달하는 전문 항원제시세포이다. 수지상세포의 체외 배양 시스템은 수지상세포 연구에 널리 쓰이는 바 있으며 수지상세포의 분화에 중요한 조혈 사이토카인인 과립구 대식세포 콜로니 자극인자 (GM-CSF)와 FMS-유사 티로신 키나제 3 리간드 (FLT3L)가 이에 사용된다. GM-CSF 조건 하에 골수세포를 배양할 경우 염증환경에서 생성되는 수지상세포와 대식세포가 생성된다. 반면, FLT3L의 존재 하에 배양된 골수세포로부터는 형질세포양 수지상세포 (plasmacytoid DCs, pDCs)와 정통 수지상세포 (classical DCs, cDCs)가 생성된다. 본 연구에서는 두 사이토카인을 사용하여 배양한 골수세포 배양에서 CD11c와 MHC II 표지분자를 발현하는 새로운 아형세포를 발굴하였다. 특히 FLT3L 골수 배양에서는 기존에 정통 수지상세포 2 아형으로 알려졌던 세포집단에서 CD11c와 MHC II 표지분자를 높게 발현하면서 항원제시능력이 낮은 수지상세포의 전구세포와, 항원제시능력이 높으면서 T 세포의 분화를 잘 유도할 수 있는 활성 수지상세포2 아형을 발굴하였다. 이 새로운 세포집단을 각각 분리하여 세포의 형태를 관찰하고 수지상세포로서의 기능을 평가하였으며, 수지상세포의 전구세포의 경우 여러 자극인자를 사용하여 수지상세포로의 분화능을 평가하였다. 또한 새로 발굴한 수지상세포 아형세포들의 유래와 개체발생을 확인하기 위해, 수지상세포의 전구세포로 알려진

조혈 전구세포들을 분리 배양하여 이를 확인하였다. 결과적으로, 수지상세포의 체외 배양을 통해 수지상세포의 전구세포를 생성할 수 있음을 증명하였다.

핵심되는 말 : 수지상세포, 항원제시세포, FLT3L, GM-CSF, 골수 세포, 골수 세포 배양, 수지상세포의 전구세포

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