國立臺灣大學醫學院免疫學研究所

碩士論文

Graduate Institute of Immunology College of Medicine

National Taiwan University

Master Thesis

*Mef2c*轉錄因子調控漿狀樹突細胞發育

Transcription factor *Mef2c* regulates the development of plasmacytoid dendritic cells

林怡文

Yi-Wen Lin

指導教授：李建國 博士

Advisor: Chien-Kuo Lee, Ph.D.

中華民國 106 年 7 月

July 2017

doi:10.6342/NTU201703495

致謝

時光飛逝，兩年碩班生活就這樣結束了。我並非一個極其刻苦耐勞的人，實驗不順遂的時候很常就會想放棄，但因為身邊許多人的鼓勵與幫助才能這樣撐過來。首先要感謝的是李老師，在我碩士班的兩年裡面教會我許多東西，不只在免疫學上的知識、實驗設計的邏輯，甚至在做學問的態度上老師都給了我很好的榜樣，也很感激老師都願意花時間與我討論，並循循善誘指引我方向讓我實驗能夠更進一步。然後衷心感謝實驗室的明勳學長，學長不只教導我許多實驗，並且總是很照顧我，就像是我在實驗室的家人。謝謝苡帆，幫助我許多，好多實驗上困難的部分都多虧你的幫忙，在實驗部分也是有你的討論才能夠激發我一些新的想法。謝謝婷婷學姐在動物實驗以及細胞實驗上總是不厭其煩的回答我好多問題，並且也教會我許多做人做事的態度。謝謝京育學姐總是很關心我，也都在我實驗極其不順的時候傾聽並且鼓勵我。謝謝學妹又綾在我碩二崩潰的一年裡陪我一起發牢騷一起吃飯減輕我不少壓力。謝謝思穎、宇瑞學姐以及 Chris 學長在這些日子裡總是一起分享熱量與負能量，實驗之餘有你們的陪伴很開心。謝謝實驗室的大家在各項大小事情上面的幫助與照顧；也謝謝免疫所的同學一起努力跨越這些難關相互砥礪。

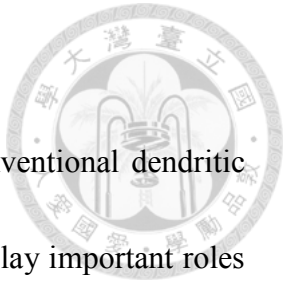
最後很真心的感謝在我情緒失落的時候總是願意在我身邊耐心陪伴傾聽的朋友彥蓉、亭君、尹媛、智慧、霍俊宇，沒有你們的安撫與鼓勵我可能撐不到今天。謝謝大學老師黛苹在我曾經最徬徨的時候義不容辭地幫助我。感謝我的父母親總是無條件包容關心我在這兩年內的負面情緒並且支持我完成學業。最後謝謝一切的緣分讓我來到台北學習與成長，經歷這一切，蛻變成現在的模樣。

doi:10.6342/NTU201703495

摘要

樹突狀細胞 (Dendritic cell, DC)主要分為傳統樹突細胞以及漿狀樹突細胞，而他們兩者的功能是連結先天免疫以及適應免疫，因此對於免疫的調節極為重要。雖然我們已知樹突細胞對於免疫調節的重要性，但相關於樹突細胞的分化途徑以及其調控的機轉仍然不清楚。而在這裡我們利用 shRNA 去靜默造血幹細胞與前期細胞株 (immortalized stem and progenitor cell line, iHSPC)的基因表現量，以此實驗方法去作為篩選策略，研究這些基因對於樹突細胞發育的影響。我們總共篩選了 14 個在漿狀樹突細胞中相較於傳統樹突細胞有較高量表現的轉錄因子，接著我們發現 *Mef2c* 跟 *Tcf12* 這兩個基因對於漿狀樹突細胞的生成有非常明顯的影響，因此我們以這兩個基因作為我們主要研究的目標。我們抑制了這兩個基因在 iHSPC 細胞株裡的表現量，並且利用 MS-5 滋養層細胞去進行培養，也發現漿狀樹突細胞的生成有減少的情形。有關於機制層面的研究則顯示了在此兩基因被抑制的 iHSPC 細胞株發育過程中 *Tcf4* (目前已知影響漿狀樹突細胞最重要的轉錄因子)的表現量有降低的傾向。並且我們利用報導基因系統分析，當 *Mef2c* 被過度表現的時候其 *Tcf4* 的活性相較於控制組增加了近一倍。最後，我們分析了 *Mef2c* 基因剔除的小鼠裡樹突細胞的分群，並且發現漿狀樹突細胞在骨髓、脾臟、淋巴結都有明顯減少的情形，而且在脾臟以及淋巴結內的減少比骨髓更顯著，暗示著漿狀樹突細胞可能有遷徙的缺陷。而我們利用慢病毒感染小鼠骨髓細胞，以進行體外剔除基因的方式，也觀察到漿狀樹突細胞生成的減少。除此之外，*Mef2c* 也會正向調控 *Flt3* 受器的表現，我們在靜默 *Mef2c* 的 iHSPC 細胞株內也觀察到 *Flt3* 表現量下降的情形。藉由這些機制層面，我們認為 *Mef2c* 對於漿狀樹突細胞的調節機轉可能有二個層面分別是透過 *Tcf4/Flt3* 來控制生成及未知因素來調控細胞自骨髓遷移到周邊。

doi:10.6342/NTU201703495



Abstract

Dendritic cells (DC) can classified into two subsets, namely conventional dendritic cells (cDC) and plasmacytoid dendritic cells (pDC), both of which play important roles in bridging the innate and adaptive immunity. Although the functions of DCs are critical for immunomodulation, the regulatory mechanisms of DC developmental process still unclear. Here, we performed a powerful screening strategy and identified *Mef2c* as a transcription factor which regulates DC development. Through shRNA-mediated knockdown of target genes in immortalized hematopoietic stem and progenitor (iHSPC) cell line, we have screen 14 transcription factors that are preferentially expressed in pDC versus cDC, we identified two transcription factors *Mef2c* and *Tcf12*, which significantly affected pDCs generation in feeder free culture system. Knockdown of *Mef2c* and *Tcf12* in iHSPC cell line decrease pDC generation in MS-5 feeder system. Mechanistically, expression of *Tcf4* (encodes E-2.2), a master regulation of pDC development, was reduced in iHSPC stably express shMef2*c* and sh*Tcf12*. Reporter assay also showed the *Tcf4* reporter activity was up-regulated by overexpression of *Mef2c*. We analyzed the DC populations from Mef2c$^{f/f}$ Tie2-Cre mice and proved that *Mef2c* deficiency indeed alter DCs generation, it reduce pDCs generation from bone marrow, spleen to lymph node. Also, in vitro deletion of *Mef2c* in primary bone marrow cell decreased pDCs frequency compared to control which show coherence to ex vivo

doi:10.6342/NTU201703495

results. Moreover, *Mef2c* also positively regulate Flt3 receptor expression. *Mef2c* knockdown decreased Flt3 expression in iHSPC. These results suggest *Mef2c* may regulate pDC development through control the expression of *Tcf4*.

doi:10.6342/NTU201703495

Table of contents

doi:10.6342/NTU201703495

doi:10.6342/NTU201703495

Chapter I Introduction

doi:10.6342/NTU201703495

1.1 Dendritic cell subsets

Dendritic cells (DCs) were first discovered by Dr.Ralph Steinman who named the cells after its dendrite-like morphology (Steinman and Cohn 1973). DCs resident in lymphoid tissues can be classified into conventional DCs (cDCs) and pDCs (pDCs) by different surface markers under steady state (Belz and Nutt, 2012). DCs including cDC and pDC can express MHC class I and high levels of MHC class II (Steinman, Kaplan et al. 1979). DCs have the greater antigen processing and presentation ability after they captured antigens, and then present them to naïve T cells. Therefore, DCs are known to serve a bridge between the innate and adaptive immunity (Banchereau and Steinman 1998).

cDCs constitutively express MHCII and CD11c, thus showing better performances in antigen capture and presentation (Merad, Sathe et al. 2013). Also, cDCs can be further classified in to cDC1 and cDC2 accroding to markers $CD8\alpha^+$ and $CD11b^+$respectitively (Mildner and Jung 2014). Since CD8 expresion is not found on in vitro-derived cDC, CD24 is used as the surrogate marker of CD8 (Naik, Proietto et al. 2005), Sirpα is used instead of CD8.

pDCs were named after the morphology resemblance to plasma cells and featuring in the massive secretion amount of type I IFN during the infections with viruses or other microbial pathogens (Colonna, Trinchieri et al. 2004). Apart from cDCs, pDCs express

doi:10.6342/NTU201703495

CD11c at intermediate levels, B220, Ly6c, Bst2 and Siglec-H (Swiecki and Colonna 2015).

1.2 Dendritic cell progenitors

DC are short-lived and are constantly replenished from their progenitos of myeloid lineages common myeloid progenitors (CMP) and lymphoid lineages common lynphoid progenitors (CLP) (Gabrilovich et al., 2012). CLP and CMP are capable to give rise in the generation of both pDCs and cDCs (Sathe, Vremec et al. 2013). However, Common dendritic cell progenitors (CDP) , are thought to be the progenitor cell lineage which can give rise to both cDCs and pDCs (Onai et al., 2007) We have previously demonstrated that CLP show higher potential in generating pDCs (Chen, Chen et al. 2013) than do CMP and CDP.

1.3 Transcription factors and cytokines involved in dendritic cells development

GM-CSF and Flt3L are two main cytokines that are well known to be involved in DC development (Watowich and Liu 2010). GM-CSF drives the differentiation pathways to cDCs (Inaba, Inaba et al. 1992), while Flt3L show prominent effects on both pDCs and cDCs generation (Gilliet, Boonstra et al. 2002). GM-CSF drives cDCs development

doi:10.6342/NTU201703495

by the enchancing expression of ID2 protein, a transcription factor depends on STAT5 signaling. FLT3L then enchances pDCs proliferation and differentiation by up-regulating the expression of transcription factor E2-2 via STAT3 signaling (Li, Yang et al. 2012). E2-2 is a family member of E-protein family while ID2 belongs to ID protein family (Kee 2009). E2-2 plays an important role in regulating many pDCs-related gene, such as *SpiB*, *Irf8*, and *Runx2*. These results identify E2-2 as a specific transcriptional regulator of the pDC lineage (Cisse, Caton et al. 2008). Whereas, ID2 shows influence on decreased cDCs number and deficiency in Langerhan cells are found in $Id2^{-/-}$ mice. Also, ID2 inhibits pDCs development by antagonizing E2-2. (Hacker, Kirsch et al. 2003)

1.4 *Mef2c*

Mef2c (Myocyte Enhancer Factor 2C, also known as MADS box transcription enhancer factor 2) is a member of the Mef2 family of transcription factors. In previous studies a central role for the MEF2 family of transcription factors has been linked to calcium-dependent signaling pathways to the genes responsible for cell division, differentiation and cell death (McKinsey, Zhang et al. 2002). There are four members in the family, from *Mef2a, 2b, 2c* and *2d*. But among Mef2 family, only *Mef2c* has been found to involve in hematopoiesis. Most aboundant researches about *Mef2c* is related to B cell development. Mef2c deficient will cause B cell development blockage from the

doi:10.6342/NTU201703495

stage pro-B to pre-B (Wang et al., 2016) . But there were no further studies about Mef2c in DCs.

1.5 *Tcf12*

Tcf12 belongs to E protein family, it encodes the protein TCF12 (transcription factor12), which previously named as HEB protein. The members of E protein family all contain helix-loop-helix (HLH) domain and the domain binds to Ephrussi-box (E-box) sequences(CANNTG), their function were first indefinied in dimerization for regulating transcription in B cells (Murre et al., 1989). Tcf12 has beeen discovered to have playing roles in regulating B-lymphocyte development in combined dosage of three E protein, which is E2A, E2-2, and HEB (Zhuang et al., 1996). Also HEB are found required to block thymocyte proliferation prior to pre-TCR expression (Wojciechowski et al., 2007).

1.6 Rationale

DCs bridge the innate and the adaptive immune response. cDCs, one of the DC subsets, display excellent antigen presenting ability. While pDCs are critical for the secretion of large amounts of type I interferon for combaring virus infection. Recent studies also diplaying evidences that population of DCs are getting complicated. And the populaion all having different charasteristc in regulating immune responses. However, the life span of DC are short, eventhough DCs comes from multiple origins.

doi:10.6342/NTU201703495

Thus, by knowing the developmental process of dendritic cells might help improve immune modulation questions.

Specific aims

1. To screen for the potential genes which regulate DC differentiaion..
2. To confirm genes that have effects on pDCs generation.
3. Find out the specific roles how *Mef2c* demonstrated in regulating dendritic cells development.

doi:10.6342/NTU201703495

Chapter II Materials and Methods

doi:10.6342/NTU201703495

Antibodies, plasmid and animals.

The amounts of antibodies were used for flow cytometry 0.2 μL for 10^6 cells. The cells stained with staining buffer (0.5% FBS and 0.1% NaN_3 in PBS) and mixed up with Fc blocker (hybridoma 2.4G2) for 15 minutes. The stained cells were washed once with staining buffer before analysis. The flow data were analyzed by FlowJo.

All antibodies used for flowcytomerty staining and cell sorting were listed in Table 1.

Ets-1 knock out mice and their heterozygous littermates were kindly provided from Dr. Tai Tzung Shiuan, E-DA Medical Research Department.

Tcf4 reporter plasmid containing −1026 to +27 bp of the promoter region (kindly provieded by Stephanie Watowich MD, Anderson cancer center) was subcloned to drive expression of eGFP. *Tcf4* reporter plasmid, Mef2c pcDNA3.1, pcDNA3 were purified by Qiagen midi kits.

Production of iHSPC cell line

Murine bone marrow cells were collected by flushing femurs and the cells were infected with virus expressing ERHBD-Hoxb8, a fusion construct of Hoxb8 and the hormone-binding domain of the estrogen receptor. After infection, cells were culture in

doi:10.6342/NTU201703495

the presence of estradiol and Flt3L. Once the cells were stably expanding, clones with high pDC potential were chosen. As described previously, the cell line is culture in the prescence of estradiol and Flt3L.

In vitro DC culture from iHSPC cell line

For feeder-free system iHSPC cells after lentiviral transduction and puromycin selection were plated $5x10^5$ cells/well in 12-well tissue culture plate with the prescence of Flt3L 100 ng/ml. At day 3 fresh medium with Flt3L in final concentration 100 ng/ml was supplemented. DC analysis were performed at day 6 with the following antibodies: APC-cy7-Streptavidin, FITC anti-CD11b, PE anti-B220, APC anti-CD24, PE-cy7 anti-SIRPα and Percp-cy5.5 anti-Siglec-H. cDC was defined as $CD11c^+CD11b^+B220^-$ and pDC was defined as $CD11c^+CD11b^-B220^+$. The cDC was further divided into cDC1($CD24^+Sirp\alpha^-$) and cDC2 ($CD24^-Sirp\alpha^+$).

For MS-5 feeder culturd system, MS-5 were plated $5.9x10^4$ cells into 12 well-plate 1 day before to reach a confluency of 80%, followed by γ-irradiation with 3,000 rads.

One thousand iHSPC cells per well were co-cultured with the MS-5 cells supplied with the Flt3L 100 ng/ml for 6 days. The derivied cells were subjected to DC staining and analyzed by flow cytometry.

doi:10.6342/NTU201703495

Lentiviral transduction

Lentivirus carrying shRNA were purchased from Academia Sinica RNAi Core Facility. iHSPCs were resuspend in OPTI-MEM medium, with 100 ng/ml Flt3L and 1mM/ml estrodiol, and plated in 24-well tissue culture plate. The iHSPCs were infected with lentiviruses at an M.O.I=100 in 500 µl medium containing polybrene 0.8µg/ml. The cells and viruses were subjected to spin infection centrifugation at 2000 g, 25℃ for 2 hr. After the spin infection, 500µl medium were added to dilute the toxicity of polybrene. The medium was refreshed day after spin infection, and puromycin selection (0.2µg/ml) were added to select transduced cells on day 3 for at least one week.

RT-qPCR

Total RNA from iHSPC cells was prepares using Trisure reagent (Bioline). Following extraction by using choloroform, isopropanol, and the RNA was resolved in 0.1 %DEPC water. One to three µg of RNA were used for the generation of cDNA using reverse transcriptase. The expression levels of *Mef2c*, *Tcf12* and *Rpl7* were analyzed by quantitative PCR and relative expression normalized to *Rpl7*.

From sorted primary pDCs and cDCs mRNA isolation from primary pDCs and cDCs was prepared by using TurboCapture kit (QIAAGEN), followed by cDNA synthesis via HiScript I reverse transcription (BIONOVAS) using random primer. The

doi:10.6342/NTU201703495

primer sets were listed in Table 2.

Sorting of primary progenitor cells and analysis of ex vivo cDC and pDC

Bone marrow were first flushed out from tibiae and femurs of mice and RBC were lysed by ACK buffer.

Bone marrow cells were stained for FITC anti-Sca-1, Percp-cy5.5 anti-c-kit, PE-cy7 anti-IL7Rα, APC anti-MCSFR and lineage markers including CD3, CD8, CD19, NK1.1, CD11b, MHCII, Ter119, B220 and Gr1. CLP, the population is defined as Lin^- $MCSFR^-$ $IL7R\alpha^+$ $c\text{-}kit^{int}$ $Sca\text{-}1^{int}$ is sorted with BD FACSAriaIII.

For cDC, bone marrow and spleenic cells were stained for PE anti-B220, PE anti-CD3ε, PE anti-GR-1, PE anti-NK1.1, APC-cy7 anti-CD11c, FITC anti-MHC II. And the cDC population is defined as Lin^- $CD11c^+$ MHC II^+.

For pDC, bone marrow and spleenic cells were stained for APC-CY7 anti-CD11C, PE anti-CD11b, APC anti-B220, Percp-cy5.5 anti-SiglecH. The population is defined as $CD11c^{int}$ $CD11b^-$ $B220^+$ $SiglecH^+$.

doi:10.6342/NTU201703495

Reporter assay

293T cells were seeded at $7x10^5$ cells in 6-well tissue culture plates for 16hrs before transfection. Total amount of plasmids 3 μg were transfected with 2.5 M $CaCl_2$ and BBS buffer. Chloroquine (100 nm) was added 10 minutes later and medium was refreshed 6hr after transfection.

Lentivirus production and concentration

Three-plasmid system for lentivirus production procedure. Briefly, 293T cells (7 x 10^5 cells in 6-well plate) were seeded 16 h before the transfection. Lentiviral vectors (2 μg), PMD2G(0.2 μg), Delta - 8.91(1.8 μg) were mixed up in 300 μl serum-free DMEM medium, with 4 μl of Maestrofectin transfection regeant and then added into the mixture, vortexed immediately up to 15 seconds. Let the mixture stand for 20 minutes and then added to 293T cells. Supernant was removed and replaced new medium 18 h later. Virus soup collected day 1 and day 2 after changing medium (40 h /68 h post transfection). Virus supernantant was concentrated by PEG 6000.

In vitro Cre-mediated deletion in primary bone marrow cells

The production of Cre-GFP and PGK-GFP lentivirus were carried out first in HEK 293T cells first using 3-plasmid system as described. GP+E86 were spin-infected by

doi:10.6342/NTU201703495

CRE-lentivirus or PGK-lentivirus, centrifugation 2000 g , RT for 2hr. Stem cells were enriched by intra-peritoneal injection (150 mg/kg) of 5-FU (dissolved in dPBS a day before injection) to $Mef2c^{f/f}$ mice 5 days before harvesting. The 5-FU enriched bone marrow cells ($5x10^5$ cells) were co-cultured with γ-irradiated (2,000 rads) GFP^{hi}GP+E86 cells (1×10^5 cells/well in 24-well tissue culture plate) for 2 days in the presence of IL-3 (6 ng/ml), SCF (500 ng/ml) and IL-6 (500ng/ml). The cultured medium was replaced after 24 hrs with supplemented Flt3L 100 ng/ml. Adding 1ml RPMI medium containing Flt3L 100 ng two days later. The cells were culture for 3 days further before subjected to DCstaining.

doi:10.6342/NTU201703495

Table 1. Antibodies for flow cytometry.

Antibody	Company	Clone number	Catalog number
Biotin-anti CD11c	Biolegend	N418	117304
APC-Cy7 streptavidin	Bio-legend	-	405208
PE-anti-CD11b	Bio-legend	M1/70	101208
PE-anti-MHC II	Bio-legend	M5/114.15.2	107608
PE-anti-NK1.1	Bio-legend	PK136	108708
PE-anti-CD3ε	Bio-legend	145-2C11	100308
PE-anti-CD8α	Bio-legend	53-6.7	100707
PE-anti-TER119	Bio-legend	TER119	116208
PE-anti-GR1	Bio-legend	RB6-8C5	108408
PE-anti-CD19	Bio-legend	6D5	115508
PE-anti-B220	Bio-legend	RA3-6B2	103208
PE-anti-Flt3	Bio-legend	A2F10	135305
PerCp/Cy5.5-anti-C-kit	Bio-legend	2B8	105824
PerCp/Cy5.5-anti-SiglecH	Bio-legend	551	129614
PE-CY7-anti-Sirpα	Bio-legend	P84	144007

doi:10.6342/NTU201703495

PE-CY7-anti-IL7α	Bio-legend	A7R34	135014
FITC-anti-CD11b	Bio-legend	M1/70	101206
FITC-anti-Scal-1	Bio-legend	D7	108106
FITC-anti-MHCII	e-Biosceince	NIMR-4	11-5322-82
APC-anti-CD24	Bio-legend	M1/69	101813
APC-anti-CSFR	Bio-legend	AFS-98	135510
APC-anti-B220	Bio-legend	RA3-6B2	103212
BV421-antiCD45.2	Bio-legend	104	109831

doi:10.6342/NTU201703495

Table 2.

Gene	Forward	Reverse
Rpl7	5’-TCAACAAGGCTTCAATTAACA T-3’	5’-CAATCAAGGAATTATCTGTCAA-3’
Tcf4	5’-AGAAGGAACGGATGG-3’	5’-CTTGTCGCTCTTCAGGTG-3’
Id2	5’-AACATGAACGACTCCTACTC-3’	5’-CTGACGATAGTGGGATGC-3’
Mef2c	5’-CAGTGTCCAGCCATAACAG-3’	5’-GGTTGCCGTATCCATTCC-3’
Tcf12	5’-TCTGCCTACTAGCCACAG-3’	5’-ATTCAGACTGACTGAATCTTCC-3’

Reagent formula

Complete medium:

(1) RPMI + 10%FBS + $5x10^{-5}$ β-ME + 10 μg/ml Gentamicin

(2) DMEM + 10%FBS + 10 μg/ml Gentamicin

PE selection buffer: 2%FBS + 1mM EDTA + 1xdPBS

ACK buffer: 0.15M NH_4Cl + 10Mm KHCO3 + 0.1mM EDTA +1xdPBS

doi:10.6342/NTU201703495

Chapter III Results

doi:10.6342/NTU201703495

iHSPCs is able to differentiate into cDC and pDC in response to Flt3L.

An immortalized hematopoietic progenitor cell line(iHSPC) with myeloid and lymphoid potential was established by retroviral transduction form of estrogen-regulated form Hobx8 into primary bone marrow cells (Redecke, Wu et al. 2013). In the presence of estrodiol, Hoxb8 is able to translocate into nucleus,maintaining the iHSPC in underdifferentiated stage. However when estradiol was removed Flt3L was able to stimulate and differentiate iHSPC into cDCs and pDCs after 5-6 days later. The derived cells were about 20% pDCs and 70% cDCs (Fig.1A).

To investigate if the iHSPC was able to reflect the requirement of TFs for DC development, we knockdown *Tcf4* and *Id2*, two TFs critical for pDC and cDC development respectively. Indeed iHSPC stably transduced with sh*Tcf4* show decreased on both pDCs differentiation percentages and cell number compared to sh*LacZ* control. Likewise sh*Id2* knockdown also inhibited cDC generation but enhanced pDC development (Fig.1B-C). These results suggest that iHSPC can faithfully reflect the transcriptional regulatory mechanism of DC development.

We next took advantage of the iHSPC to screen the novel TFs that mayregulate pDC development. We reasoned that TFs preferentially expressed in pDC development as compared to cDC may plays a role in pDC development. We analyzed the data sets provided by Immunological Genome project (immgen.org). Two criteria were set up for

doi:10.6342/NTU201703495

choosing the genes. The first one was that fold changes between pDC and cDC should be greater than 2, and the second one is that transcription factors were chosen.

In total 14 transcription factors were selected, using lentivirus carrying short hairpin RNA were transduced into iHSPC cell line to observe the effects on DC development (Fig2B-O). Interestingly deficiency of genes cause less pDCs generation from iHSPC cells compared to the sh*LacZ* control(Fig.2A-O) The relative percentage (Fig.2P,Q) and cell numbers (Fig. 2R,S) also show a similar trend. Among them, *Mef2c* (Fig.2E) and *Tcf12* (Fig.2F) show a significant phenotypical changes on both pDCs percentages and cell numbers. Therefore we focused these two genes for further studies.

Ets-1 is not involved in pDCs development.

It has been reported that *Ets-1* is required for functions in lymphoid cells (Robinette, Fuchs et al. 2015). *Ets-1* is also preferentially expressed in pDC as opposed to cDC. We next analyzed DCs from bone marrow and spleen of *Ets-1* knock out mice. The heterozygous littermates mice were used as control. While there is no significance difference in pDCs in both bone marrow and spleen (Fig.3C-H), the cDC frequency was slightly increased of *Ets-1* knock out mice (Fig3 B,H).

doi:10.6342/NTU201703495

We reasoned that CLP shows a greater potential in generating pDCs under Flt3L stimulation (Chen, Chen et al. 2013), thus, we next examined whether *Ets-1* deficiency affected pDC potential from lymphoid progenitor. We sorted out CLP from both *Ets-1* heterozygous mice and knockout mice and performed in vitro development to culture with Flt3L. there was no significant difference in percentage and numbers of pDC and cDC derived from CLP of *Ets-1* heterozygous and homozygous knock out mice (Fig.4B-C).

Knockdown of *Mef2c* and *Tcf12* gene in iHSPCs decrease pDCs generation

During the initiation screen using shRNA to knockdown different genes, we have shown that sh*Mef2c* and sh*Tcf12* displayed the most server reduction of pDC generation from iHSPCs. We next confirmed knockdown of the *Mef2c* and *Tcf12* in iHSPCs indeed significantly decreased generation of pDCs from precursor cells, both in the relative percentages and cell numbers (Fig. 5A-B). To clarify whether this significant phenomenon is specific and not an off target effect; here we examined the knockdown efficiencies by performing qPCR. Indeed the levels of *Mef2c* and *Tcf12* were lower compared to sh*LacZ* control (Fig.5C-D). Moreover, we next confirmed for the highly expression levels of *Mef2c* and *Tcf12* in primary pDCs. Indeed both Mef2c and Tcf12

doi:10.6342/NTU201703495

were highly expressed in pDC from BM and spleen. However, BM nor splenic cDCs show expression levels of *Mef2c* when compared to pDCs (Fig. 5H). While there was a low amount of *Tcf12* detected in splenic cDCs (Fig. 5I).

MS-5, a murine stromal cell line, has been shown to exhibit an ability to support the colony-forming unit-spleen (CFU-S) maintenance both in short-term colony assays and long-term cultures, thus can be used as an approach for characterizing the differentiation of hematopoietic stem cells (Kobari, Dubart et al. 1995). Therefore we next examined the role of *Mef2c* and *Tcf12* knockdown in MS-5 dependent feeder culture system. Following co-cultured with shMef2c or shTcf12 of MS-5 feeder cell, decrease pDCs generation was observed compared to sh*LacZ* control (Fig. 6A-B). The results from feeder-free and MS-5 feeder culture system suggest that *Mef2c* and *Tcf12* may be required for pDC development.

Mef2c regulates the expression of *Tcf4* and Flt3 recepor

To investigate the underlying mechanisms for *Mef2c* or *Tcf12* regulated pDC development from iHSPC. We first examined the expression of *Tcf4*, a master regulation of pDC. Reduced expression of *Tcf4* was observed in iHSPCs that have been stably transduced with sh*Mef2c* and sh*Tcf12* during the developmental process (Fig.7A-D). Moreover, *Tcf4*-driven reporter activity was enhanced 2-fold after overexpression of

doi:10.6342/NTU201703495

Mef2c (Fig. 7E-F). These results imply that important to imply that *Mef2c* regulate DC development through modulating *Tcf4* expression in precursor cells stages.

Previous studies have shown that *Mef2c* deficiency in multipotent progenitor cells (MPPs) results in down-regulation of Flt3 (Stehling-Sun, Dade et al. 2009). Flt3 is the receptor for Flt3L, an important cytokine directing DC development. Therefore, we also examined the expression levels of Flt3. While iHSPCs were usually maintained in Flt3L and estradiol, the receptor may be internalized because of the persistent stimulation of the ligand. To circumvent this possibility, the iHSPCs were maintained by stem cell factor (SCF) for 24 hours. The results show that Flt3 receptor was up-regulated after the SCF culture. Moreover the expression levels of Flt3 receptor was decreased in iHSPC stably transduced with sh*Mef2c* (Fig. 8A-C).

Mef2c deficiency decreases generation of pDCs in primary bone marrow and blocks pDC migration to periphery

To confirm that *Mef2c* did play a role in DC development, we performed in vitro deletion of *Mef2c* in the primary progenitor cells. Lentiviruses containing Cre recombinase and eGFP were used for transduction. GP+E86 package cells used to facilitate infection by co-culturing with primary bone marrow cells from Mef2c $^{f/f}$ mice (Fig. 9D). Interestingly, primary bone marrow cells which were infected with Cre-GFP virus decreased pDC potential when compared to PGK-GFP control (Fig. 9A). The

doi:10.6342/NTU201703495

relative percentages (Fig. 9B) and cell numbers (Fig. 9C) of pDC were significantly lower when Cre was expressed which presumably *Mef2c* was deleted in precursor cells, suggesting that *Mef2c* is a critical transcription factor for pDCs development.

Conditional knockout of *Mef2c* did not show effects on cDCs in bone marrow (Fig. 10A). However, *Mef2c* deficiency reduced bone marrow pDC generation when compared to control mice (Fig. 10D), both in percentages (Fig. 10G) and cell numbers (Fig. 10H). Interestingly, *Mef2c* deficiency dramatically altered splenic DCs generation. Apart from the reduction in spleen cDC (Fig. 10B), the population of pDC nearly disappeared in the spleen (Fig. 10E) and lymph nodes (Fig. 10F). These results suggest that *Mef2c* plays a role in pDC development, especially in pDCs migration.

doi:10.6342/NTU201703495

Chapter IV Discussion

doi:10.6342/NTU201703495

Dendritic cells are distinct populations which play important roles on bridging the innate and adaptive immune response. Because of the short lifespan, the DC population is consistently replenished from precursor cells, which make it an important question to understand mechanism regulating developmental processes. Previous studies recognized that all DCs originate from the bone marrow progenitor cells, and starting with the macrophage/dendritic cell progenitor (MDP) (Fogg, Sibon et al. 2006), which then gives rise to CDP (Naik, Sathe et al. 2007). However, the consensus pf present knowledge is that DCs are from different origins, including CMP and CLP (Sathe, Vremec et al. 2013). Moreover progress research even shows that a group of precursor cells express lower levels of M-CSFR, which show higher pDC potential (Onai, Kurabayashi et al. 2013). Besides, another article supports that the absence of GM-CSF signaling (which induces STAT5 phosphorylation) may also promote pDCs development. For these lines of evidence imply that mechanism regulating DCs development still remain largely unclear. Here we performed screening strategy to show that transcription factors are capable of regulating DCs development.

doi:10.6342/NTU201703495

Transcription factors highly expressed in pDCs are playing roles in dendritic cell development.

Two criteria were set up for choosing our screening targets; one is that the gene should be preferentially expressed in pDCs, and the other is that the genes of interest are transcription factors. Based on these criteria we have found many potential targets, and some of them were already known to regulate pDC development. For instance, *Runx2*, is required for the expression of *Ccr2* and *Ccr5*, two chemokine receptors important for mature pDCs to migrate to the periphery (Sawai, Sisirak et al. 2013). Another example *Zeb2*, is recently found to interact with ID2 protein and enhance pDCs development by preventing ID2 binding to E2-2 (Scott, Soen et al. 2016). Therefore these examples suggest that our screening strategy is working. Interestingly, we found that knockdown of target genes in iHSPCs, did affect DCs development when compared to *sh*LacZ control. For example the expression levels of CD11b were impaired in the knockdown of target genes (Fig2.). Besides CD11b as a marker to distinguish cDCs from pDCs, we further examined on two subsets of cDC namely cDC1 (express XCR1 or CD24) and cDC2 (which express Sirpα instead) (Guilliams, Ginhoux et al. 2014). cDC1s can recognize intracellular pathogens and meanwhile initiate type 1 immune responses (Mashayekhi, Sandau et al. 2011). Apart from cDC1, cDC2s govern type 2 immune responses against parasites in which they activate ILC2s and Th2 cells (Tussiwand,

doi:10.6342/NTU201703495

Everts et al. 2015). Besides CD11b expression levels, subtypes of cDCs were also altered by genes knockdown. Deficiency in *Duxbl*, *Rbm15b* and *Ets-1* show different distribution pattern in cDC1 and cDC2 compared to *LacZ* control (data not shown). All of them show a tendency in shifting the population from cDC2 to cDC1, even though the generation of cDCs frequencies was not affected. Therefore to study on fate determination of cDC1 and cDC2 remains an interesting issue even though they did not altered pDCs development.

Ets-1 deficiency display alteration in lymphoid cell lineage but not DC population

Ets1 is the founding member of the family of *Ets* transcription factors. Loss of Ets1 has effects on T, B, and NK cells, all of which express high levels of *Ets1* under normal physiological conditions (Garrett-Sinha 2013). *Ets1* knockout mice have multiple defects in the T cell lineage including aberrant thymic differentiation, reduction in the peripheral T cell numbers, and reduced IL-2 production (Muthusamy, Barton et al. 1995). There was an article also shown that Ets1 is important in maintaining the expression of CD127 (IL7Rα) in peripheral T cells (Grenningloh et al., 2011). As to B cells, partial defect in transitioning from pro-B to pre-B cell stage is observed in *Ets-1* deficiency (Eyquem et al., 2004). Most importantly, there are reduced numbers of NK

doi:10.6342/NTU201703495

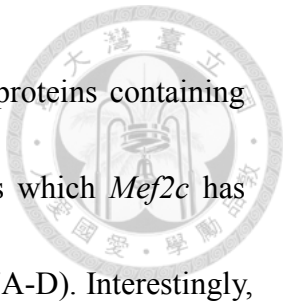
cells and NK progenitors in the bone marrow and reduced numbers of NK T cells in the thymus, spleen, and liver of *Ets1*$^{-/-}$ mice (Barton et al., 1998). However, none of the study on DCs was published.

Since *Ets-1* is one of the screening targets, we examined DCs population in *Ets-1* knockout mice. Even though the screening result showed that there was a slightly reduction of pDC frequencies in iHSPCs expressing sh*Ets-1* (Fig. 2G), there was no difference in pDCs population when compared to their heterozygous littermates (Fig. 3A-D). Since *Ets-1* highly expressed levels in CLP, we sorted out CLPs from *Ets-1* knockout mice and in vitro culture to examine the effects. Apparently, there was no defect in pDC or cDC potential from CLP (Fig. 4).

Mef2c and *Tcf12* play a role in dendritic cell development.

We have demonstrated that knockdown of *Mef2c* or *Tcf12* in iHSPCs strikingly decreased the generation of pDCs (Fig. 5A-E). The MEF2 family members have multiple splicing variants and they all share a conserved N-terminal domain. These domains are required for DNA binding in the promoter regions and interaction with myogenic basic helix–loop–helix (bHLH) proteins (Molkentin et al., 1996). To the knowledge we know is that bHLH proteins act as heterodimers with members of a class of ubiquitous partners, the E proteins (Kee, 2009), therefore we hypothesized that *Mef2c*

doi:10.6342/NTU201703495



may enhance *Tcf4* expression through recruitments of some other proteins containing bHLH domains (Fig. 7E). *Tcf4* expression was reduced in iHSPCs which *Mef2c* has been knocked down during the developmental process of DCs (Fig. 7A-D). Interestingly, the phosphorylation of *Mef2c* can be regulated by p38 kinase under inflammation conditions (Han et al., 1997). p38 is also a downstream molecule of Flt3L signaling (Stirewalt and Radich, 2003). However, whether *Mef2c* could be a downstream regulator through Flt3L-P38 axis pathway remains unknown.

Tcf12, also known as HEB, which belongs to E protein family. E protein family involves an important part in lymphoid lineage (Kee, 2009). One of the members, E2-2 is known as the master transcription factor regulating pDCs development (Cisse et al., 2008). All the members of E protein family are highly expressed in pDCs. Moreover, previous researches even revealed that B cell development can also be regulated by the combined dosage of all the members in E protein family, E2A, E2-2, and HEB (Zhuang et al., 1996). Therefore we hypothesize that there should be some roles played by HEB for pDCs development. Whether it might regulate pDCs development by its own or by cooperation with E2-2 remain unclear.

doi:10.6342/NTU201703495

Mef2c regulate many lymphoid-associated genes, and its deficiency down-regulate Flt3 receptor expression

As mentioned in the previous paragraph transcription factors of MEF2 family is essential for the development of many cell types. Previous studies have shown that *Mef2c* also involves in the cardiac and skeletal muscle lineage differentiation during mouse embryogenesis (Molkentin et al., 1996). Moreover, *Mef2c* is the family member which found crucial to hematopoiesis. Earlier study has demonstrated that *Mef2c* processes all the developmental progression of B cell and progenitor cells in myeloid lineage by regulating many lymphoid genes transcription. The interesting part is that *Mef2c* express high levels in peripheral B cells (Swanson et al., 1998), which show importance in B cell development. Meanwhile it also preferentially express in pDCs, which also indicates that it should be important for pDC. Further evidence suggests that Genes important in lineages development such as IL-7Rα were also decreased in *Mef2c* deficiency (Gerstein et al., 2009). In other words, *Mef2c* is required for the formation of lymphoid lineage during bone marrow hematopoiesis. Indeed we also found that cell surface expression of the Flt3 receptors was down-regulated in iHSPCs transduced with sh*Mef2c* (Fig. 8). Moreover, it is suggested that *Mef2c* expression depends on sufficiency in the PU.1 transcription factor, suggesting that *Mef2c* is a target of PU.1 (Stehling-Sun et al., 2009). PU.1 (encoded by *Sfpi1*) has been reported to be a crucial

doi:10.6342/NTU201703495

for DCs development in vivo and that conditional ablation of PU.1 in defined precursors, such as CDP, blocked Flt3 ligand induced DC generation in vitro (Carotta et al., 2010). These researches support our hypothesis that *Mef2c* regulates DCs development. However, the regulatory mechanisms remain to be determined. We next further confirmed the results of iHSPC by in vitro deletion of *Mef2c* in primary bone marrow cells. Following the transduction of Cre into $Mef2c^{f/f}$ primary bone marrow cells. Therefore it appears that *Mef2c* indeed regulates pDCs development probably through controlling *Tcf4* and *Flt3* expression.

Mef2c deficiency not only reduces bone marrow pDCs generation it also dramatically reduced the frequency of pDC in the periphery, such as spleen and lymph nodes (Fig. 10E-F). Apart from regulating *Tcf4* and *Flt3*, *Mef2c* might also have a role in regulating *Runx2*, as it is also a downstream gene regulated by *Tcf4* and affects pDCs migration (Swiecki et al., 2015). *Runx2* has recently been reported to be a crucial transcription factors for pDCs migrating to peripheral organs through affecting the expression of chemokine receptor CCR2 and CCR5 (Sawai et al., 2013). So, we hypothesize that *Mef2c* not only directly regulates *Tcf4* but also regulates *Runx2* to affect pDCs generation and migration.

doi:10.6342/NTU201703495

Mef2c regulates pDCs generation by multifaceted ways

Deficiency of *Mef2c* shows reduction in pDCs population, especially in peripheral organs. We have shown that *Mef2c* regulates the expression levels of Flt3, and therefore *Mef2c* is a positive regulator. Interestingly, *Mef2c* is shown to be a direct target of PU.1 (Gerstein et al., 2009), which is also a transcription factor important in regulating DCs development through *Flt3* (Carotta et al., 2010). Moreover, we have found that during the developmental processes, deficiency of *Mef2c* in iHSPC decreases *Tcf4* expression. Most importantly, there is a *Mef2c* binding motif within 240bp upstream of the promoter sequence of *Tcf4*. These results suggest that *Mef2c* influences generation of pDC not only by regulating the expression of Flt3 but also by enhancing the expression of *Tcf4*. Further, analysis of DC populations in peripheral organs indicates the migration of pDC is also affected by Mef2c. *Runx2*, a direct target of *Tcf4* has recently been defined as an important transcription factor controlling pDCs migration through Ccr2 and Ccr5 (Sawai et al., 2013). Therefore, we hypothesize that *Mef2c* may influence the migration of pDC through *Runx2*.

In conclusion, our studies using iHSPCs to screen potential transcription factors have revealed *Mef2c* and *Tcf12* as important transcription factors regulating DC development. Conditional knockout of *Mef2c* show alteration in pDC development, not only in the bone marrow, but also in the spleen and lymph nodes. *Mef2c* is critical for

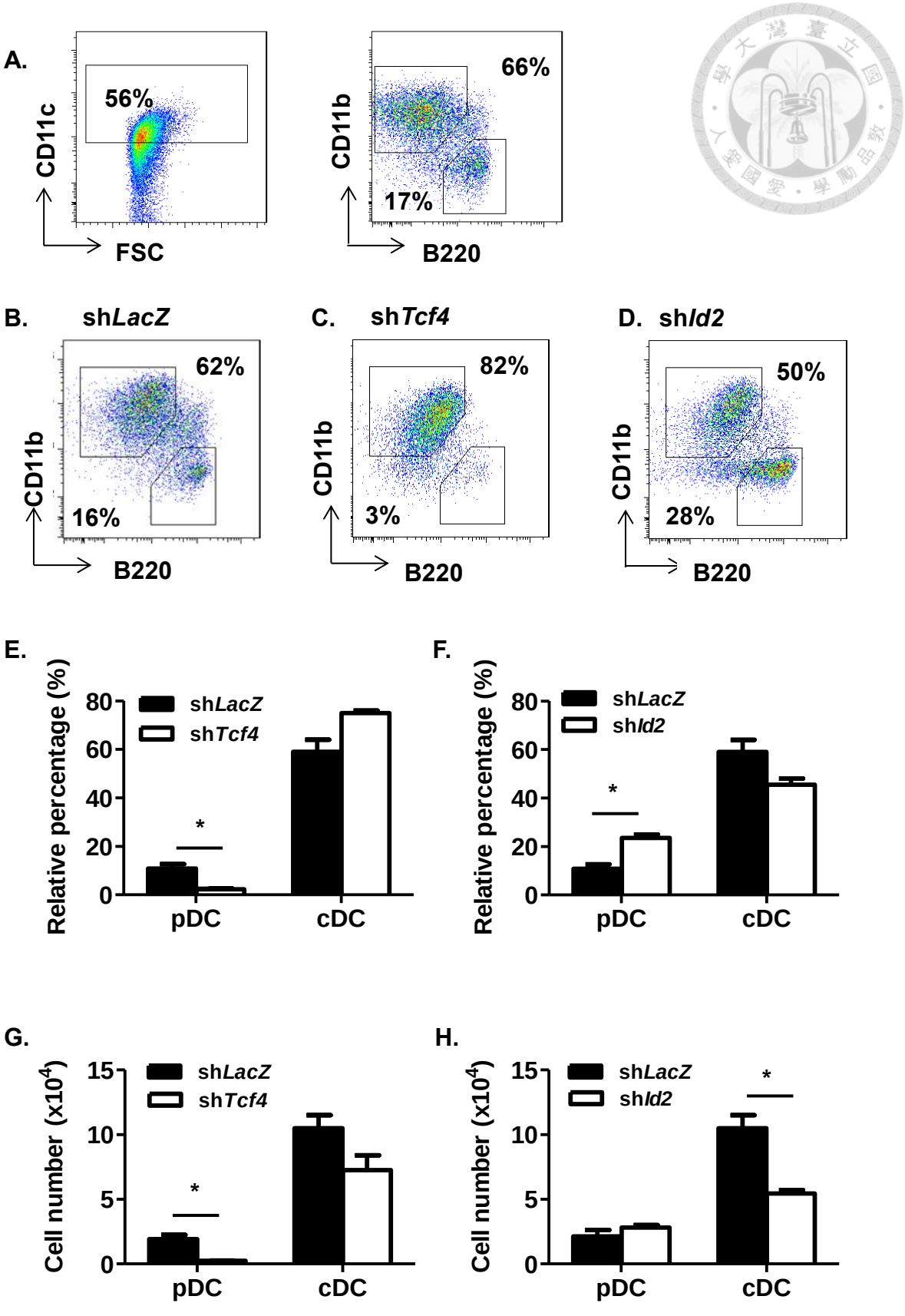
doi:10.6342/NTU201703495

DC development because it displays many possible regulation mechanisms by up-regulating Flt3 receptor expression to alter the response in the precursor cells to stimulation of Flt3L, or by enhancing *Tcf4* expression to promote pDCs generation.

doi:10.6342/NTU201703495

Chapter V Figures

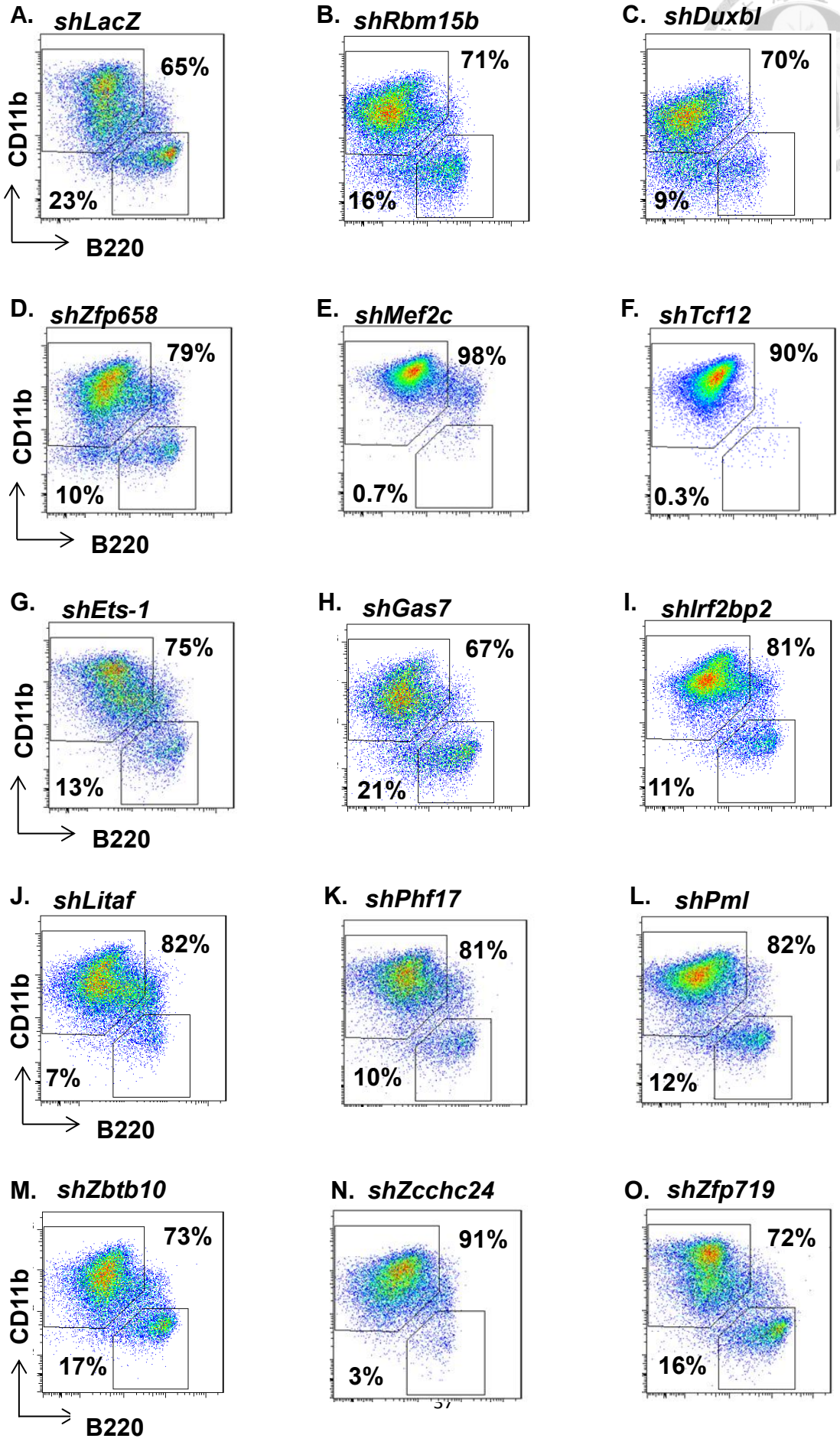
doi:10.6342/NTU201703495



doi:10.6342/NTU201703495

Figure 1. Immortalized hematopoietic stem and progenitor (iHSPC) cell line is able to differentiate into cDC and pDC in response to Flt3L. (A) $5x10^5$ iHSPC cells were cultured for 5 days in the presence of 100 ng/ml Flt3L. The derived cells were stained with antibodies to CD11c, CD11b and B220. cDCs were defined as $CD11c^+CD11b^-B220^+$ and pDCs were defined as $CD11c^+CD11b^-B220^+$. iHSPC cell lines were stably transduced with lentivirus carrying shRNA to *LacZ* control (B), *Tcf4* (C) and *Id2* (D) were subjected to in vitro culture differentiation as described in (A). The relative percentage and cell numbers of sh*LacZ* and sh*Tcf4* (E,G) or sh*Id2* (F,H) are shown. N=2 (*, $P<0.05$).

doi:10.6342/NTU201703495



doi:10.6342/NTU201703495

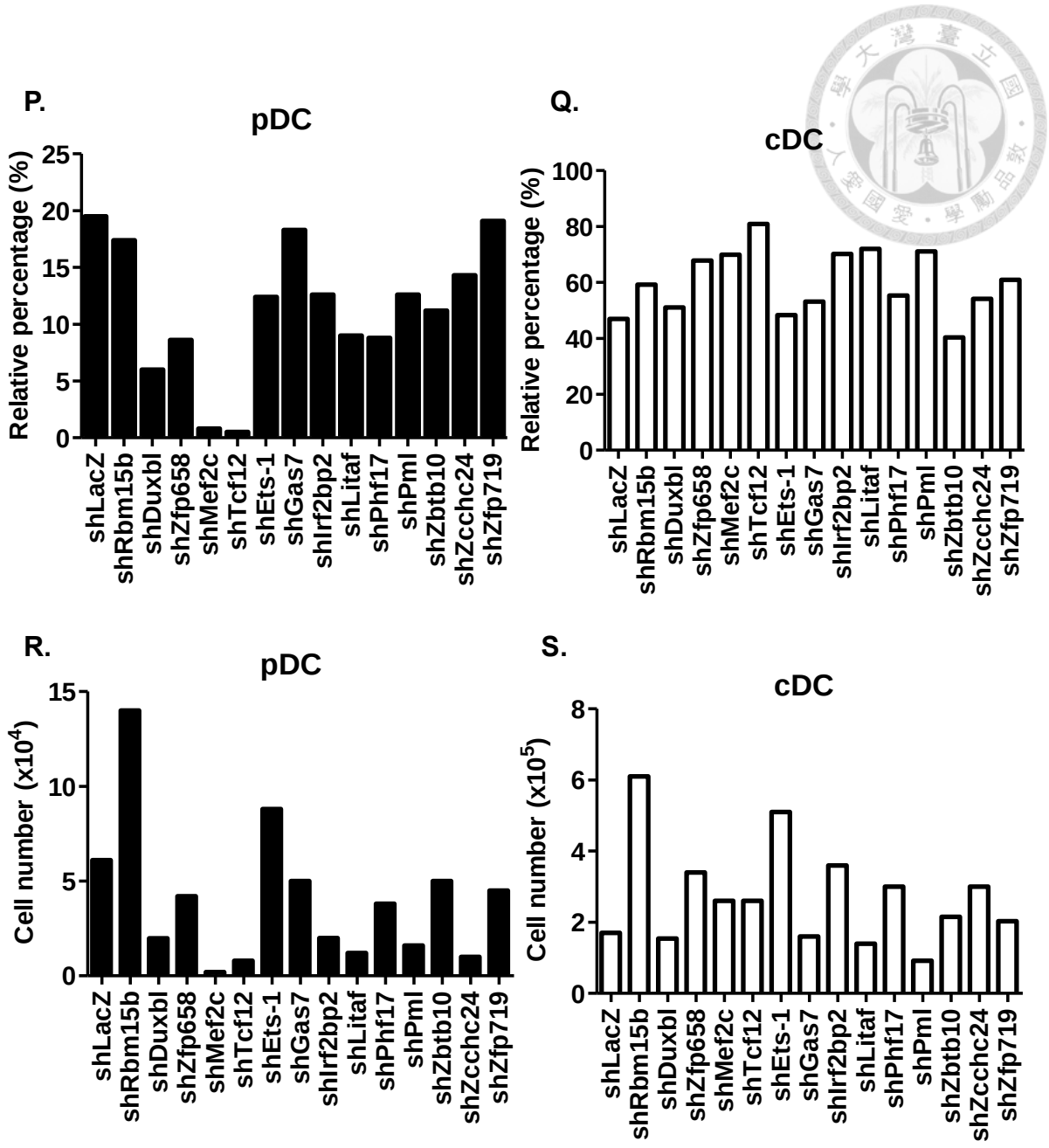
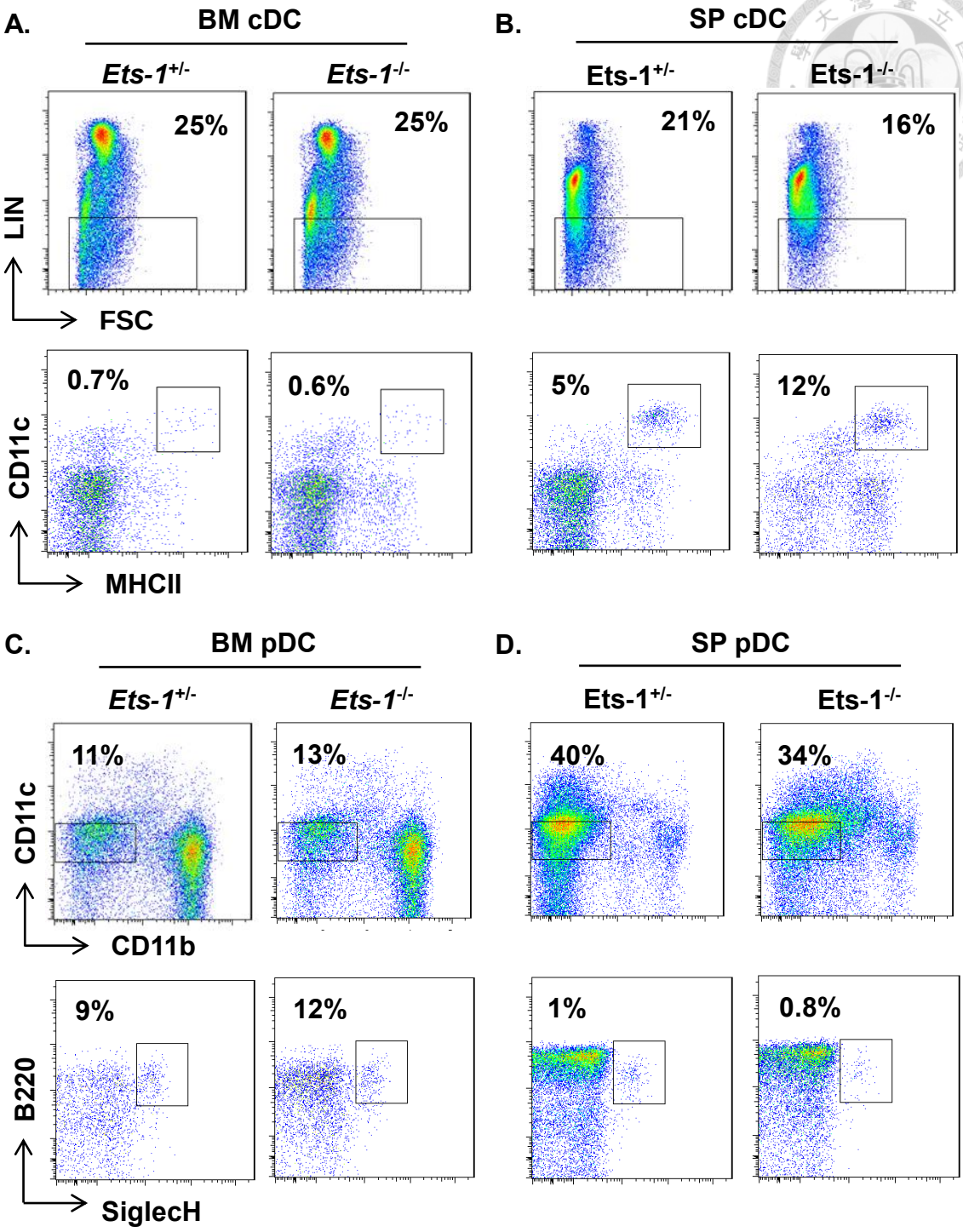


Figure 2. Knockdown of *Mef2c* and *Tcf12* severely impairs pDC development from iHSPC. iHSPC cell lines were stably transduced with lentiviruses carrying shRNA to *LacZ* (A) control, *Rbm15b* (B), *Duxbl* (C), *Zfp658* (D), *Mef2c* (E), *Tcf12* (F), *Ets-1* (G), *Gas7* (H), *Irf2bp2* (I), *Litaf* (J), *Phf17* (K), *Pm l*(L), *Zbtb10* (M), *Zcchc24* (N), *Zfp719* (O). After the lentiviral transduction iHSPC cells ($5x10^5$) were cultured for 5 days in the

doi:10.6342/NTU201703495

presence of 100 ng/ml Flt3L. The derived cells were stained with antibodies to CD11c, CD11b and B220. cDCs were defined as $CD11c^{+}CD11b^{-}B220^{+}$ and pDCs were defined as $CD11c^{+}CD11b^{-}B220^{+}$. Relative percentages (P.Q) and cell numbers (R.S) of pDCs and cDCs are shown.

doi:10.6342/NTU201703495



doi:10.6342/NTU201703495

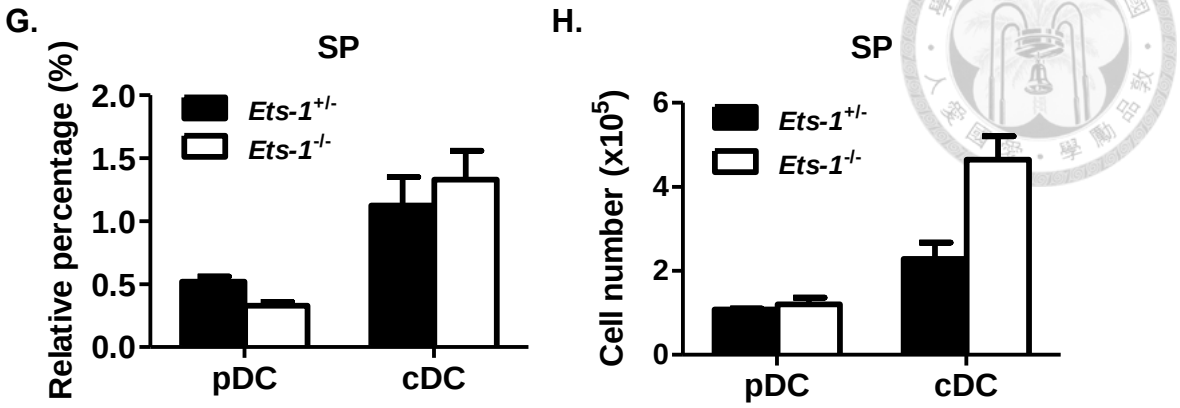


Figure 3. *Ets-1* deficiency did not show alteration in DCs development. Bone marrow cells and splenocytes were isolated from *Ets-1* heterozygous and knockout mice, and stained with antibodies to lineage markers, MHC II and CD11c for cDCs, and CD11c, CD11b, B220 and SiglecH for pDCs. cDCs were defined as lineage$^-$(CD3, B220, NK1.1, GR-1) CD11c$^+$MHC II$^+$. pDCs from bone marrow (C) and spleen (D), and the population were defined as CD11b$^-$CD11c^{int} B220$^+$SiglecH$^+$. The relative percentages (E-G) and cell numbers (F-H) of bone marrow and spleen are shown. N=2.

doi:10.6342/NTU201703495

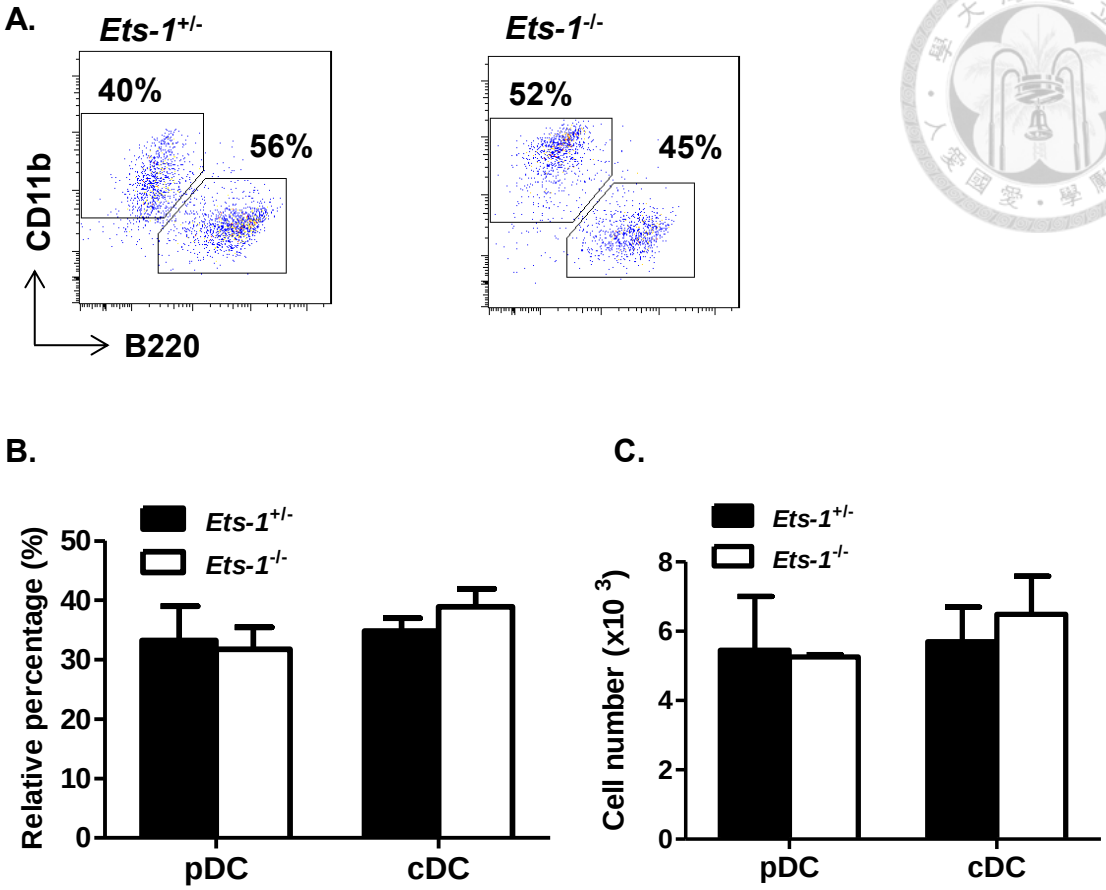
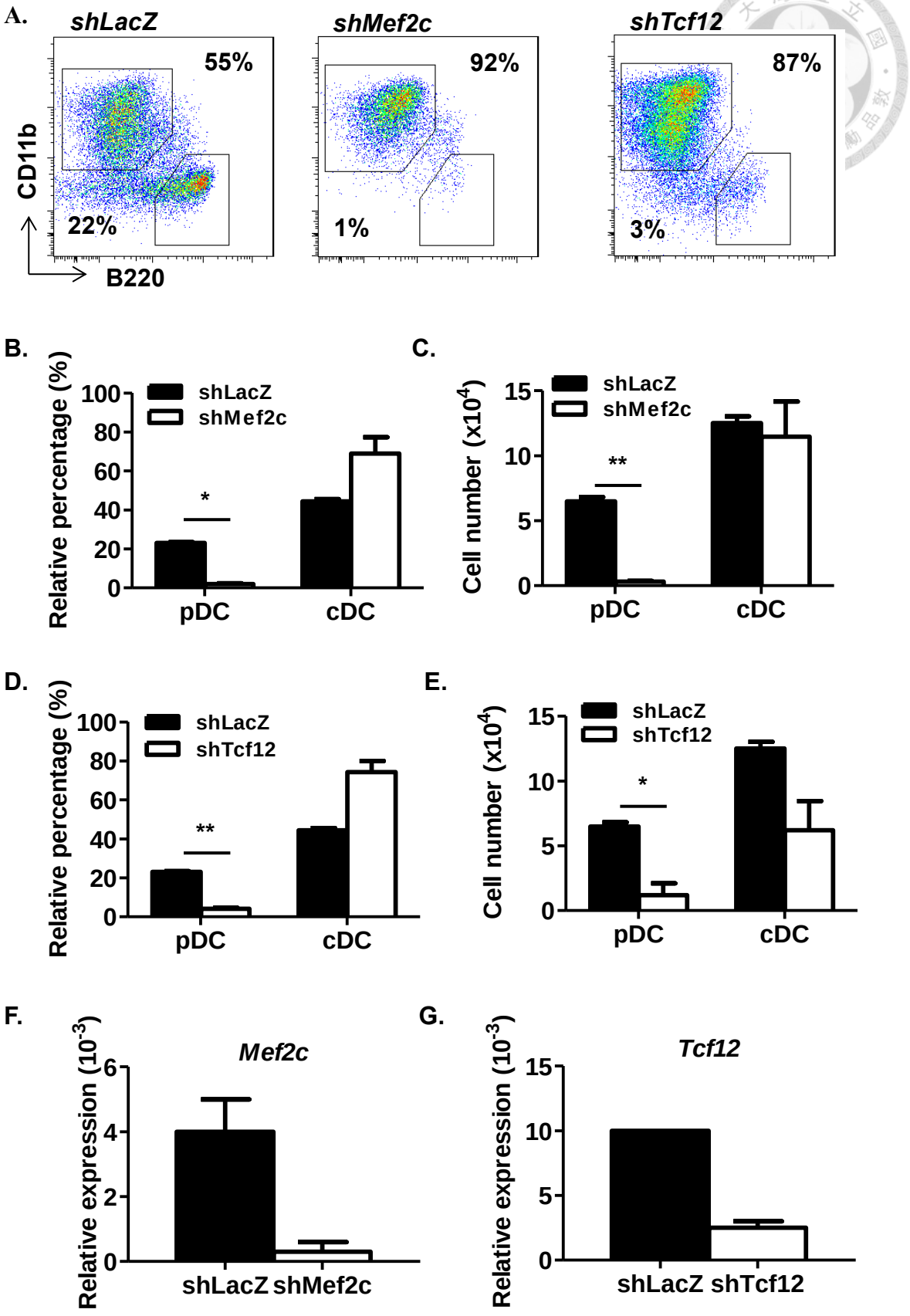


Figure 4. *Ets-1* deficiency did not alter the pDC potential of CLP in vitro. (A) CLPs (Lin^- $MCSFR^-$ $IL7R\alpha^+$ $c\text{-}kit^{int}$ $Sca\text{-}1^{int}$) were sorted out from bone marrow of *Ets-1* heterozygous and knockout mice, and $2x10^4$ cells/well were seeded in 96U-bottom plates for 6 days, with the presence of Flt3L 100 ng/ml. The derived cells are stained with antibodies to CD11c, CD11b, B220 and analyzed by flow cytometry. cDCs are defined as $CD11c^+CD11b^+B220^-$, and pDCs are defined as $CD11c^+CD11b^-B220^+$. The relative percentages (B) and cell numbers (C) are shown. N=2.

doi:10.6342/NTU201703495



doi:10.6342/NTU201703495

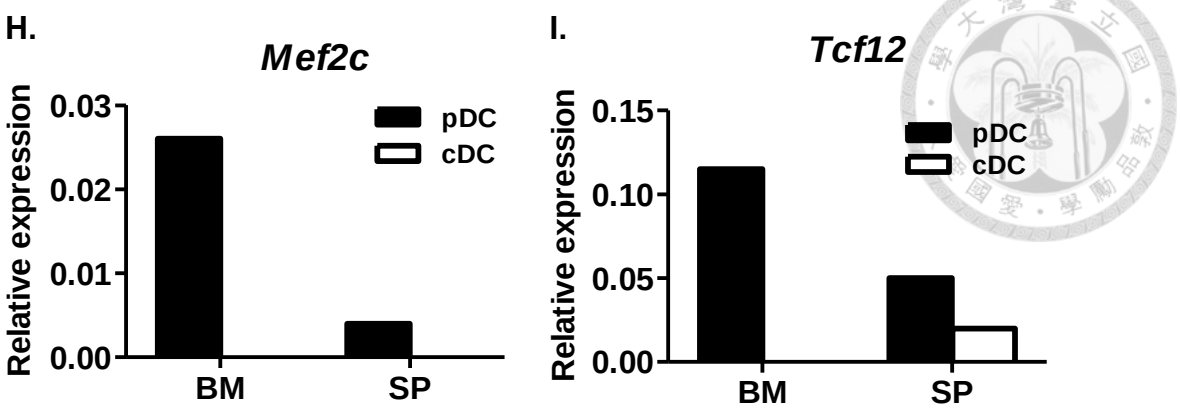


Figure 5. Knockdown of *Mef2c* or *Tcf12* gene in iHSPC cell line decreases pDCs generation. iHSPC cell lines were stably transduced with lentivirus carrying shRNA to *LacZ* control (A), *Mef2c* (B), and *Tcf12* (C). iHSPC cells stably transduced with shRNA were seeded $5x10^5$ cells in vitro cultured for 5 days in the presence of 100 ng/ml Flt3L. The derived cells were stained with antibodies to CD11c, CD11b and B220 and analyzed by flow cytometry. cDCs were defined as $CD11c^+CD11b^-B220^+$ and pDCs were defined as $CD11c^+CD11b^-B220^+$. The relative percentages and cell numbers of knockdown in *Mef2c* (B-C) and knockdown in *Tcf12* (D-E) are shown. RNA isolated from iHSPC cells had treated with sh*Mef2c* (F) or sh*Tcf12* (G) was subjected to RT-qPCR using primers to *Mef2c*, *Tcf12* and *Rpl7*. Relative expression was normalized to *Rpl7*. N=2-4. (*,$p<0.05$ and **,$p<0.01$). Primary pDCs and cDCs were sorted out from bone marrow or spleen from wild-type mice, mRNA from primary cells were extracted subjected to RT-qPCR the relative gene expression levels of *Mef2c* (H) and *Tcf12* (I) is shown.

doi:10.6342/NTU201703495

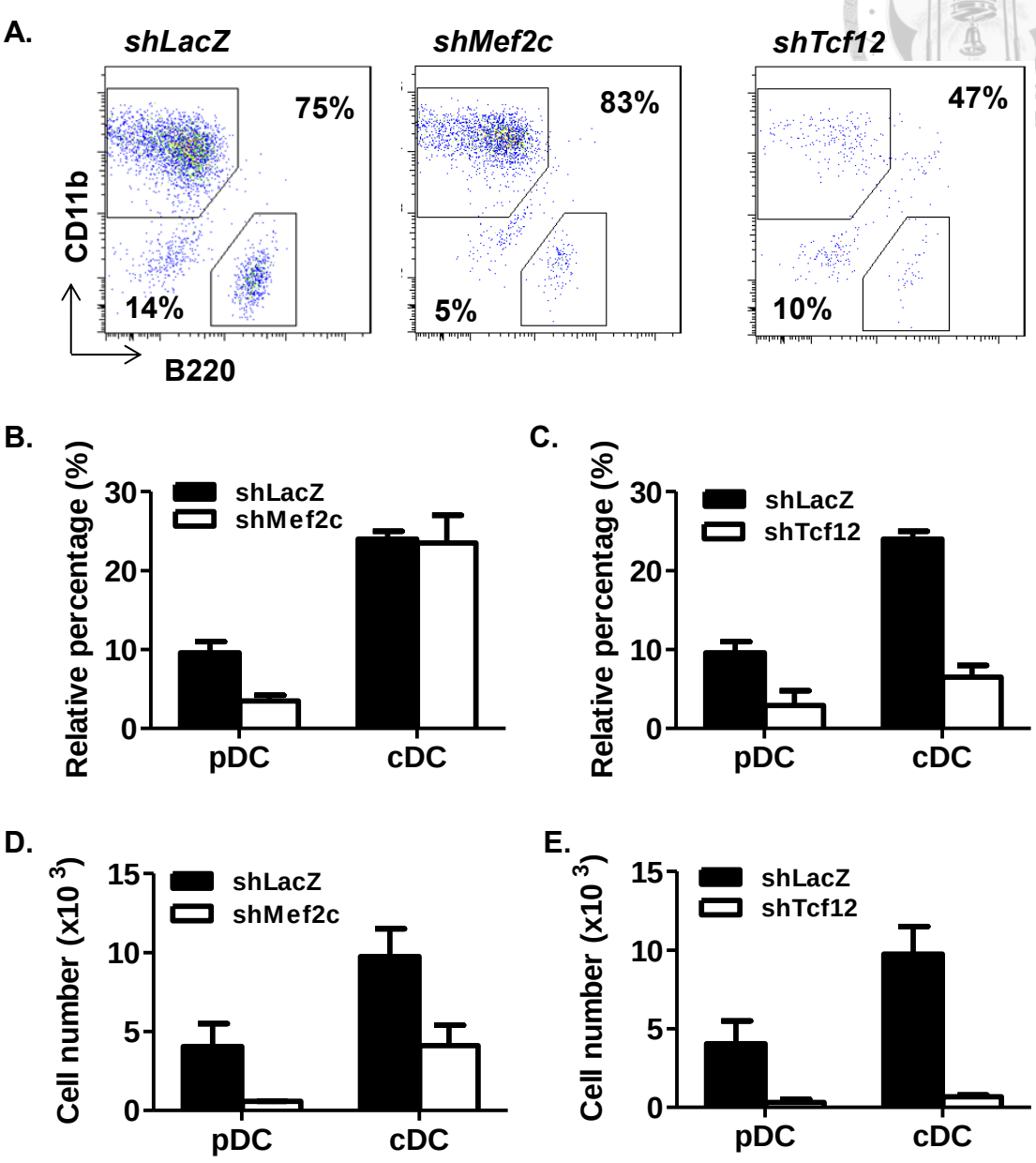


Figure 6. Knockdown of *Mef2c* or *Tcf12* in iHPC cell line also show decreases pDC generation in MS-5 feeder culture system. (A) MS-5 stromal cells were seeded at 5.9 × 10^4 into 12-well plates for 1 d before, followed by γ-irradiation (3,000 rad). Then one thousand of iHSPC cells that had been stably transduced with sh*LacZ,* sh*Mef2c,* sh*Tcf12* were cultured together in the presence of Flt3L for 6 days. The derived cells were

doi:10.6342/NTU201703495

stained with antibodies to CD11c, CD11b and B220, and analyzed by flow cytometry. cDCs were defined as $CD11c^{+}$ $CD11b^{-}$ $B220^{+}$ and pDCs were defined as $CD11c^{+}CD11b^{-}B220^{+}$. Relative percentages (B-C) and cell numbers (D-E) are shown. N=2.

doi:10.6342/NTU201703495

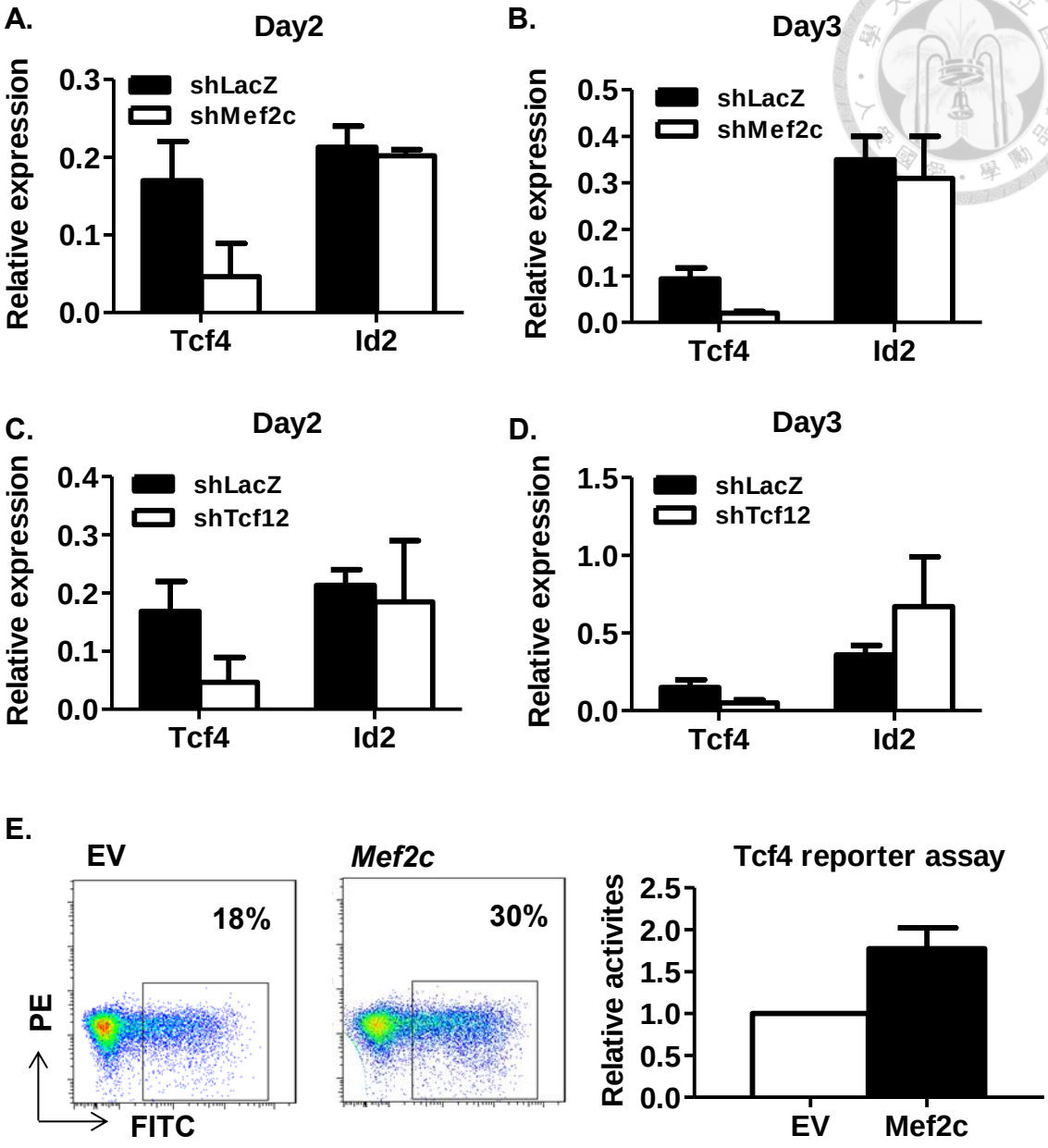
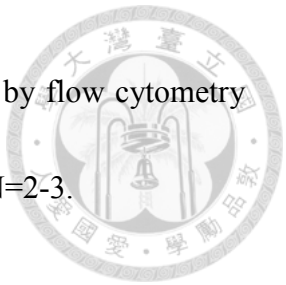


Figure 7. *Mef2c* positively regulates *Tcf4* expression. (A-D) iHSPC cells that were treated with sh*LacZ* control, sh*Mef2c*, or sh*Tcf12* were cultured in vitro for 2 days or 3 days in the presence of Flt3L. RNA was prepared and was subjected to RT-qPCR, using primers to *Tcf4*, *Id2* and *Rpl7*. Relative expression was normalized to *Rpl7*. (E) Co-transfection using empty vector or plasmid overexpress *Mef2c* with *Tcf4*-GFP

doi:10.6342/NTU201703495

reporter plasmid into 293T cells. Expression off GFP was analyzed by flow cytometry 18h later. The fold change induction was normalized to EV control. N=2-3.



doi:10.6342/NTU201703495

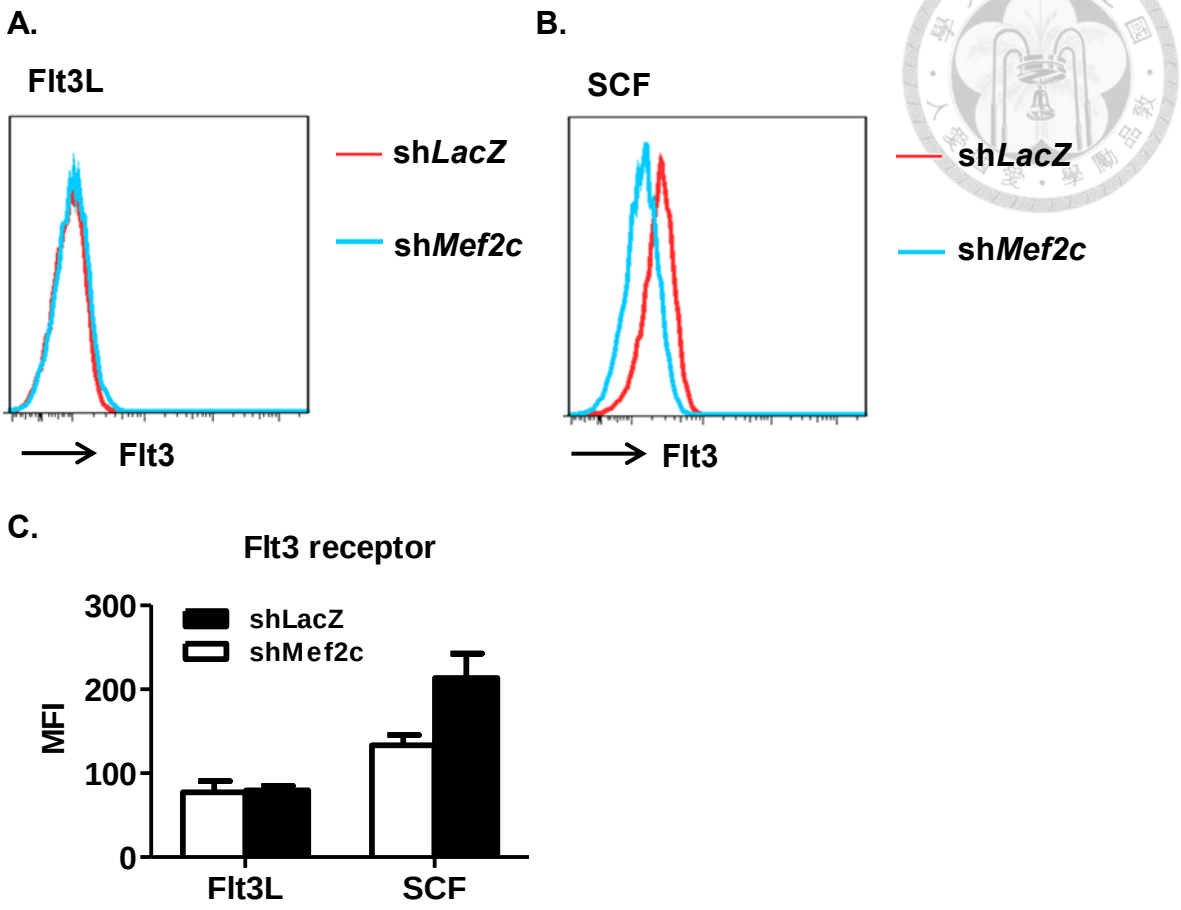


Figure 8. *Mef2c* positively regulates Flt3 receptor expression in iHSPC. iHSPC cell lines were stably transduced with sh*LacZ* control or sh*Mef2c*, cultured in Flt3L (100 ng/ml) (A) or SCF(100 ng/ml) (B) for 24 h and were stained anti-Flt3 antibodies follow by flow cytometer analysis. The MFI of Flt3 expression following Flt3 or SCF treatment is shown. N=4.

doi:10.6342/NTU201703495

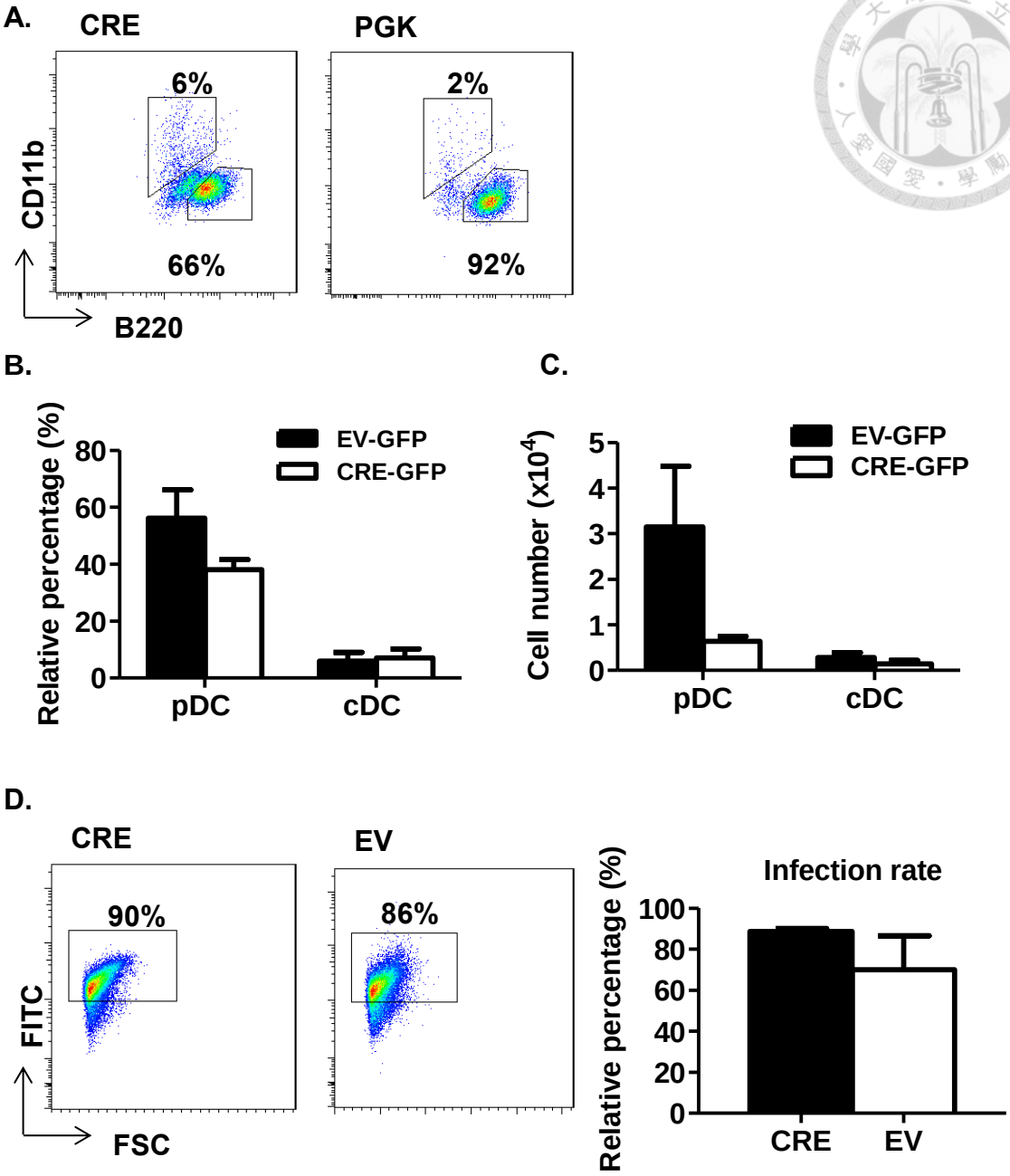
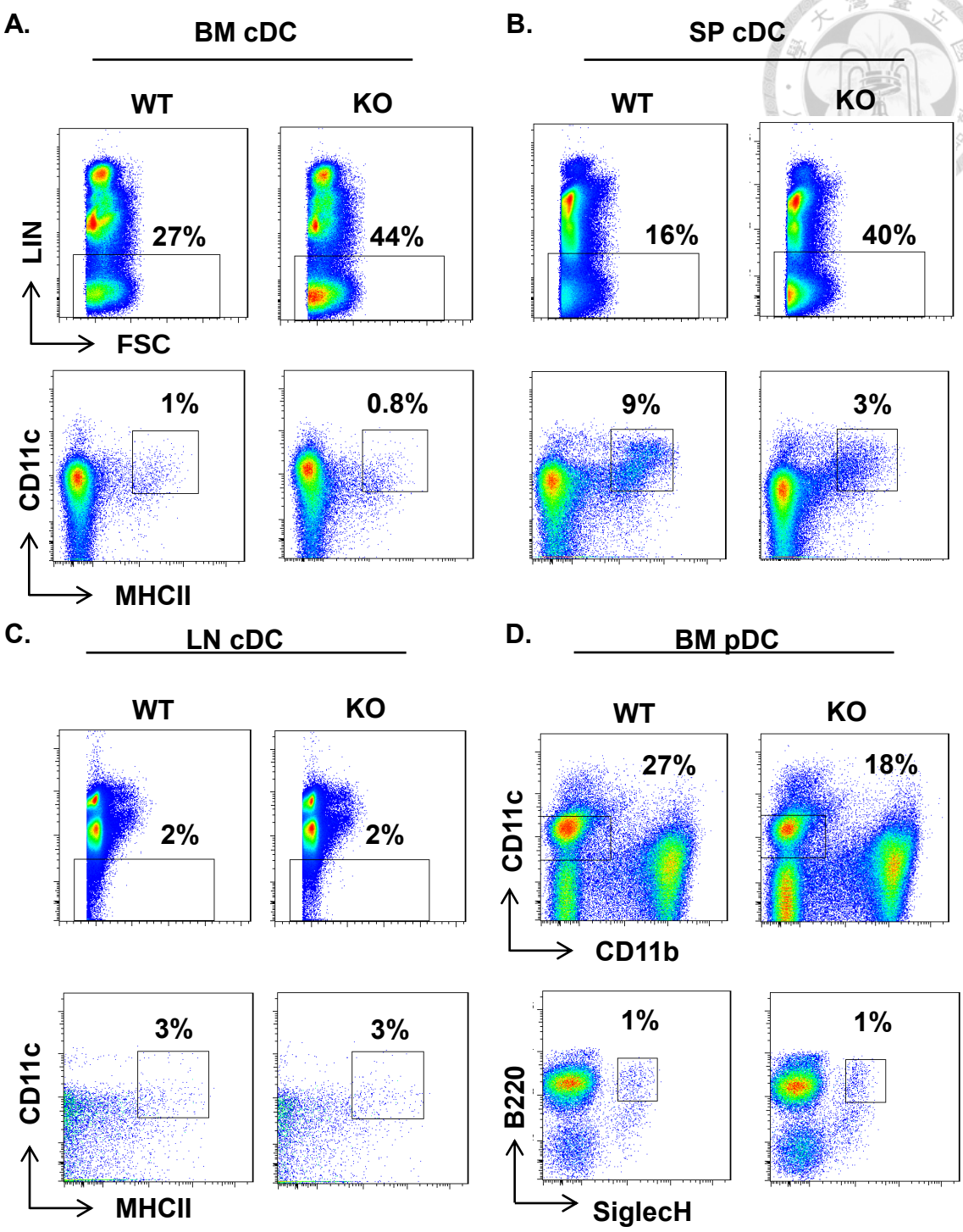


Figure 9. *Mef2c* deletion from primary bone marrow cells decrease generation of pDCs. (A) Primary bone marrow cells from Mef2c $^{f/f}$ mice were transduced with PGK-GFP as an empty vector control (EV) or CRE containing GFP (PGK-GFP) using GP+E86 packaging cell system as described in material and methods. The transduced bone marrow cells were in vitro differentiation 6 days using Flt3L, and the relative

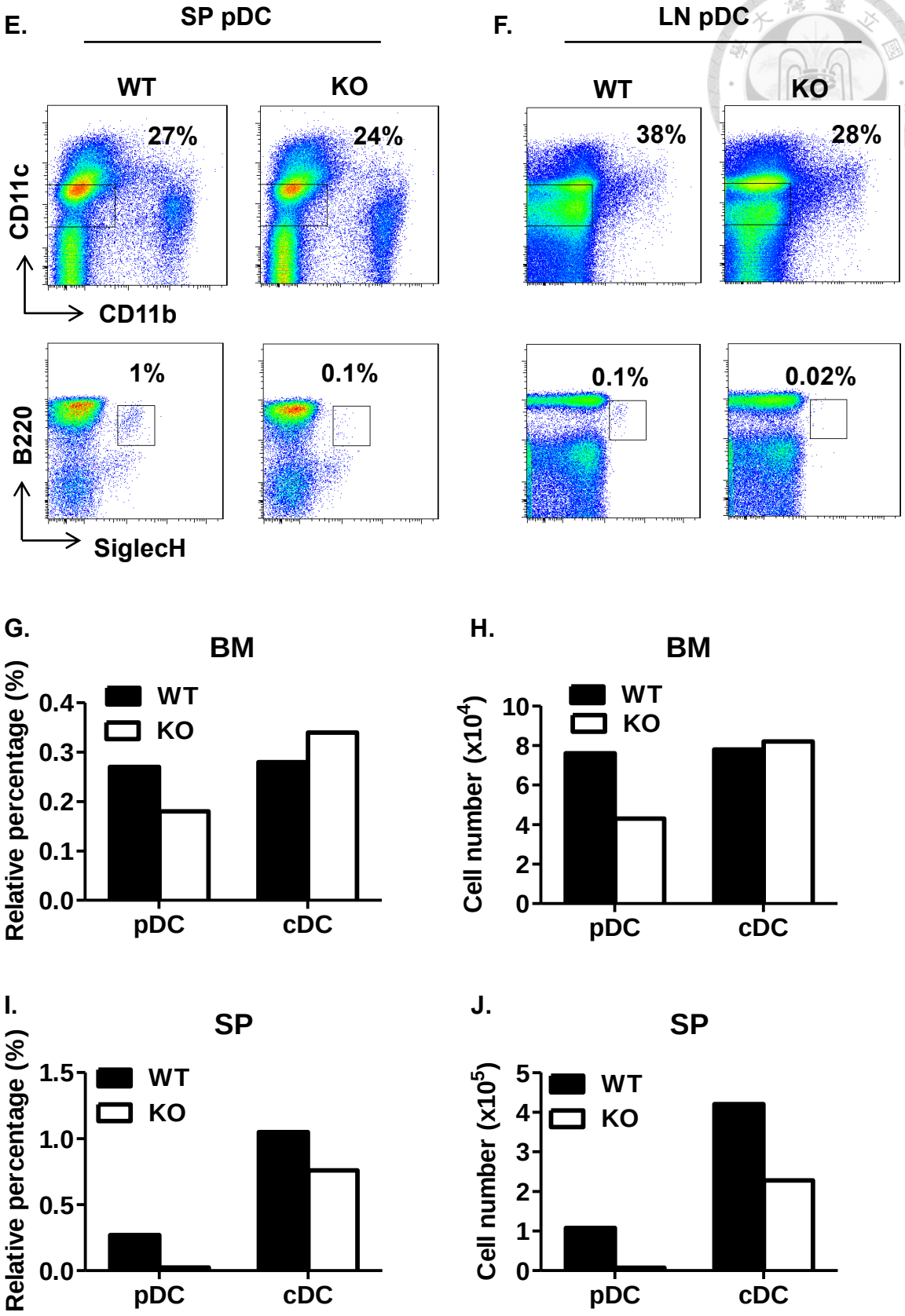
doi:10.6342/NTU201703495

percentages (B) and cell numbers (C) are shown. (D) Infection rates were calculated according to GFP fluorescence. N=3.

doi:10.6342/NTU201703495



doi:10.6342/NTU201703495



doi:10.6342/NTU201703495

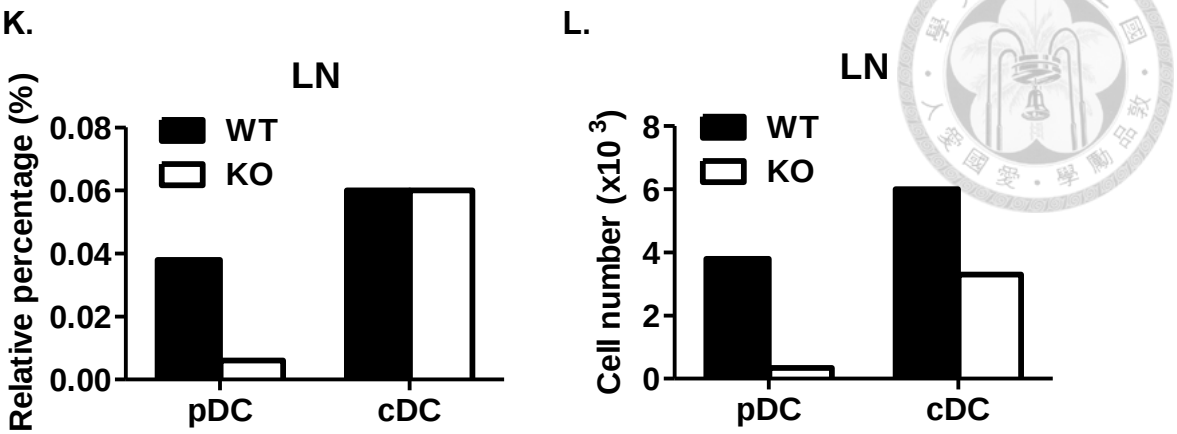


Figure 10. *Mef2c* deficiency decreases pDC generation and migration. Bone marrow cells(A) and splenocytes(B) and lymph node(C) were isolated from *Mef2c*$^{f/f}$ (WT) *and* *Mef2c*$^{f/f}$ Tie2-Cre (KO) mice, and stained with antibodies to lineage markers (CD3, B220, NK1.1, GR-1), MHC II and CD11c for cDCs, and CD11c, CD11b, B220 and SiglecH for pDCs. cDCs were defined as lineage^{-} CD11c^{+}MHC II^{+}. pDCs were defined as CD11b^{-}CD11c^{int} B220^{+}SiglecH^{+}. The relative percentages and cell numbers of bone marrow DCs (G-H), spleen DCs (I-J) and lymph node pDC (K-L) are shown. N=1.

doi:10.6342/NTU201703495

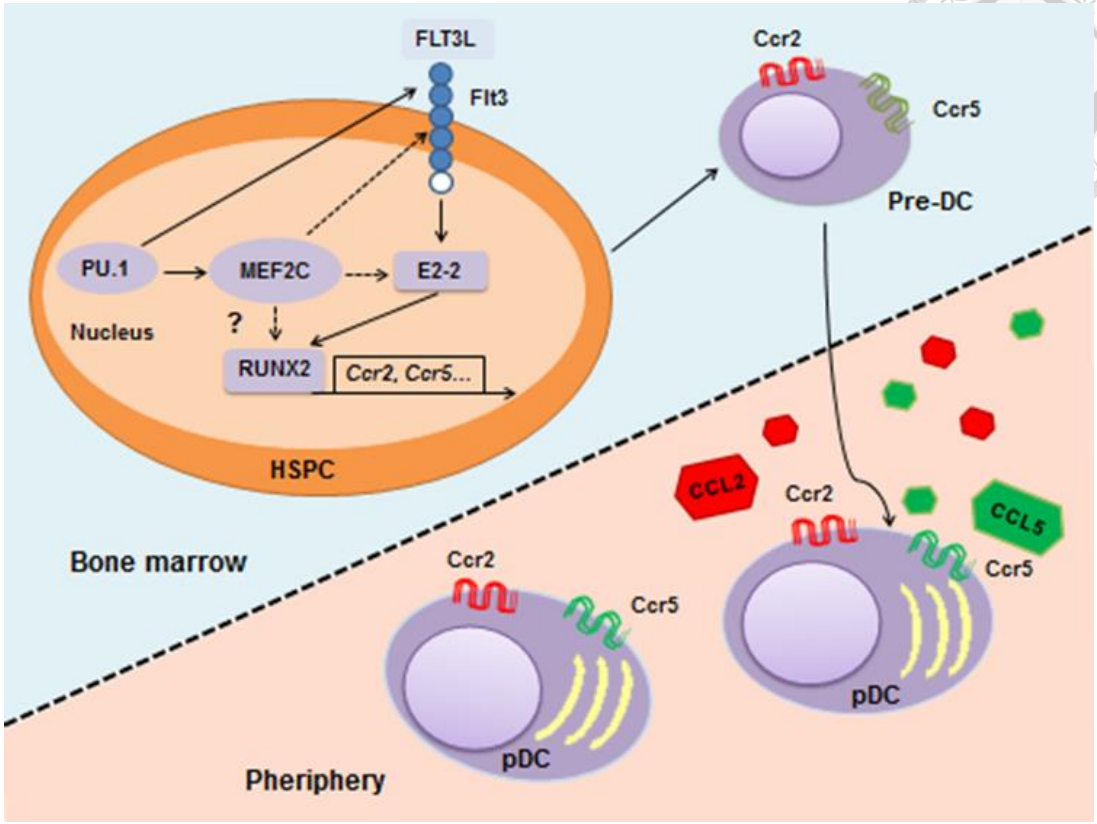


Figure 11. Hypothetic model of *Mef2c* dependent pDC development and migration.

PU.1 regulates DCs development in a Flt3-dependent manner. MEF2C is a downstream target of PU.1. PU.1 may regulate FLT3 through a MEF2C dependent or independent maner. Further, MEF2C also controls E2-2 expression, which is important for the generation of pDC. *Runx2* is one of E2-2 downstream target genes, and is important for pDCs migration to periphery through up regulate of Ccr2 and Ccr5. We hypothesize that *Mef2c* regulates *Runx2* to alter the migration of pDC.

doi:10.6342/NTU201703495

Chapter VI References

doi:10.6342/NTU201703495

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Barton, K., Muthusamy, N., Fischer, C., Ting, C.N., Walunas, T.L., Lanier, L.L., and Leiden, J.M. (1998). The Ets-1 transcription factor is required for the development of natural killer cells in mice. Immunity *9*, 555-563.

Carotta, S., Dakic, A., D'Amico, A., Pang, S.H., Greig, K.T., Nutt, S.L., and Wu, L. (2010). The transcription factor PU.1 controls dendritic cell development and Flt3 cytokine receptor expression in a dose-dependent manner. Immunity *32*, 628-641.

Chen, Y.L., Chen, T.T., Pai, L.M., Wesoly, J., Bluyssen, H.A., and Lee, C.K. (2013). A type I IFN-Flt3 ligand axis augments plasmacytoid dendritic cell development from common lymphoid progenitors. J Exp Med *210*, 2515-2522.

Cisse, B., Caton, M.L., Lehner, M., Maeda, T., Scheu, S., Locksley, R., Holmberg, D., Zweier, C., den Hollander, N.S., Kant, S.G.*, et al.* (2008). Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. Cell *135*, 37-48.

Colonna, M., Trinchieri, G., and Liu, Y.J. (2004). Plasmacytoid dendritic cells in immunity. Nat Immunol *5*, 1219-1226.

Eyquem, S., Chemin, K., Fasseu, M., Chopin, M., Sigaux, F., Cumano, A., and Bories, J.C. (2004). The development of early and mature B cells is impaired in mice deficient for the Ets-1 transcription factor. Eur J Immunol *34*, 3187-3196.

doi:10.6342/NTU201703495

Fogg, D.K., Sibon, C., Miled, C., Jung, S., Aucouturier, P., Littman, D.R., Cumano, A., and Geissmann, F. (2006). A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. Science *311*, 83-87.

Gabrilovich, D.I., Ostrand-Rosenberg, S., and Bronte, V. (2012). Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol *12*, 253-268.

Garrett-Sinha, L.A. (2013). Review of Ets1 structure, function, and roles in immunity. Cell Mol Life Sci *70*, 3375-3390.

Gilliet, M., Boonstra, A., Paturel, C., Antonenko, S., Xu, X.L., Trinchieri, G., O'Garra, A., and Liu, Y.J. (2002). The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. J Exp Med *195*, 953-958.

Grenningloh, R., Tai, T.S., Frahm, N., Hongo, T.C., Chicoine, A.T., Brander, C., Kaufmann, D.E., and Ho, I.C. (2011). Ets-1 maintains IL-7 receptor expression in peripheral T cells. J Immunol *186*, 969-976.

Guilliams, M., Ginhoux, F., Jakubzick, C., Naik, S.H., Onai, N., Schraml, B.U., Segura, E., Tussiwand, R., and Yona, S. (2014). Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. Nat Rev Immunol *14*, 571-578.

Hacker, C., Kirsch, R.D., Ju, X.S., Hieronymus, T., Gust, T.C., Kuhl, C., Jorgas, T., Kurz, S.M., Rose-John, S., Yokota, Y., and Zenke, M. (2003). Transcriptional profiling

doi:10.6342/NTU201703495

identifies Id2 function in dendritic cell development. Nat Immunol *4*, 380-386.

Han, J., Jiang, Y., Li, Z., Kravchenko, V.V., and Ulevitch, R.J. (1997). Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. Nature *386*, 296-299.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med *176*, 1693-1702.

Kee, B.L. (2009). E and ID proteins branch out. Nat Rev Immunol *9*, 175-184.

Kobari, L., Dubart, A., Le Pesteur, F., Vainchenker, W., and Sainteny, F. (1995). Hematopoietic-promoting activity of the murine stromal cell line MS-5 is not related to the expression of the major hematopoietic cytokines. J Cell Physiol *163*, 295-304.

Li, H.S., Yang, C.Y., Nallaparaju, K.C., Zhang, H., Liu, Y.J., Goldrath, A.W., and Watowich, S.S. (2012). The signal transducers STAT5 and STAT3 control expression of Id2 and E2-2 during dendritic cell development. Blood *120*, 4363-4373.

Mashayekhi, M., Sandau, M.M., Dunay, I.R., Frickel, E.M., Khan, A., Goldszmid, R.S., Sher, A., Ploegh, H.L., Murphy, T.L., Sibley, L.D., and Murphy, K.M. (2011). CD8alpha(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by Toxoplasma gondii tachyzoites. Immunity *35*, 249-259.

doi:10.6342/NTU201703495

McKinsey, T.A., Zhang, C.L., and Olson, E.N. (2002). MEF2: a calcium-dependent regulator of cell division, differentiation and death. Trends Biochem Sci *27*, 40-47.

Merad, M., Sathe, P., Helft, J., Miller, J., and 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.

Mildner, A., and Jung, S. (2014). Development and function of dendritic cell subsets. Immunity *40*, 642-656.

Molkentin, J.D., Black, B.L., Martin, J.F., and Olson, E.N. (1996). Mutational analysis of the DNA binding, dimerization, and transcriptional activation domains of MEF2C. Mol Cell Biol *16*, 2627-2636.

Muthusamy, N., Barton, K., and Leiden, J.M. (1995). Defective activation and survival of T cells lacking the Ets-1 transcription factor. Nature *377*, 639-642.

Naik, S.H., Proietto, A.I., Wilson, N.S., Dakic, A., Schnorrer, P., Fuchsberger, M., Lahoud, M.H., O'Keeffe, M., Shao, Q.X., Chen, W.F.*, et al.* (2005). Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. J Immunol *174*, 6592-6597.

Naik, S.H., Sathe, P., Park, H.Y., Metcalf, D., Proietto, A.I., Dakic, A., Carotta, S., O'Keeffe, M., Bahlo, M., Papenfuss, A.*, et al.* (2007). Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in

doi:10.6342/NTU201703495

vivo. Nat Immunol *8*, 1217-1226.

Onai, N., Kurabayashi, K., Hosoi-Amaike, M., Toyama-Sorimachi, N., Matsushima, K., Inaba, K., and Ohteki, T. (2013). A clonogenic progenitor with prominent plasmacytoid dendritic cell developmental potential. Immunity *38*, 943-957.

Redecke, V., Wu, R., Zhou, J., Finkelstein, D., Chaturvedi, V., High, A.A., and Hacker, H. (2013). Hematopoietic progenitor cell lines with myeloid and lymphoid potential. Nat Methods *10*, 795-803.

Robinette, M.L., Fuchs, A., Cortez, V.S., Lee, J.S., Wang, Y., Durum, S.K., Gilfillan, S., Colonna, M., and Immunological Genome, C. (2015). Transcriptional programs define molecular characteristics of innate lymphoid cell classes and subsets. Nat Immunol *16*, 306-317.

Sathe, P., Vremec, D., Wu, L., Corcoran, L., and Shortman, K. (2013). Convergent differentiation: myeloid and lymphoid pathways to murine plasmacytoid dendritic cells. Blood *121*, 11-19.

Sawai, C.M., Sisirak, V., Ghosh, H.S., Hou, E.Z., Ceribelli, M., Staudt, L.M., and Reizis, B. (2013). Transcription factor Runx2 controls the development and migration of plasmacytoid dendritic cells. J Exp Med *210*, 2151-2159.

Scott, C.L., Soen, B., Martens, L., Skrypek, N., Saelens, W., Taminau, J., Blancke, G., Van Isterdael, G., Huylebroeck, D., Haigh, J.*, et al.* (2016). The transcription factor

doi:10.6342/NTU201703495

Zeb2 regulates development of conventional and plasmacytoid DCs by repressing Id2. J Exp Med *213*, 897-911.

Stehling-Sun, S., Dade, J., Nutt, S.L., DeKoter, R.P., and Camargo, F.D. (2009). Regulation of lymphoid versus myeloid fate 'choice' by the transcription factor Mef2c. Nat Immunol *10*, 289-296.

Steinman, R.M., and Cohn, Z.A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. The Journal of experimental medicine *137*, 1142-1162.

Steinman, R.M., Kaplan, G., Witmer, M.D., and Cohn, Z.A. (1979). Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers, and maintenance in vitro. The Journal of experimental medicine *149*, 1-16.

Stirewalt, D.L., and Radich, J.P. (2003). The role of FLT3 in haematopoietic malignancies. Nat Rev Cancer *3*, 650-665.

Swanson, B.J., Jack, H.M., and Lyons, G.E. (1998). Characterization of myocyte enhancer factor 2 (MEF2) expression in B and T cells: MEF2C is a B cell-restricted transcription factor in lymphocytes. Mol Immunol *35*, 445-458.

Swiecki, M., and Colonna, M. (2015). The multifaceted biology of plasmacytoid dendritic cells. Nat Rev Immunol *15*, 471-485.

doi:10.6342/NTU201703495

Tussiwand, R., Everts, B., Grajales-Reyes, G.E., Kretzer, N.M., Iwata, A., Bagaitkar, J., Wu, X.D., Wong, R., Anderson, D.A., Murphy, T.L., *et al.* (2015). Klf4 Expression in Conventional Dendritic Cells Is Required for T Helper 2 Cell Responses. Immunity *42*, 916-928.

Watowich, S.S., and Liu, Y.J. (2010). Mechanisms regulating dendritic cell specification and development. Immunol Rev *238*, 76-92.

Xu, Y., Zhan, Y., Lew, A.M., Naik, S.H., and Kershaw, M.H. (2007). Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. Journal of immunology *179*, 7577-7584.

Zhuang, Y., Cheng, P.F., and Weintraub, H. (1996). B-lymphocyte development is regulated by the combined dosage of three basic helix-loop-helix genes, E2A, E2-2, and HEB. Molecular and Cellular Biology *16*, 2898-2905.

doi:10.6342/NTU201703495