의학석사 학위논문

# Effects of TLR5 and TLR7 Ligands on Myelopoiesis during Bone Marrow Cell Culture

# TLR5 L와 TLR7 L가 골수성 세포 분화에 미치는 영향

2020 년 1 월

서울대학교 대학원
의과학과 의과학 전공
정민호

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이 논문을 의학석사 학위논문으로 제출함

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의과학과 의과학 전공

정 민 호

정민호의 석사 학위논문을 인준함

2020 년 1 월

위 원 장 _____ (인)

부 위 원 장 _____ (인)

위 원 _____ (인)

# Abstract

# Effects of TLR5 and TLR7 Ligands on Myelopoiesis during Bone Marrow Cell Culture

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Balance between immune activation and immune suppression is important for immune homeostasis. Toll-like receptors (TLRs) play key roles in the innate immune system, initiating inflammatory responses against pathogen infections by recognizing various TLR ligands (TLR L). TLR signaling through pathogen infection not only causes the activation of already-differentiated myeloid cells, but also results in emergency myelopoiesis of hematopoietic stem and progenitor cells (HSPCs) to cope with infection. However, distinct myeloid cells are differentiated by different TLR-TLR L interactions. For example, TLR5 is well known for immunosuppression, and TLR7 for immune activation. Even though TLR5 and TLR7 share a common signaling pathway, such as MyD88-adaptor mediation, they show opposite immune responses, with immune suppression and activation,

respectively. The specific cellular mechanisms of TLR5 L, flagellin, and TLR7 L, gardiquimod, related to myeloid cell differentiation remain unknown. This study confirmed that TLR5 L causes immune suppression by increasing not only polymorphonuclear myeloid derived suppressor cells (PMN-MDSCs) but also monocytic myeloid derived suppressor cells (M-MDSC) and by decreasing dendritic cell (DC)-poiesis. In contrast, TLR7 L induces the activation of $CD11b^{+}Ly6C^{high}Ly6G^{int}$ monocytic cell differentiation, triggers immune activation, and subsequently enhances differentiation into macrophages. TLR5 L promotes $Ly6G^{high}$ granulocyte differentiation through TLR5 expressed by granulocyte precursors (GP), while TLR7 L promotes $Ly6C^{high}$ monocyte differentiation through TLR7 expressed by monocyte precursors. These results indicate TLR5 L promotes GP-$Ly6G^{high}$ granulocyte lineage differentiation via PMN-MDSCs enhancement, resulting in immunosuppression effect, but TLR7 L enhances monocyte precursor (MP)-$Ly6C^{high}$ monocyte lineage differentiation, facilitating immune activation. Altogether, these results demonstrated that the differential expression of TLR5 and TLR7 along the early myeloid precursors causes the differential outcomes of the respective stimulations.

Key Words: TLR5, TLR7, myeloid, myelopoiesis, progenitor
Student number: 2016-22007

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# List of Abbreviations and Symbols

**B**

BM: Bone marrow

**C**

cDC: classical dendritic cell

CDP: Common dendritic cell progenitor

CMP: Common myeloid progenitor

**D**

DC: dendritic cell

**G**

GMP: Granulocyte-monocyte progenitor

GP: Granulocyte progenitor

GvHD: Graft versus host disease

**H**

HSC: Hematopoietic stem cell

HSPC: Hematopoietic stem and progenitor cell

**M**

M-MDSC: Monocytic myeloid derived suppressor cell

MDP: Monocyte and dendritic cell progenitor

MDSC: Myeloid derived suppressor cell

MEP: Megakaryocyte-erythrocyte progenitor

mo-DC: Monocytic dendritic cell

MP: Monocytic progenitor

MPP: Multi potent progenitors

MyD88: Myeloid differentiation primary response 88

**P**

pDC: plasmacytoid dendritic cell

PMN-MDSC: Polymorphonuclear myeloid derived suppressor cell

PRR: Pattern recognition receptor

**T**

TLR: Toll-like receptor

# Introduction

Innate immune response is the crucial first line of defense against foreign antigens such as bacteria and viruses. Myeloid cells, such as neutrophil, monocytes, and macrophages play critical roles in inflammation and anti-microbial defense, as well as tissue remodeling and repair. [1]

Myeloid cells, especially monocytes and neutrophils, recognize the molecular pattern of microbes by using pattern recognition receptors (PRRs). The best-known PRR is the Toll-like receptor (TLR). [2] TLRs are classified depending on the ligands they encounter. TLRs transmit signals through adaptor proteins, and one of the most important adaptor proteins is myeloid differentiation primary response 88 (MyD88), the universal adaptor molecule in all TLRs except TLR3. [3] MyD88 signaling induces the expression of inflammatory cytokines to initiate immune responses against infections and pathogens. [4]

In physiological condition, hematopoietic stem and progenitor cells (HSPCs), which are undifferentiated cells, can supply myeloid populations to tissue and lymphoid organ for immune surveillance. [5] Under acute infection, such as bacterial or viral infection, the demand for myeloid cells increases. Murine and human HSPCs express functional TLRs, and those TLR/PRR signals provoke emergency myelopoiesis. [6] It is reported that LPS, which is a TLR4 L, induces granulocyte-monocyte progenitor (GMP)-derived cells, such as $Ly6C^{high}$ monocyte and $Ly6G^{high}$ neutrophil. CpG as known as TLR9 L treatment increases monocyte-DC progenitor (MDP)-derived cells, such as, classical dendritic cells (cDCs). [7] However, it is not well established what kind of ligands act on HSPCs to enhance emergency myelopoiesis.

Acute infections can be resolved by myeloid cells from emergency myelopoiesis. However, chronic infections and pathological conditions are not solved in the immediate stage, and can even enter the sustained stage. [8] In such chronic infections, the nature of signals that are activating myeloid cells differs. The signals in chronic condition are relatively weak and long-lasting and often appear in the form of inflammatory mediators and growth factors, such as SCF, GM-CSF, IL-3, and IL-6. [9,10] Neutrophils and monocytes generated under these conditions have an immature and suppressive phenotype, such as weak phagocytic activity and high expression of arginase1 and IDO1. [10, 11] Furthermore, these immature cells tend to either suppress adaptive immunity or support tumor progression rather than eliminate the cause of unresolved inflammation. [9] Putting these together, these cells with immune suppressive activity are called myeloid-derived suppressor cells (MDSCs).

Traditionally, MDSCs are identified as $CD11c^{-}CD11b^{+}Gr\text{-}1^{+}$ cells. MDSCs are sub-classified as polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs); each has a more specific phenotype, $Ly6G^{high}Ly6C^{int}$ and $Ly6G^{lo}Ly6C^{high}$, respectively. [9] However, even with this phenotype subclassification, they are still heterogeneous because neutrophil and monocyte express those same phenotypic markers. Further studies have reported that CD244 is expressed in PMN-MDSCs but not in neutrophils and the CD101 is expressed exclusively in mature neutrophils. [12] M-MDSC is defined by adding low side scatter and $CD115^{+}$ to the existing phenotype. [9]

It has been shown that transplantation of MyD88-deficient bone marrow (BM) results in aggravation of GvHD. MyD88 KO BM-derived $CD11b^{+}Gr\text{-}1^{+}$ cells were highly apoptotic cell with

deceased cell expansion unlike WT. [13] As a result, alloreactive T cells are unable to be suppressed by KO donor $CD11b^{+}Gr\text{-}1^{+}$ cells and the GvHD symptoms deteriorated. However, the response on physiological and pathological events of TLR with MyD88 adapter protein varies according to TLR. TLR7 KO mouse decreases lymphocyte activation and improved symptom of lupus disease[44], whereas transgenic mice with a overexpression of Tlr7 have raised development of autoimmune disease. [43] TLR7 agonists cause anti-tumor activity and induced type I interferon, [16,17] which suggests that the response induced through TLR7 engagement is pro-inflammatory. Unlike TLR7, in TLR5-deficient mice, pro-inflammatory cytokine increases or colitis tends to occur. [17] Contrary to this, intestinal DC expresses inflammatory cytokine via TLR5 signaling. [18] Thus, the response of physiological and pathological events to TLR5 is still controversial.

The effects of TLR5 and TLR7 on myelopoiesis, as well as the effects on the physiological and pathological events, are different. It is known that pDC expresses TLR7 and TLR7 agonist treatment induces pDC activation. [19, 20] On the other hand, TLR7 agonist treatment was reported to induce differentiation of BM CD34+ progenitors into macrophage and monocytic dendritic cell (mo-DC) precursors in human. [20] On the contrary, TLR7 agonist treatment impairs mo-DC differentiation in human PBMCs. [41] Although it has been identified that TLR7 treatment induces pro-inflammatory condition, the effects of TLR7 L on mo-DC/Macrophage-myelopoiesis remain controversial. It has been reported that TLR5 L treatment induces PMN-MDSC in human PBMC [21] and could not make murine splenic DC mature. These results indicate that TLR5 L treatment induces myeloid cells

differentiation which has anti-inflammatory status. However, there is an opposite report that intestinal DC is activated through TLR5 L, resulting in a pro-inflammatory condition. [22] Not only is the immune response of myeloid cells being formed through TLR5 L controversial, but also the whole process of differentiation from early progenitor in TLR5 L-treated condition is not yet known.

The present study investigated the cellular mechanisms by which TLR5 L and TLR7 L facilitate different myeloid lineages. Even though TLR5 and TLR7 share the same MyD88 signaling, TLR5 L treatment enhanced PMN-MDSC, while TLR7 L treatment increased immune activating monocytes that are differentiated into macrophages. Moreover, TLR5 L facilitated GMP-GP-Ly6G$^{high}$ granulocytes with high expression of TLR5 in GP. On the other hand, TLR7 treatment increased differentiation of progenitors into Ly6C$^{high}$ monocytic cells. These results show that TLR5 and TLR7 induce differentiation into different lineages of myeloid cells from progenitors to differentiated cells.

# Materials and Methods

## Mice

The C57BL/6 (B6) and B6.PL-Thy1$^a$/CyJ (Thy1.1) mouse strains were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were maintained at the Biomedical Center for Animal Resource Development of the Seoul National University, College of Medicine under specific pathogen-free conditions (SPF). The mice were used at between 8 and 12 weeks of age. All experiments were performed after approval from the Seoul National University Institutional Animal Care and Use Committee (IACUC) was obtained and in accordance with the IACUC guidelines. (SNU-170816-8-8)

## Bone marrow cell culture

Bone marrow cells in the femurs and tibia femurs and tibias of 8-week old B6 mice were isolated. Isolated BM cells were lysed with ACK lysis buffer to remove red blood cells. $5\times10^6$ cells BM cells were cultured in the 10% FBS media with 40ng/mL recombinant murine (rm) GM-CSF (PrimeGene, Shanghai, PRC). The standard concentration of supplement for BM culture with rmGM-CSF was as follows: 50ng/mL TLR5 L (RecFLA-ST) or 1μg/mL TLR7 L (Gardiquimod). In addition, $5\times10^6$ cells BM cells were cultured in the 10% FBS media with 40ng/mL rmSCF (PrimeGene, Shanghai, PRC), 20ng/mL rmIL-3 (PrimeGene, Shanghai, PRC), and 50ng/mL rmIL-6 (PrimeGene, Shanghai, PRC). Plates were incubated at 37°C with 5% $CO_2$.

## Immune suppression assay

Thy1.1$^+$ CD4$^+$ or Thy1.1$^+$ CD8$^+$ T cells from lymph nodes were purified using MACS negative isolation method. These MACS-purified T cells were labeled with CFSE (2.5μM; Invitrogen), stimulated with coated anti-CD3e (2μg/mL; eBioscience) plus soluble anti-CD28 (1μg/mL; eBioscience) antibodies. MACS-purified T cells were co-cultured with BM-derived cells at a 1:0.1 (T cells: BM cells) ratio. The cells were incubated at 37°C in an incubator with 5% $CO_2$. After 72 hours, CFSE dilution of Thy1.1$^+$ T cells was analyzed by flow cytometry.

## Antibodies and flow cytometric analysis

Single-cell suspensions were stained at 4°C for 30 min in staining buffer (1*X* phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) and 0.1% sodium azide) containing the appropriate antibody cocktail. For the flow cytometric analysis of BM-derived cells, CD3e, CD19, CD40, CD127, H-2Kb, Ly6G, Sca1, and CD244.2 (2B4) conjugated with FITC; NK1.1-AF488; CD70, CD80, CD86, CD11b, CD101, CD285 (TLR5), CD287 (TLR7), and MERTK conjugated with PE; CD11c, and CD117 conjugated with APC; Ly6C-P.Cy7; B220-AF700; CD11b, CD11c, and CD16/CD32 conjugated with APC-Cy7; Ly6G-eFluor450; CD135-BV421; Streptavidin and CD11b conjugated with BV605; XCR1 and CD11c conjugated with BV650; CD115-BV711; MHCII-BV785; and biotinylated anti-CD3e, CD19, NK1.1, and Ter-119 were used. To analyze the intra-cytoplasmic protein staining in BM-derived cells, BM-derived cells were fixed and permeabilized to stain with Arginase1-FITC, and IDO1-AF647. After immune suppression assay, T cells were analyzed with Thy1.1-APC, CD44-APC-Cy7, CD4-BV605, and CD8a-BV711. Data were collected using LSRII Green (BD Biosciences) and were analyzed using FlowJo

software (Tree Star, Ashland, OR, USA).

## tSNE analysis of cultured BM cells

For t-distributed Stochastic Neighbor Embedding (t-SNE), tSNE function was analyzed using Flowjo software. All downsampled data from each culture condition were concatenated into one file. Myeloid$^{+}$ [Dump (CD3e, NK1.1, CD19)$^{-}$] population were analyzed with tSNE dimensionality reduction (using the Barnes-Hut implementation of t-SNE from Rtsne R package [45]) based on five expression markers, clustering into eight populations.

## Statistical analysis

Prism (GraphPad Software Inc., La Jolla, CA, USA) was used for the statistical analysis. Data were presented as means ± standard error of the mean (SEM). P values were determined by unpaired Student's *t*-tests; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

# Results

## Fewer colonies in TLR7 L-treated condition

Most studies have been conducted on the TLR ligand treatment on differentiated cells, such as DC or pDC, in GM-CSF or Flt3 L culture. [23, 24] However, few studies have revealed myelopoiesis by treating TLR ligands on early progenitors. [6, 7]

To identify the effects of TLR5 L and TLR7 L on myelopoiesis, total BM cells were cultured using GM-CSF. TLR ligands were treated from the beginning of culture; cell counting was performed from day 1 to day 5 and microscopy and flow cytometry analysis on day 3 to day 5. (Figure 1A) The total cell number was mostly similar from day 1 to day 5 regardless of culture conditions. (Figure 1B) The number of colonies in the GM-CSF group and GM-CSF+TLR5 L group were similar. However, the colony number was significantly lower in the GM-CSF+TLR7 L group. (Figure 1C) Based on these results, GM-CSF and TLR5 L treatment seemed to produce earlier stages of cells with colony-forming capacity.

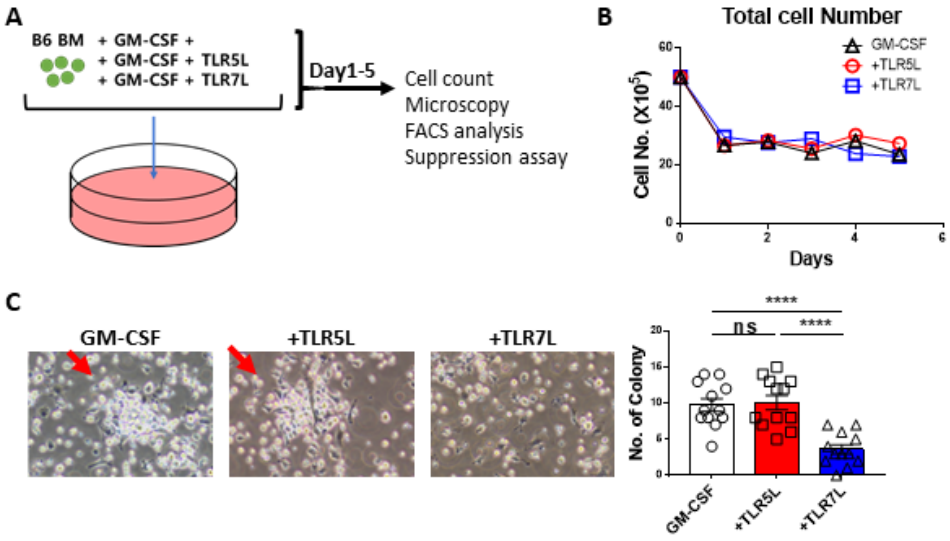



**Figure 1. Fewer colonies in TLR7 L-treated condition**

(A). Experimental design. Femur and tibia from B6 male mice were used for BM extraction. BM cells were lysed with ACK lysis buffer to remove RBCs. BM cells were cultured in the 10% FBS media with $40ng/mL$ rmGM-CSF. Additionally, TLR5 L (RecFLA-ST) or TLR7 L (gardiquimod) was added to BM culture media for 3 days. Cell counting was done daily up to day 5. Microscopic and FACS analysis were done from day 4 to day 5. BM-cultured cells on day 3 were used for the suppression assay. (B). Total cellularity kinetics after culture BM cells with TLR5 L or TLR7 L. (C). Analysis of BM-derived cell morphologic on day 3 of GM-CSF culture with TLR5 L or TLR7 L by light microscope (Nikon ECLIPSE Ts2R). The number of colonies in unit area was counted in three different snapshots in one independent experiment and pooled with four independent experiments. All data are representative of four independent experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

**TLR5 L treatment increases Ly6G$^{high}$ granulocytes, while TLR7 L treatment enhances Ly6C$^{high}$ monocytic cells**

The number of colony decreased in TLR7 L-treated culture. I assumed that the different colony numbers was caused by the different composition of cell population in each culture condition. To test this cell composition, each BM culture was identified through flow cytometry analysis. T, NK cell, B cell, pDC, DC, and Gr-1+ subset were gated on according to current gating strategy described in Figure 2A. The result demonstrated that myeloid cells accounted for the largest number in each culture condition. However, the numbers of T, NK cells, and B cells were relatively increased in TLR7 L-treated group (Figure 2B), which is consistent with previous studies showing that TLR7 L activates B cells and enhances effector T cells. [27, 28] Similarly, these results seem to correlate with low colony count in TLR7 L-treated group. (Figure 1C)

After gated on myeloid population, the myeloid compositions of GM-CSF treatment only and TLR5 L supplemented group were similar. Unexpectedly, the TLR7 L-treated group had a smaller fraction of DC and larger proportion of progenitor population and CD11c$^{-}$CD11b$^{+}$Gr-1$^{-}$ population. CD11c$^{-}$CD11b$^{+}$Gr-1$^{+}$ (Gr-1$^{+}$) population was similar in all three different culture conditions. (Figure 2C) There was no difference in the fraction of Gr-1$^{+}$ in the myeloid upon further analysis using Ly6C and Ly6G plots to determine whether the composition differs by the culture condition. The fraction and cell number of Ly6G$^{high}$ granulocytic population were found to be higher significantly in TLR5 L-supplemented group compared to GM-CSF only condition; on the other hand the fraction of Ly6C$^{high}$ monocytic population was higher in the TLR7 L-supplemented group. (Figure 2C)

These data indicate that BM cells undergo different myelopoiesis depending on TLR ligands.

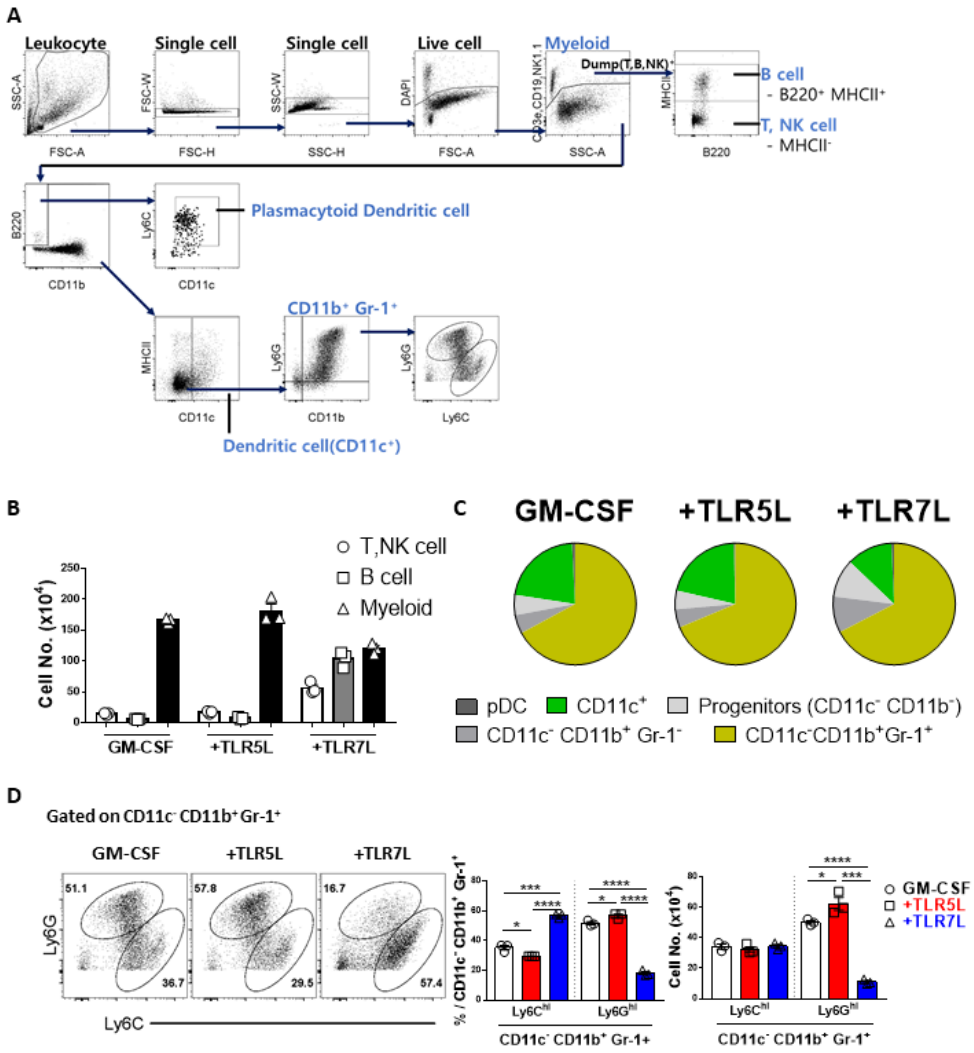


**Figure 2. TLR5 L treatment increases Ly6G$^{high}$ granulocytes, while TLR7 L treatment enhances Ly6C$^{high}$ monocytic cells**

(A). Gating strategy for BM-derived cell analysis. On day 3, BM cells were harvested after treatment of Trypsin-EDTA. BM-cultured cells were analyzed with flow cytometry. (B). Dump (CD3e CD19 NK1.1 TER119)$^{+}$ B220$^{-}$ T,NK cells, Dump$^{+}$B220$^{+}$MHCII$^{+}$ B cells, and Dump$^{-}$ Myeloid subsets were assessed after GM-CSF culture for 3 days with TLR5 L or TLR7 L. Data were presented as means plus standard deviations of three independent experiments. (C). The relative proportion of the Myeloid subsets were measured in 3 days after culture. (D). Flow cytometric analysis of CD11c$^{-}$CD11b$^{+}$Gr-1$^{+}$ BM-derived cell profile on day 3 of GM-CSF culture with TLR5 L or TLR7 L. BM-MDSCs from day 3 were harvested and analyzed using flow cytometry. CD11c$^{-}$CD11b$^{+}$Gr-1$^{+}$ populations were plotted by using Ly6G and Ly6C. The percentage of Ly6G$^{high}$ and Ly6C$^{high}$ cells in the CD11c$^{-}$CD11b$^{+}$Gr-1$^{+}$. The numbers of Ly6G$^{high}$ and Ly6C$^{high}$ cells were also shown. All data are representative of three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

**TLR5 L-treated myeloid cells are immune suppressive and TLR7 L-treated myeloid cells are immune activating**

The $CD11b^{+}Gr\text{-}1^{+}$ cells are the MDSC population. MDSC population consists of $Ly6C^{high}$ monocytic MDSC and $Ly6G^{high}$ PMN-MDSC. However, $Ly6C^{high}$ and $Ly6G^{high}$ are also relevant to monocytes and neutrophils. To determine whether the TLR5 L-treated and TLR7 L-treated myeloid cells are actually suppressive cells, T cell suppression assay was performed. Myeloid cells from each culture condition were co-incubated with activated T cells. Although both TLR5 L-treated and TLR7 L-treated myeloid cells showed PMN-MDSC and M-MDSC phenotype, only the TLR5 L-treated myeloid cells could suppress the activated T cells. On the contrary, the TLR7 L-treated myeloid cells significantly increased the proliferation of T cells compared to GM-CSF only treated cells. (Figure 3A)

In order to confirm the suppressive feature of the samples used for T cell suppression assay, intra-cellular staining for expression of arginase1 and IDO1 which are well-known suppressive markers was performed. [10,11] $Ly6G^{high}$ granulocytic cells and $Ly6C^{high}$ monocytic cells from TLR5 L-treated cells showed no difference in arginase1 compared to the GM-CSF only samples, whereas TLR7 L-treated $Ly6G^{high}$ granulocytic cells showed significantly decreased IDO1 expression levels compared to GM-CSF only samples. (Figure 3B, C)

Figure 3A showed that TLR7 L-treated myeloid cells made T cell more proliferate. CD40, CD80, CD86, and MHCII expressions were examined to determine whether myeloid cells formed by TLR7 L treatment are immune activating. The markers described above are activation markers of monocytes or granulocytes as well as T cell costimulatory factors. [29,30] Only TLR7 L-treated myeloid cells had

the increased frequency of $CD40^+$, $CD80^+$, $CD86^+$ or $MHCII^+$ cells, except for $CD80^+$ $Ly6C^{high}$ monocytic cell. Though myeloid cells from each culture condition had phenotypically similar, expressing MDSC markers ($CD11b^+Gr\text{-}1^+Ly6C^+Ly6G^+$), TLR5 L-treated and TLR7 L-treated myeloid cells were in immune suppressive and activating status, respectively.

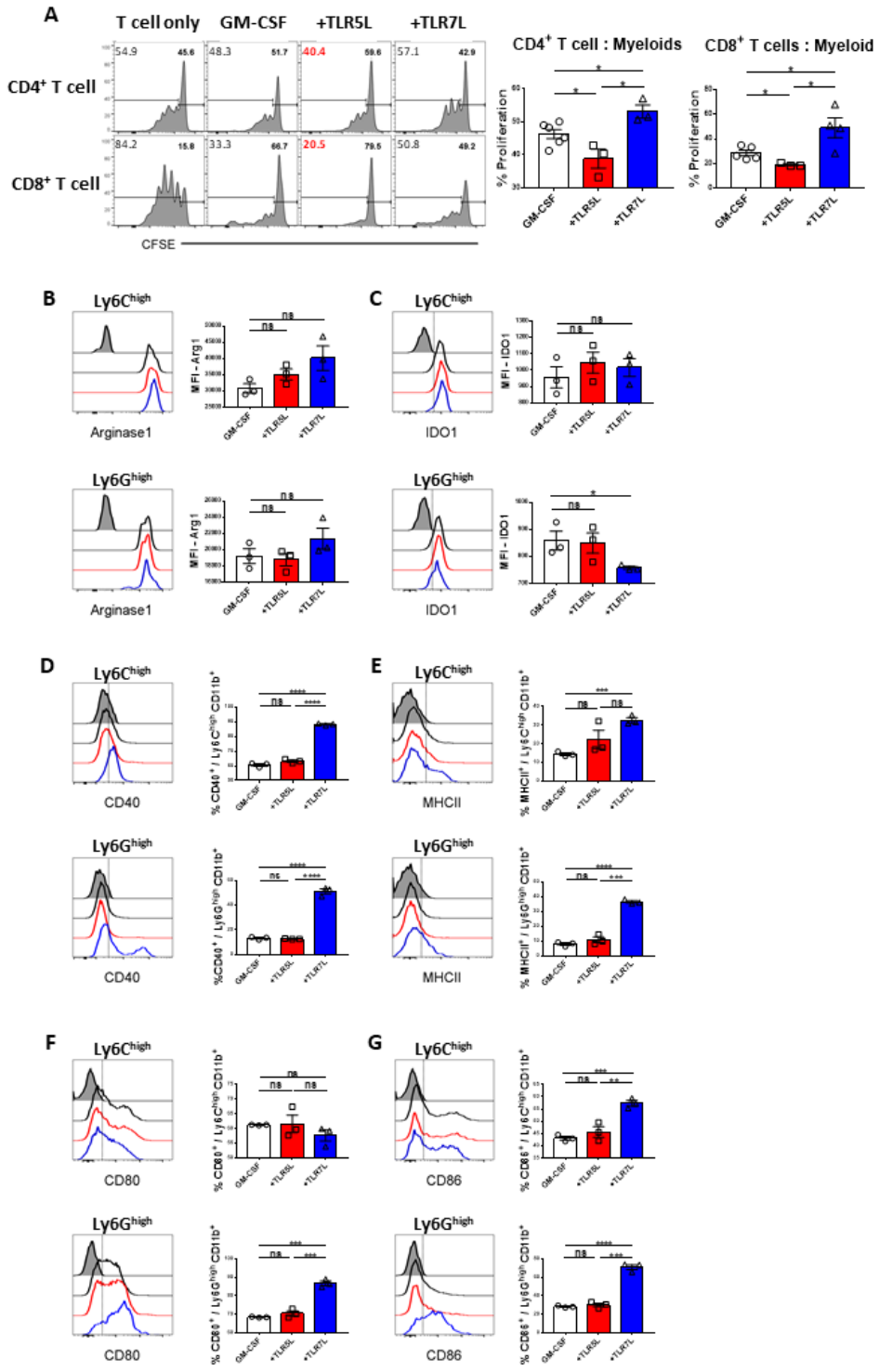

A
T cell only
GM-CSF
+TLR5L
+TLR7L
CD4+ T cell
CD8+ T cell
54.9
45.6
48.3
51.7
40.4
59.6
57.1
42.9
84.2
15.8
33.3
66.7
20.5
79.5
50.8
49.2
CFSE
CD4+ T cell : Myeloids
CD8+ T cells : Myeloid
% Proliferation
GM-CSF
+TLR5L
+TLR7L
B
Ly6Chigh
Arginase1
MFI - Arg1
C
IDO1
MFI - IDO1
Ly6Ghigh
D
CD40
%CD40+ / Ly6Chigh CD11b+
%CD40+ / Ly6Ghigh CD11b+
E
MHCII
% MHCII+ / Ly6Chigh CD11b+
% MHCII+ / Ly6Ghigh CD11b+
F
CD80
% CD80+ / Ly6Chigh CD11b+
% CD80+ / Ly6Ghigh CD11b+
G
CD86
% CD86+ / Ly6Chigh CD11b+
% CD86+ / Ly6Ghigh CD11b+
ns

**Figure 3. TLR5 L-treated myeloid cells are immune suppressive and TLR7 L-treated myeloid cells are immune activating.**

(A). Immune suppression assays of $Thy1.1^{+}CD4^{+}$ T cells and $CD8^{+}$ T cells isolated from lymph nodes of the naïve mice. CFSE dilution by CFSE-labeled T cells was analyzed via flow cytometry after CD3/CD28 stimulation in the presence of BM cells from each condition. The proportions of proliferated $Thy1.1^{+}CD4^{+}$ and $Thy1.1^{+}CD8^{+}$ T cells were indicated and plotted. (B, C) Arginase1 (B) and IDO1 (C) expression by $Ly6G^{high}$ and $Ly6C^{high}$ in $CD11c^{-}CD11b^{+}Gr\text{-}1^{+}$population from GM-CSF culture with TLR5 L or TLR7 L on day 3. (D, E, F, G) CD40 (D), MHCII (E), CD80 (F), and CD86 (G) histograms of $Ly6G^{high}$ and $Ly6C^{high}$ in $CD11c^{-}$ $CD11b^{+}$ $Gr\text{-}1^{+}$ population from GM-CSF culture with TLR5 L or TLR7 L on day 3. The numbers of each molecules-positive in $Ly6G^{high}$ and $Ly6C^{high}$ cells were shown. All data are representative of three independent experiments. ns, no significance; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$

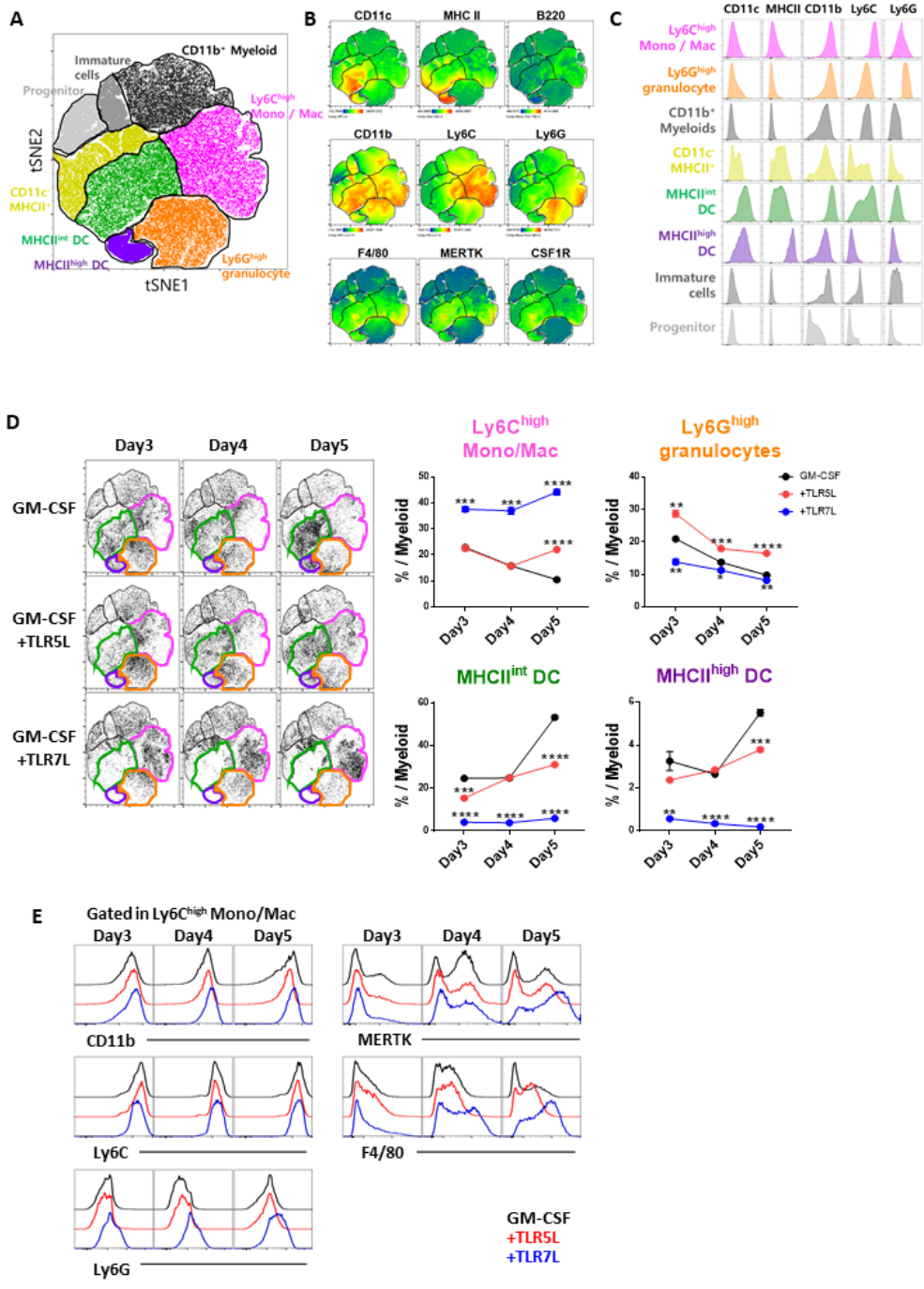
**Enhanced macrophage differentiation by TLR7 L and increased MDSC by TLR5 L**

Previous studies have reported that PMN-MDSC is formed through TLR5 L treatment in human. In the case of TLR7 L, the differentiation into mo-DC and macrophage through TLR7 L treatment is still controversial. [20,31] The effects of TLR5 L and TLR7 L on the formation of specific myeloid populations have been reported. However, they are still controversial and have not been identified from multiple angles in the myelopoiesis of various populations in BM cells. t-SNE analysis was conducted on day3-day5 samples to identify the differentiation lineages in different between TLR5 L or TLR7 L samples. Clustering was performed using CD11b, CD11c, MHCII, Ly6C, and Ly6G which were differentially expressed in myeloid populations. Cell subsets were divided into eight clusters by the indicated markers (Figure 4A), and each cluster was identified based on the expression of cell-subset phenotypic markers. (Figure 4B, C)

In TLR7 L-treated cells, $Ly6C^{high}$ Mono/Mac population ($CD11c^{-}$ $MHCII^{-}$ $CD11b^{high}Ly6C^{high}Ly6G^{int}$) increased most, whereas $MHCII^{int}$ DC ($CD11c^{+}MHCII^{low}CD11b^{high}Ly6C^{int/high}Ly6G^{low}$) and $MHCII^{high}$ DC populations ($CD11c^{+}MHCII^{high}CD11b^{+}Ly6C^{-}$) decreased significantly compared with other culture conditions (Figure 4D). Although it was the highest frequency for $Ly6C^{high}$ Mono/Mac populations in the TLR7 L-treated group, this population increased significantly compared to GM-CSF and TLR5 L-treated groups at day 5. From day 3 to day 5, MERTK and F4/80 expressions of $Ly6C^{high}$ Mono/Mac populations in TLR7 L-treated group were increased and higher compared to other conditions. (Figure 4E) These results show that DC-poiesis was reduced and monocytes were differentiated into macrophages by TLR7

L treatment. In TLR5 L-treated group, MHCII$^{int}$ DC and MHCII$^{high}$ DC were decreased compared to GM-CSF group.

As shown in Figure 2, Ly6G$^{high}$ granulocytes had the highest frequency from day 3 to day 5 in TLR5 L-treated group. (Figure 4D) CD101 is known as a mature neutrophil marker and CD244 as a PMN-MDSC marker. [9,12] To determine whether TLR5 L-treatment enhances PMN-MDSC or neutrophil differentiation, it was first verified that the CD101$^{-}$Ly6G$^{high}$ granulocytes were PMN-MDSC with CD244$^{+}$ (Figure 4F), and analyzed Ly6G$^{high}$ granulocytes with CD101 marker. The frequency of PMN-MDSC (CD101$^{-}$Ly6G$^{high}$ granulocyte) in TLR5 L-treated group was higher than in other groups (Figure 4G). M-MDSC differentiates into PMN-MDSC. [32] Therefore, whether TLR5 L treatment increases M-MDSCs was investigated as well. M-MDSCs were identified to be Ly6C$^{high}$CD115$^{+}$SSC$^{low}$. [9] In the TLR5 L-treated group, the fraction of M-MDSC increased significantly than in GM-CSF group. (Figure 4H) In summary, with TLR5 L treatment, DC-poiesis was decreased and PMN-MDSCs and M-MDSCs were increased.


A
Immature cells
CD11b+ Myeloid
Progenitor
Ly6Chigh Mono / Mac
tSNE2
CD11c+ MHCII+
MHCIIint DC
MHCIIhigh DC
Ly6Ghigh granulocyte
tSNE1
B
CD11c
MHC II
B220
CD11b
Ly6C
Ly6G
F4/80
MERTK
CSF1R
C
CD11c MHCII CD11b Ly6C Ly6G
Ly6Chigh Mono / Mac
Ly6Ghigh granulocyte
CD11b+ Myeloids
CD11c+ MHCII+
MHCIIint DC
MHCIIhigh DC
Immature cells
Progenitor
D
Day3 Day4 Day5
GM-CSF
GM-CSF +TLR5L
GM-CSF +TLR7L
Ly6Chigh Mono/Mac
Ly6Ghigh granulocytes
% / Myeloid
GM-CSF
+TLR5L
+TLR7L
MHCIIint DC
MHCIIhigh DC
E
Gated in Ly6Chigh Mono/Mac
Day3 Day4 Day5
CD11b
MERTK
Ly6C
F4/80
Ly6G
GM-CSF
+TLR5L
+TLR7L

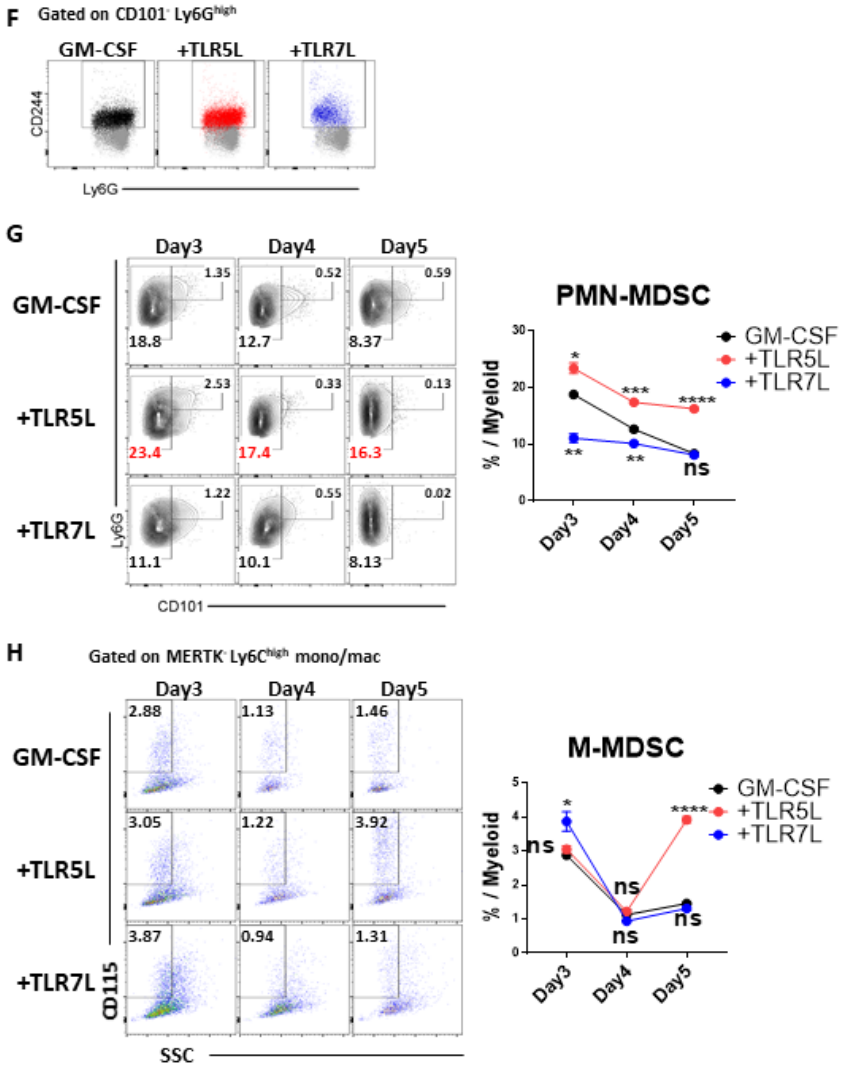

F
Gated on CD101- Ly6Ghigh
GM-CSF
+TLR5L
+TLR7L
CD244
Ly6G
G
Day3
Day4
Day5
GM-CSF
+TLR5L
+TLR7L
1.35
0.52
0.59
18.8
12.7
8.37
2.53
0.33
0.13
23.4
17.4
16.3
1.22
0.55
0.02
11.1
10.1
8.13
Ly6G
CD101
PMN-MDSC
% / Myeloid
GM-CSF
+TLR5L
+TLR7L
*
***
****
**
**
ns
H
Gated on MERTK- Ly6Chigh mono/mac
Day3
Day4
Day5
GM-CSF
+TLR5L
+TLR7L
2.88
1.13
1.46
3.05
1.22
3.92
3.87
0.94
1.31
CD115
SSC
M-MDSC
% / Myeloid
GM-CSF
+TLR5L
+TLR7L
*
****
ns
ns
ns
ns

**Figure 4. Enhanced macrophage differentiation by TLR7 L and increased MDSC by TLR5 L**

(A, B, C) tSNE dimensionality reduction and clustering of Dump (CD3e NK1.1 CD19)$^-$ populations based on the relative expression of five phenotypic markers, which were CD11c, MHCII, CD11b, Ly6G, and Ly6C. Cell subsets were delineated into eight groups (C) based on the expression of cell-subset phenotypic markers. The name of each population was annotated on tSNE graph (A). (D). BM cells cultured with GM-CSF on day 3 to day 5 were analyzed by tSNE. Among Dump$^-$ cells concatenated data, distribution of cells from three different culture conditions was shown. Data focused on four populations on each day: pink (Ly6C$^{high}$ Mono/Mac), orange (Ly6G$^{high}$ granulocytes), green (MHCII$^{int}$ DC), and purple (MHCII$^{high}$ DC). The percentage of each population in Dump$^-$ populations. (E). Expression of CD11b, Ly6C, Ly6G, MERTK, and F4/80 in Ly6C$^{high}$ Mono/Mac subset defined in (C) was assessed by flow cytometry. (F). CD244 expression of CD101$^-$Ly6G$^{high}$CD11c$^-$CD11b$^+$Gr-1$^+$ population from GM-CSF culture with TLR5 L or TLR7 L on day 3. (G). Ly6G$^{high}$ granulocytes subset was plotted by CD101 and Ly6G. The percentage of each populations in Dump$^-$ populations. (H). MERTK$^-$Ly6C$^{high}$ Mono/Mac subset were analyzed by CD115$^+$ and SSC. The percentage of each populations in Dump$^-$populations. (D, G, H) The significance of the graph in figures D, G, and H represented the TLR ligands treated groups compared to the GM-CSF group. All data are representative of three independent experiments. ns, no significance; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

**Increased CMP-GMP-GP lineage through TLR5 L and Reduction of overall progenitor through TLR7 L**

TLR5 L or TLR7 L treatment each led to different myelopoiesis, resulting in different myeloid cell differentiation. To determine what stage TLR5 L and TLR7 L act on myeloid cells to induce distinguished differentiation, SCF, IL-3 and IL-6 were used for culture to expand early progenitors, with additional TLR5 L or TLR7 L treatment.

According to myelopoiesis lineage of common myeloid progenitor (CMP) for DC-poiesis [5,7] (Figure 5A), the cell number of CMP increased, but the number of monocyte-DC progenitor (MDP) decreased significantly in TLR5 L-treated group. A slight decrease in the number of common DC progenitor (CDP) was observed in TLR5 L-treated group on day 1. Subsequently, the number of DCs and pDCs were significantly decreased on day 3 compared to the SCF+IL-3+IL-6 only (SCF mix) control. (Figure 5B, C, E)

In granulocyte differentiation lineage, the cell number of GMP and granulocyte progenitor (GP) was significantly increased in TLR5 L-treated group. (Figure 5D) Later, on day 3, $Ly6G^{high}$ granulocytes were significantly enhanced in TLR5 L-treated group compared to in SCF+IL-3+IL-6 only group. (Figure 5F) There was no significant difference in monocyte progenitor (MP), but a tendency to decrease slightly. (Figure 5D) The day 3 culture showed that $Ly6C^{high}$ monocytic cells diminished in TLR5 L-treated group. (Figure 5F) In summary, DC-poiesis was reduced by TLR5 L treatment and CMP-GMP-GP-granulocytes lineage was enhanced.

Unlike TLR5 L-treated group, the number of progenitors in TLR7 L-treated group all decreased except for GMP. (Figure 5B-D) The

numbers of DCs and pDCs also diminished in the TLR7 L-treated group on day 3 (Figure 5E), which was consistent with previous results in GM-CSF group. (Figure 2C) However, the number of $Ly6C^{high}$ cells in the TLR7 L-treated group increased significantly compared to other groups. From these results, it can be inferred that $Ly6C^{high}$ monocytic cell differentiation was induced by TLR7 L treatment.

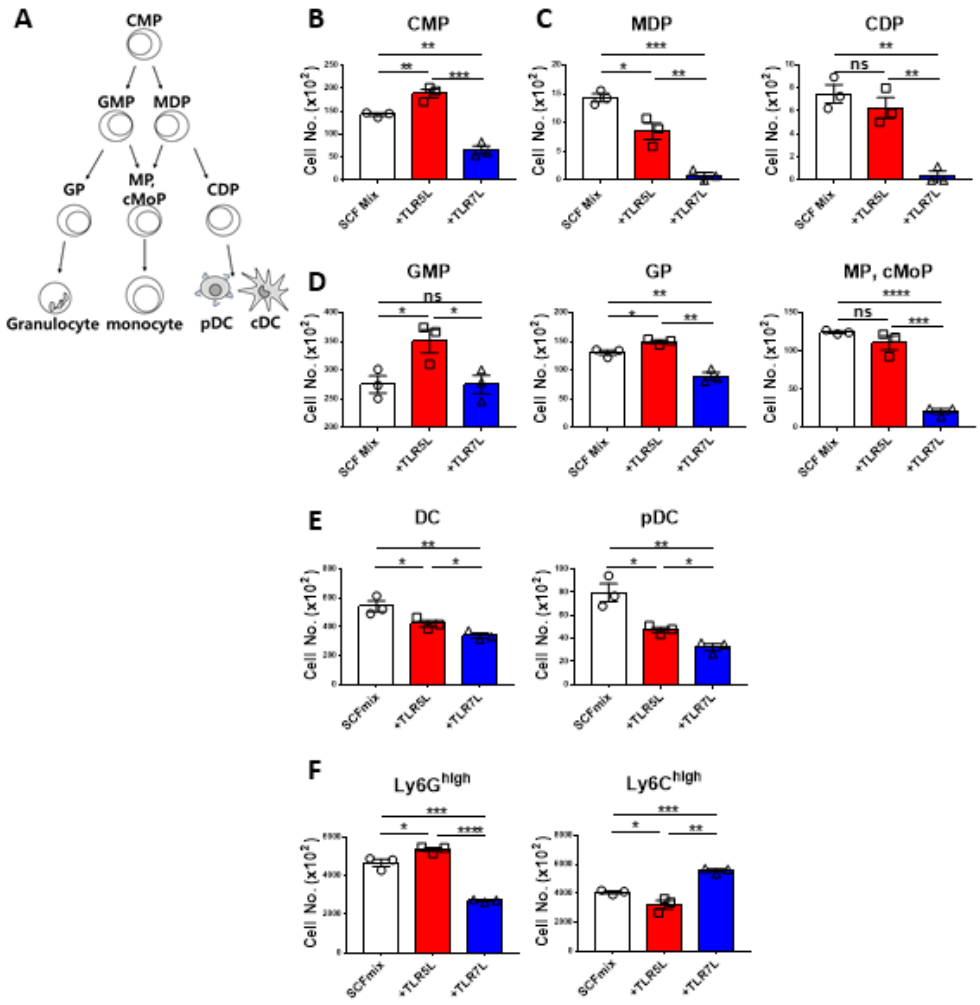

A
CMP
GMP
MDP
GP
MP, cMoP
CDP
Granulocyte
monocyte
pDC
cDC
B
CMP
C
MDP
CDP
D
GMP
GP
MP, cMoP
E
DC
pDC
F
Ly6G^high
Ly6C^high
Cell No. (x10^2)
SCF Mix
SCFmix
+TLR5L
+TLR7L
ns

**Figure 5. Increased CMP-GMP-GP lineage through TLR5 L and reduction of overall progenitor through TLR7 L**

(A). Hematopoietic tree of common myeloid progenitor (CMP) based on previous reports [5,7]. (B, C, D) Progenitors were basically gate in lineage $(CD3e\ CD19\ NK1.1\ Gr\text{-}1\ Sca\text{-}1\ CD127\ CD11b\ B220)^-$. CMP ($Lin^-\ CD117^+\ CD16/32^{int}\ CD115^-$), MDP ($Lin^-\ CD117^+\ CD16/32^{int}\ CD115^+$), CDP ($Lin^-\ CD117^-\ CD115^+\ CD135^+$), GMP ($Lin^-\ CD117^+\ CD16/32^{high}\ Ly6C^-\ CD115^-$), GP ($Lin^-\ CD117^+\ CD16/32^{high}\ Ly6C^+\ CD115^-$), and MP, cMoP ($Lin^-\ CD117^+\ CD16/32^{high}\ Ly6C^+\ CD115^+$) were assessed after SCF+IL-3+IL-6 culture for 1 day with TLR5 L or TLR7 L. Data are presented as means plus standard deviations of three independent experiments. (E, F) DC ($CD11c^+$), pDC ($B220^+CD11b^-Ly6C^+CD11c^+$), $Ly6G^{high}$ ($CD11c^-CD11b^+Gr\text{-}1^+Ly6G^{high}$), and $Ly6C^{high}$ ($CD11c^-CD11b^+Gr\text{-}1^+Ly6C^{high}$) were assessed after SCF+IL-3+IL-6 culture for 1 day with TLR5 L or TLR7 L. Data are presented as means plus standard deviations of three independent experiments. All data are representative of three independent experiments. ns, no significance; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$

**Highest expression level of TLR5 in GP and TLR7 in MP, cMoP**

Figure 5 showed that different stages of myeloid cells expanded depending on what TLR ligand was treated. Based on the results, it was hypothesized that the expression of TLR5 and TLR7 on different stages of progenitor made contrasting consequences on myelopoiesis. To verify this, the expression and the level of TLR5 and TLR7 in naive BM progenitors were analyzed.

Among the progenitors of BM cells in naive state, the relative MFI of TLR5 in GP was highest compared to other progenitors. Unlike GP, CDP hardly expressed TLR5. Among differentiated cells, relative MFI of TLR5 was highest in $Ly6G^{high}$ granulocytes. (Figure 6A) In terms of TLR7, MP and cMop expressed the highest level compared to other progenitors. As previously shown, TLR7 was highly expressed in $Ly6C^{high}$ monocytic cells and pDC. (Figure 6B) [33,34]

In summary, GP with high TLR5 expression seemed to differentiate into $Ly6G^{high}$ granulocytes when treated with TLR5 L. The MP and cMoP with the highest TLR7 L expression appear to differentiate into $Ly6C^{high}$ monocytic cells when treated with TLR7 L. Combined with results of Figure 5, these results suggest a strong correlation between the TLR expression of progenitors and the consequences of the differentiation.

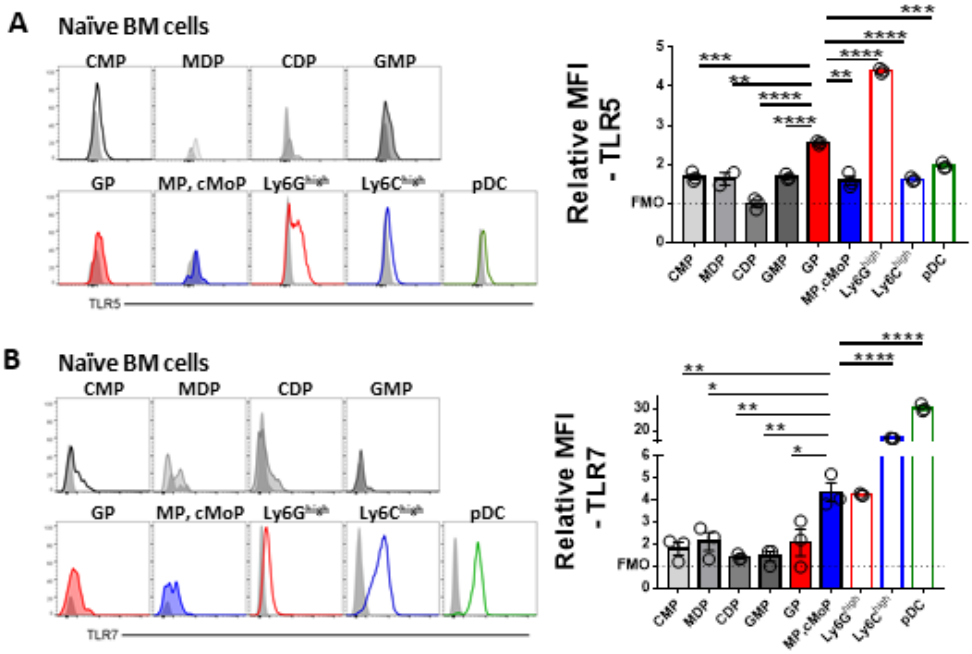



**Figure 6. Highest expression level of TLR5 in GP and TLR7 in MP, cMoP**

(A, B) Flow cytometric analysis of the expression of TLR5 (A) and TLR7 (B) on the various populations from naive B6 mouse. Gray filled histograms represent FMO controls for each population. The value of relative MFI shows the ratios of expression level to FMO control level. All data are representative of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001

**TLR5 L-treated myeloid cells are immune suppressive and TLR7 L-treated myeloid cells have immune activating status in SCF+IL-3+IL-6 culture**

Although SCF and IL-6 are well known hematopoietic stem cell expansion cytokines, previous research revealed that those growth factors induce MDSC expansion. [38,39] IL-3 induces the production of ROS, which contributes to the suppressive activity of MDSCs. [40] Figure 6 results suggest that the composition of $Ly6G^{high}$ granulocytes and $Ly6C^{high}$ monocytic cells in SCF+IL-3+Il-6 condition with TLR ligands recapitulated GM-CSF culture condition. As in the GM-CSF culture condition, T cell suppression assay was performed to determine whether the TLR5 L-treated myeloid cells could suppress activated T cell, though TLR7 L-treated myeloid cells could not. Consistent with the GM-CSF results, TLR5 L treatment made the myeloid cells more immune-suppressive than SCF+IL-3+IL-6 treated group. Unlike the results of TLR5 L treatment, TLR 7 L treatment made the myeloid cells more immune activate than SCF+IL-3+IL-6 treated group (Figure 7A) Consistent with the GM-CSF results, in the $CD11b^{+}$ $Gr-1^{+}$ population, $Ly6G^{high}$ granulocyte frequency was significantly higher in TLR5 L-treated culture, while $Ly6C^{high}$ monocytic cell frequency was significantly higher in TLR7 L-treated culture. (Figure 7B) Intra-cellular staining with arginase1 and IDO1 was performed to determine whether the myeloid cells from each culture condition are functionally suppressive. Arginase1 freqeuency of $Ly6C^{high}$ monocytic cell and $Ly6G^{high}$ granulocyte, and $IDO1^{+}$ frequency of $Ly6G^{high}$ granulocyte were similar between TLR5 L and TLR7 L-treated groups. On the contrary, the $IDO1^{+}$ frequency of $Ly6C^{high}$ monocytic cells was significantly decreased at TLR7 L-treated group. (Figure 7C)

TLR7 L-treated myeloid cells were less suppressive, and the frequency of stimulatory factors (CD40, CD80, CD86, MHCII) positive cells were also significantly elevated. In summary, in SCF+IL-3+IL-6 culture, suppressive Ly6G$^{high}$ granulocytes were enhanced in TLR5 L-treated group while the immune activating Ly6C$^{high}$ monocytic cell was increased in the TRL7 L-treated group, which was in line with results from GM-CSF-based culture.

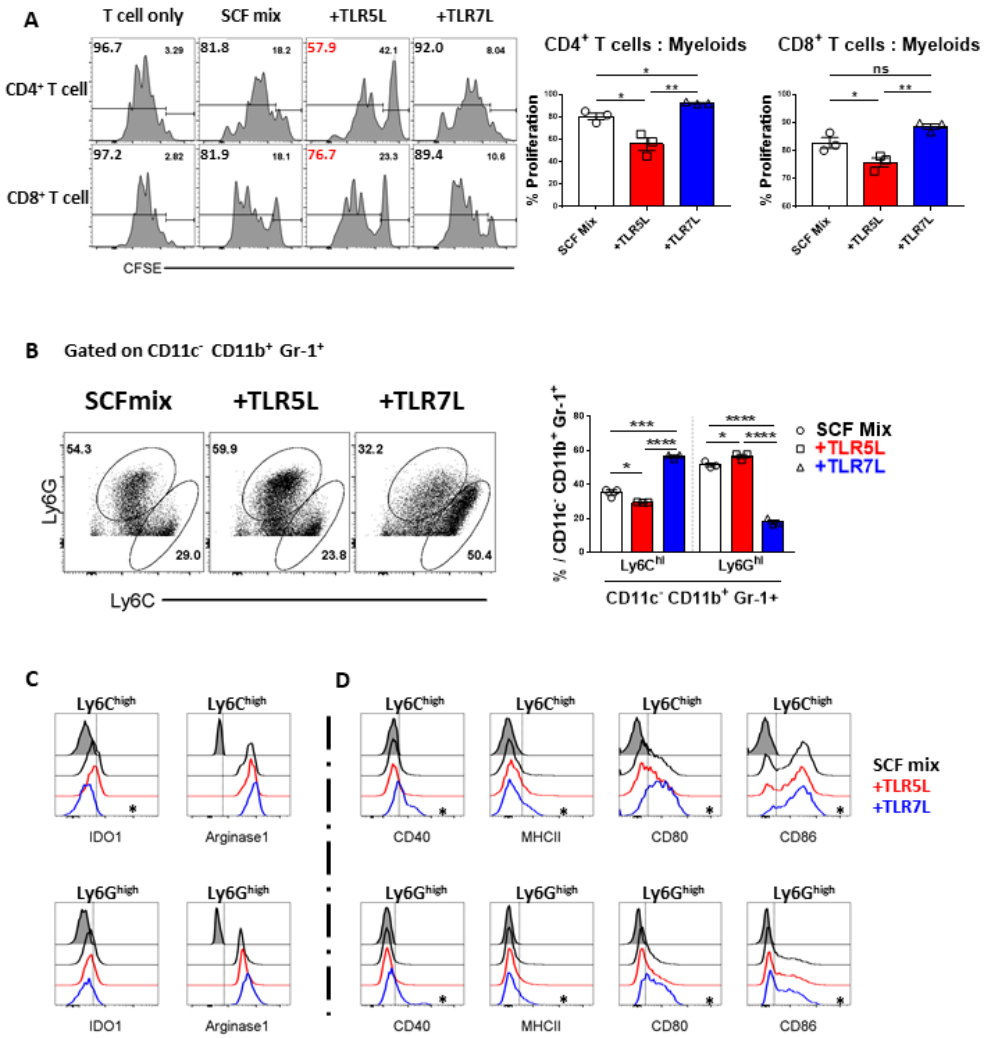

A
T cell only
SCF mix
+TLR5L
+TLR7L
CD4+ T cell
CD8+ T cell
96.7
3.29
81.8
18.2
57.9
42.1
92.0
8.04
97.2
2.82
81.9
18.1
76.7
23.3
89.4
10.6
CFSE
CD4+ T cells : Myeloids
CD8+ T cells : Myeloids
% Proliferation
SCF Mix
+TLR5L
+TLR7L
ns
B
Gated on CD11c- CD11b+ Gr-1+
SCFmix
+TLR5L
+TLR7L
Ly6G
Ly6C
54.3
29.0
59.9
23.8
32.2
50.4
% / CD11c- CD11b+ Gr-1+
SCF Mix
+TLR5L
+TLR7L
Ly6Chi
Ly6Ghi
CD11c- CD11b+ Gr-1+
C
D
Ly6Chigh
Ly6Ghigh
IDO1
Arginase1
CD40
MHCII
CD80
CD86
SCF mix
+TLR5L
+TLR7L

**Figure 7. TLR5 L-treated myeloid cells are immune suppressive and TLR7 L-treated myeloid cells have immune activating status in SCF+IL-3+IL-6 culture**

(A). Immune suppression assays of $Thy1.1^{+}CD4T$ cells and CD8T cells isolated from lymph node of the naïve mice. CFSE dilution was analyzed via flow cytometry after CD3/CD28 stimulation in the presence of BM cells from each condition. The proportions of proliferated $Thy1.1^{+}CD4^{+}$ and $Thy1.1^{+}CD8^{+}$ T cells were presented in histogram. (B). Flow cytometric analysis of $CD11c^{-}CD11b^{+}Gr\text{-}1^{+}$ BM-derived cell profiles on day 3 of SCF+IL-3+IL-6 culture with TLR5 L or TLR7 L. BM-MDSCs from day 3 were harvested and analyzed using flow cytometry. $CD11c^{-}CD11b^{+}Gr\text{-}1^{+}$ populations were plotted by using Ly6G and Ly6C. The percentage of $Ly6G^{high}$ cells and $Ly6C^{high}$ cells in the $CD11c^{-}CD11b^{+}Gr\text{-}1^{+}$ were presented. All data were representative of three independent experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$ (C). Arginase1 and IDO1 expression by $Ly6G^{high}$ and $Ly6C^{high}$ in $CD11c^{-}CD11b^{+}Gr\text{-}1^{+}$ population from SCF+IL-3+Il-6 culture with TLR5 L or TLR7 L on day 3. (D). CD40, MHCII, CD80, and CD86 histograms of $Ly6G^{high}$ and $Ly6C^{high}$ in $CD11c^{-}CD11b^{+}Gr\text{-}1^{+}$ population were presented. '*' represents the significance of the marker positive cell frequency referred to by each FACS plot. All data are representative of three independent experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$

# Discussion

MDSC was initially identified in tumor conditions. Many studies have been done on MDSC as it is a negative regulator of immune response in cancer or other disease conditions. Factors related to the expansion and activation of MDSC in cancer have been found to be GM-CSF, G-CSF, SCF, IL-6, etc. [9,10] In addition, there are reports that TLR2/6 L, TLR4 L, and TLR5 L induce MDSC. [21, 35]

In this experiment, TLR5 L treatment enhanced the progenitor of CMP-GMP-GP lineage. In addition, TLR5 L treatment promoted PMN-MDSC and M-MDSC, and thus had an immune-suppressive effect. TLR7 L treatment induced $Ly6C^{high}$ monocytic cell activation, resulting in an immune-activating effect. The cells were then identified to differentiate into macrophages.

Myeloid cells in TLR5 L- and TLR7 L-treated groups were both phenotypically $CD11b^{+}Gr\text{-}1^{+}$ cells. Although they both expressed basic MDSC markers, TLR5 L-treated myeloids could actively suppress T cells. This was because the frequency of PMN-MDSC in TLR5 L-treated group was higher than that of other groups. This result was consistent with the increase of PMN-MDSC through TLR5 L treatment, as in human results in previous studies. [21] Previous studies have reported that M-MDSCs differentiate into PMN-MDSCs. [32] PMN-MDSCs were maintained at the highest frequency compared to other groups until day 5. M-MDSC was decreased on day 4 and regenerated on day 5 in TLR5 L-treated group. This result was not a direct evidence of differentiation from M-MDSC to PMN-MDSC, but indicates that the M-MDSC on day 4 may differentiate into PMN-MDSC, thereby maintaining frequency of PMN-MDSC until day 5. In summary, MDSC generation through TLR5 L treatment showed

the potential for a new in-vitro MDSC culture condition. It is necessary to clarify and reconfirm that TLR5 L-derived MDSCs are effective suppressor cells in vivo, for example, by transferring them to graft versus host disease (GvHD) condition mice to see GvHD symptom alleviation.

Though TLR7 L-treated myeloid cells had many cells with MDSC phenotype of $CD11b^{+}Gr\text{-}1^{+}$, unlike suppressive cells, T cell proliferation was increased and stimulatory markers expression was increased in both $Ly6G^{high}$ and $Ly6C^{high}$ cells. In addition, low IDO1 level in $Ly6G^{high}$ granulocytes supported that the TLR7 L-treated myeloid cells had weak suppressive ability. It is well known that monocytes differentiate into monocytic dendritic cell and macrophage. However, in TLR7 L treatment, it is still controversial whether monocytes differentiate into macrophage or mo-DC. [20,31] In this study, it was shown that $Ly6C^{high}$ monocytic cells from TLR7 L-treated group differentiate into macrophages rather than mo-DCs. In summary, despite the MDSC culture condition, TLR7 L treatment made the myeloid cells immune stimulatory status and $Ly6C^{high}$ monocytic cell become macrophage.

I used a well-known MDSC culture method, GM-CSF BM culture. [35] In addition, a new combination of SCF, IL-3, IL-6 was used to increase the population of early progenitors, which were difficult to identify with GM-CSF cultures. As a result, myelopoiesis could be analyzed in detail.

Here, it was shown that TLR5 L and TLR7 L cause difference in myelopoiesis of HSPCs through the differential expression of TLRs in HSPC. Most of the previous studies focused on the effect of TLR ligands on already differentiated myeloid cells using growth factors

such as GM-CSF, and Flt3 ligands. [23, 24] In particular, there has not been much research on myelopoiesis of HSPCs with TLR ligands. Among them, previous reports showed that GMP-derived cells are increased through TLR4 L, LPS and that MDP-derived cells are facilitated through TLR9 L, CpG.

TLR5 L treatment increased the early progenitor of CMP-GMP-GP lineage. TLR5 expression was particularly high in GP, which was thought to contribute to the increase of $Ly6G^{high}$ granulocytes population. The expression of TLR7 in MP was the highest among progenitors, suggesting that TLR7 L treatment was responsible for the increase in $Ly6C^{high}$ monocytic cell population. It is considered that TLR7 L treatment induced $Ly6C^{high}$ monocytic cells, which expressed a high level of TLR7, activation and macrophage differentiation.

TLR7 L is known for pDC activation in Flt3L culture. [36] Unlike the Flt3L, GM-CSF and SCF+IL-3+IL-6 induced the GMP-derived myeloid differentiation. [36, 37, 42] Therefore, the effect of TLR7 L on pDC lineage was not well shown in GM-CSF and SCF+IL-3+IL-6 cultures.

Unlike TLR5 L, TLR7 L treatment significantly reduced the number of progenitors. It might be thought that the progenitors were all differentiated, resulting in decreased numbers of the progenitors. On the contrary, I assumed that this might be due to indirect effect of Type I IFN induced by TLR7 L rather than due to the direct effect of TLR7 L on HSPC. Because RNA seq of myeloid cells in the TLR7 L-treated group increased the Type I IFN (INFa, IFNb) responses. (data not shown) Previous studies have reported that type I IFNs express cell cycle inhibitors of HSPCs and suppress proliferation in in-vitro experiments. [25] In in vivo experiments, on the other hand, it has been reported that type I IFN-mediated HSPCs are activated and

enters the cell cycle. [26] Although the effect of Type I IFN on HSPC remains controversial, the results from TLR7 L-treated group were similar to the previous in-vitro reports. As it is still in the hypothesis stage, further experiments are needed to prove this.

In summary, myelopoiesis processes in which TLR5 L and TLR7 L act on early progenitors and the immune responses of TLR5 L- or TLR7 L-treated myeloid cells have been verified. Although the exact mechanism requires further testing, TLR5 L treatment increased the CMP-GMP-GP lineage, resulting in an increase in $Ly6G^{high}$ granulocytes. PMN-MDSCs and M-MDSCs formed through TLR5 L treatment were sufficient to suppress T cells. On the other hand, TLR7 L treatment decreased the number of progenitors but activated $Ly6C^{high}$ monocytic cells. $Ly6C^{high}$ monocytic cells from TLR7 L treatments subsequently differentiated into macrophages. These results indicate that the differential expression of TLR5 and TLR7 along the early myeloid precursors caused the differential outcomes of the respective stimulations. Altogether, these results provided an understanding of different myelopoiesis types by treating different TLR ligands.

# Reference


1. Mitroulis I, Kalafati L, Hajishengallis G, Chavakis T. 2018. Myelopoiesis in the Context of Innate Immunity. *J Innate Immun* 10: 365-72
2. Kawai T, Akira S. 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int Immunol* 21: 317-37
3. Vidya MK, Kumar VG, Sejian V, Bagath M, Krishnan G, Bhatta R. 2018. Toll-like receptors: Significance, ligands, signaling pathways, and functions in mammals. *Int Rev Immunol* 37: 20-36
4. Kawai T, Akira S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 11: 373-84
5. Yanez A, Goodridge HS, Gozalbo D, Gil ML. 2013. TLRs control hematopoiesis during infection. *Eur J Immunol* 43: 2526-33
6. Nagai Y, Garrett KP, Ohta S, Bahrun U, Kouro T, Akira S, Takatsu K, Kincade PW. 2006. Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. *Immunity* 24: 801-12
7. Yanez A, Coetzee SG, Olsson A, Muench DE, Berman BP, Hazelett DJ, Salomonis N, Grimes HL, Goodridge HS. 2017. Granulocyte-Monocyte Progenitors and Monocyte-Dendritic Cell Progenitors Independently Produce Functionally Distinct Monocytes. *Immunity* 47: 890-902 e4
8. Landskron G, De la Fuente M, Thuwajit P, Thuwajit C, Hermoso MA. 2014. Chronic inflammation and cytokines in the tumor microenvironment. *J Immunol Res* 2014: 149185

9. Veglia F, Perego M, Gabrilovich D. 2018. Myeloid-derived suppressor cells coming of age. *Nat Immunol* 19: 108-19
10. Gabrilovich DI, Nagaraj S. 2009. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 9: 162-74
11. Yu J, Du W, Yan F, Wang Y, Li H, Cao S, Yu W, Shen C, Liu J, Ren X. 2013. Myeloid-derived suppressor cells suppress antitumor immune responses through IDO expression and correlate with lymph node metastasis in patients with breast cancer. *J Immunol* 190: 3783-97
12. Evrard M, Kwok IWH, Chong SZ, Teng KWW, Becht E, Chen J, Sieow JL, Penny HL, Ching GC, Devi S, Adrover JM, Li JLY, Liong KH, Tan L, Poon Z, Foo S, Chua JW, Su IH, Balabanian K, Bachelerie F, Biswas SK, Larbi A, Hwang WYK, Madan V, Koeffler HP, Wong SC, Newell EW, Hidalgo A, Ginhoux F, Ng LG. 2018. Developmental Analysis of Bone Marrow Neutrophils Reveals Populations Specialized in Expansion, Trafficking, and Effector Functions. *Immunity* 48: 364-79 e8
13. Lee YK, Ju JM, Shon WJ, Oh S, Min CK, Kang MS, Shin DM, Choi EY. 2018. Skewed Dendritic Cell Differentiation of MyD88-Deficient Donor Bone Marrow Cells, Instead of Massive Expansion as Myeloid-Derived Suppressor Cells, Aggravates GVHD. *Immune Netw* 18: e44
14. Schwab L, Goroncy L, Palaniyandi S, Gautam S, Triantafyllopoulou A, Mocsai A, Reichardt W, Karlsson FJ, Radhakrishnan SV, Hanke K, Schmitt-Graeff A, Freudenberg M, von Loewenich FD, Wolf P, Leonhardt F, Baxan N, Pfeifer D,

Schmah O, Schonle A, Martin SF, Mertelsmann R, Duyster J, Finke J, Prinz M, Henneke P, Hacker H, Hildebrandt GC, Hacker G, Zeiser R. 2014. Neutrophil granulocytes recruited upon translocation of intestinal bacteria enhance graft-versus-host disease via tissue damage. *Nat Med* 20: 648-54

15. S Jensen S, Parhamifar L. 2016. Targeting Monocytes with TLR7 Ligands as a Novel Opportunity in Immuno- Oncology. *Immunotherapy: Open Access* 02
16. Feuillet V, Medjane S, Mondor I, Demaria O, Pagni PP, Galán JE, Flavell RA, Alexopoulou L. 2006. Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria. *PNAS.* 103 (33) 12487-12492
17. Vijay-Kumar M, Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S, Sitaraman SV, Knight R, Ley RE, Gewirtz1 AT. 2010. Metabolic Syndrome and Altered Gut Microbiota in Mice Lacking Toll-Like Receptor 5. *Science* 328 (5975), 228-231.
18. Maaser C, Heidemann J, von Eiff C, Lugering A, Spahn TW, Binion DG, Domschke W, Lugering N, Kucharzik T. 2004. Human intestinal microvascular endothelial cells express Toll-like receptor 5: a binding partner for bacterial flagellin. *J Immunol* 172: 5056-62
19. Shirota H, Tross D, Klinman DM. 2015. CpG Oligonucleotides as Cancer Vaccine Adjuvants. *Vaccines (Basel)* 3: 390-407
20. Sioud M, Floisand Y, Forfang L, Lund-Johansen F. 2006. Signaling through toll-like receptor 7/8 induces the differentiation of human bone marrow CD34+ progenitor cells

along the myeloid lineage. *J Mol Biol* 364: 945-54

21. Rieber N, Brand A, Hector A, Graepler-Mainka U, Ost M, Schafer I, Wecker I, Neri D, Wirth A, Mays L, Zundel S, Fuchs J, Handgretinger R, Stern M, Hogardt M, Doring G, Riethmuller J, Kormann M, Hartl D. 2013. Flagellin induces myeloid-derived suppressor cells: implications for Pseudomonas aeruginosa infection in cystic fibrosis lung disease. *J Immunol* 190: 1276-84
22. Liu H, Chen F, Wu W, Cao AT, Xue X, Yao S, Evans-Marin HL, Li YQ, Cong Y. 2016. TLR5 mediates CD172alpha(+) intestinal lamina propria dendritic cell induction of Th17 cells. *Sci Rep* 6: 22040
23. Xu Y, Zhan Y, Lew AM, Naik SH, Kershaw MH. 2007. Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. *J Immunol* 179: 7577-84
24. Macal M, Jo Y, Dallari S, Chang AY, Dai J, Swaminathan S, Wehrens EJ, Fitzgerald-Bocarsly P, Zuniga EI. 2018. Self-Renewal and Toll-like Receptor Signaling Sustain Exhausted Plasmacytoid Dendritic Cells during Chronic Viral Infection. *Immunity* 48: 730-44 e5
25. Pietras EM, Lakshminarasimhan R, Techner JM, Fong S, Flach J, Binnewies M, Passegue E. 2014. Re-entry into quiescence protects hematopoietic stem cells from the killing effect of chronic exposure to type I interferons. *J Exp Med* 211: 245-62
26. Essers MA, Offner S, Blanco-Bose WE, Waibler Z, Kalinke U, Duchosal MA, Trumpp A. 2009. IFNalpha activates dormant haematopoietic stem cells in vivo. *Nature* 458: 904-8
27. Hua Z, Hou B. 2013. TLR signaling in B-cell development and

activation. *Cell Mol Immunol* 10: 103-6

28. Li Q, Yan Y, Liu J, Huang X, Zhang X, Kirschning C, Xu HC, Lang PA, Dittmer U, Zhang E, Lu M. 2019. Toll-Like Receptor 7 Activation Enhances CD8+ T Cell Effector Functions by Promoting Cellular Glycolysis. *Front Immunol* 10: 2191
29. Soudja SM, Ruiz AL, Marie JC, Lauvau G. 2012. Inflammatory monocytes activate memory CD8(+) T and innate NK lymphocytes independent of cognate antigen during microbial pathogen invasion. *Immunity* 37: 549-62
30. Matsushima H, Geng S, Lu R, Okamoto T, Yao Y, Mayuzumi N, Kotol PF, Chojnacki BJ, Miyazaki T, Gallo RL, Takashima A. 2013. Neutrophil differentiation into a unique hybrid population exhibiting dual phenotype and functionality of neutrophils and dendritic cells. *Blood* 121: 1677-89
31. Sioud M, Floisand Y. 2007. TLR agonists induce the differentiation of human bone marrow CD34+ progenitors into CD11c+ CD80/86+ DC capable of inducing a Th1-type response. *Eur J Immunol* 37: 2834-46
32. Youn JI, Kumar V, Collazo M, Nefedova Y, Condamine T, Cheng P, Villagra A, Antonia S, McCaffrey JC, Fishman M, Sarnaik A, Horna P, Sotomayor E, Gabrilovich DI. 2013. Epigenetic silencing of retinoblastoma gene regulates pathologic differentiation of myeloid cells in cancer. *Nat Immunol* 14: 211-20
33. Merad M, Sathe P, Helft J, Miller J, Mortha A. 2013. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* 31: 563-604

34. Reizis B, Bunin A, Ghosh HS, Lewis KL, Sisirak V. 2011. Plasmacytoid dendritic cells: recent progress and open questions. *Annu Rev Immunol* 29: 163-83
35. Ribechini E, Greifenberg V, Sandwick S, Lutz MB. 2010. Subsets, expansion and activation of myeloid-derived suppressor cells. *Med Microbiol Immunol* 199: 273-81
36. Saitoh SI, Abe F, Kanno A, Tanimura N, Mori Saitoh Y, Fukui R, Shibata T, Sato K, Ichinohe T, Hayashi M, Kubota K, Kozuka-Hata H, Oyama M, Kikko Y, Katada T, Kontani K, Miyake K. 2017. TLR7 mediated viral recognition results in focal type I interferon secretion by dendritic cells. *Nat Commun* 8: 1592
37. Zhang CC, Lodish HF. 2005. Murine hematopoietic stem cells change their surface phenotype during ex vivo expansion. *Blood* 105: 4314-20
38. Bunt SK, Yang L, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. 2007. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. *Cancer Res* 67: 10019-26
39. Pan PY, Wang GX, Yin B, Ozao J, Ku T, Divino CM, Chen SH. 2008. Reversion of immune tolerance in advanced malignancy: modulation of myeloid-derived suppressor cell development by blockade of stem-cell factor function. *Blood* 111: 219-28
40. Sauer H, Wartenberg M, Hescheler J. 2001. Reactive Oxygen Species as Intracellular Messengers During Cell Growth and Differentiation. *Cell Physiol Biochem* 11:173-186

41. Assier E, Marin-Esteban V, Haziot A, Maggi E, Charron D, Mooney N. 2007. TLR7/8 agonists impair monocyte-derived dendritic cell differentiation and maturation. *J Leukoc Biol* 81: 221-8

42. Ost M, Singh A, Peschel A, Mehling R, Rieber N, Hartl D. 2016. Myeloid-Derived Suppressor Cells in Bacterial Infections. *Front Cell Infect Microbiol* 6: 37

43. Christensen SR, Shupe J, Nickerson K, Kashgarian M, Flavell RA, Shlomchik MJ. 2006. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. *Immunity* 25: 417-28

44. Deane JA, Pisitkun P, Barrett RS, Feigenbaum L, Town T, Ward JM, Flavell RA, Bolland S. 2007. Control of toll-like receptor 7 expression is essential to restrict autoimmunity and dendritic cell proliferation. *Immunity* 27: 801-10

45. https://cran.r-project.org/web/packages/Rtsne/

# 국문 초록

면역 활성화와 면역 억제 사이의 균형은 면역 항상성에 중요하다. 톨-유사 수용체 (TLRs)는 다양한 TLR 리간드를 인식하여, 선천 면역계에서 병원체 감염에 대한 염증 반응을 개시하는 결정적인 역할을 한다. 병원체 감염을 통한 TLR 신호전달은 이미 분화가 이뤄진 골수성 세포의 활성을 일으키는 것뿐만 아니라, 조혈 줄기 및 초기 세포 (HSPC)의 긴급 골수 조혈을 초래하여 감염에 대처하게 한다. 하지만 TLR-TLR 리간드의 차이에 따라 서로 다른 골수성 세포가 분화되고 이와 연계된 면역반응 또한 다르게 나타난다. 예를 들어 TLR5는 면역 억제로 잘 알려져 있고 TLR7은 면역 활성으로 잘 알려져 있다. TLR5와 TLR7은 MyD88 adaptor를 통한 공통의 신호전달 체계를 갖고 있음에도 불구하고 각각 면역 억제 또는 면역 활성 같은 서로 반대의 면역반응을 나타내는데, TLR5 리간드, flagellin과 TLR7 리간드, gardiquimod가 골수성 세포 분화과정에 미치는 구체적인 세포 기전은 아직 알려지지 않았다. 따라서, 본 연구는 TLR5 리간드가 과립구성 골수 유래 억제 세포 (PMN-MDSC) 뿐만 아니라 단핵구성 골수 유래 억제 세포를 증가시키고 수지상세포 (dendritic cell) 분화과정을 감소시키는 것을 통해 면역 억제 반응을 일으킨다는 것을 확인하였다. 이와는 반대로 TLR7 리간드가 $CD11b^{+}Ly6C^{high}Ly6G^{int}$ monocytic cell의 활성화를 유도하여 면역 활성화를 일으키며, 이후 대식세포로 분화한다는 것을 밝혔다. 과립구 전구체(GP)에서 높게 발현되는 TLR5를 통해 TLR5 리간드가 $Ly6G^{high}$ 과립구 분화과정을 촉진시키며, 단핵구 전구체 (MP)에서 높게 발현되는 TLR7을 통해 TLR7 리간드가 $Ly6C^{high}$ 단핵구 분화과정을 촉진시키는 세포 기전을 확인하였다. 이러한 결과는 초기 골수성 전구체의 TLR5 및 TLR7의 차등 발현이 각각의 자극에 따른 차등 결과를 야기한다는 것을 나타낸다. 정리하여, 위 결과를 통해 TLR5 리간드는 GP-$Ly6G^{high}$ 과립구 분화과정을 촉진을 통한 과립구성 골수 유래 억제 세포를 증가시켜 면역 억제를 유도하고 TLR7 리간드는

MP-Ly6C$^{high}$ 단핵구 분화과정을 유도하여 면역 활성화를 일으킨다는 것을 제안하였다.

주요어: TLR5, TLR7, 골수성 세포, 골수성 세포 분화, 전구체
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