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半乳糖凝集素-1和半乳糖凝集素-3在調控漿狀樹突細胞發育及功能之角色

The Role of Galectin-1 and Galectin-3 in Development and Function of Plasmacytoid Dendritic Cells

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誌謝

沒有想到兩年的碩士生涯過得如此迅速，已經到了打致謝的時候。還記得當初的我連pipetman都不會用，讀paper是一問三不知，但現在的我能夠自己設計實驗解決問題，在meeting時能夠對paper提出問題並進行討論，不但能夠教導學妹做實驗還能幫忙解決問題。對於自己的進步，雖不敢說十分滿意，但能很驕傲地說出我有成長。這一切的一切要感謝很多人的幫助，最要感謝的是我的指導老師　朱清良老師，老師總是充滿著耐心關心，一步步地教導我，從實驗上的規劃到人生經驗的分享，無所不包。實驗失敗時不曾責備過我，總是貼心的提醒我們要記得休息，養精蓄銳後再繼續跟實驗奮鬥。同時也要感謝兩位口委老師，劉扶東老師和李建國老師，在每次的進度報告時都給了我許多十分有用的建議，另外也感謝劉扶東老師提供gal-1$^{-/-}$和gal-3$^{-/-}$老鼠讓我進行實驗，而李建國老師則提供了許多funcional assay上所需的cell line，讓我實驗能順利進行，十分感謝兩位老師，不論是在實驗方面或是撰寫論文方面都幫助我將研究完成。謝謝第一共研的靜嫻學姊和志成學長幫助我sorting，也從你們身上學到很多跟flowcytometry有關的知識和技巧。謝謝小龜學長教導我各項實驗的基礎，以及在meeting時給我許多實驗上的建議。感謝亦師亦友亦學妹的亭伊，不管是實驗瓶頸或是生活苦事趣事，都有妳能一起討論和分享，妳是最好的partner。謝謝Nana，總是讓實驗室充滿著聲音，能夠熱熱鬧鬧地做實驗。謝謝怡君、牧昀、書帆一起拍送舊影片，未來也在實驗上多多加油！謝謝小巫學姊分細胞給我，也給了我許多實驗上的建議。謝謝秋月學姊和派派學姊也教會我實驗上的一些技巧。謝謝我的同學們，逸駿、孔奇、瀚遠，深夜的15F有你們不孤單；飄飄、ＹＹ、蝦子、承希、柏諺、柚子、智傑，大家感情很好，一起預報消除seminar的緊張，一起辦活動一起聊八卦，真沒想到研究所了還能這樣忙裏偷閒，希望大家在自己要走的路上都能順利。最後要感謝我的母親，你總是支持我的想法，相信我的決定。謝謝逸凡總是願意陪著我，這兩年總是在臺大醫學院約會委屈妳了，我一定會給妳一個更好的未來，一起築夢踏實。呂嘟寶下台一鞠躬！

摘要

半乳糖凝集素 (Galectins) 是一種動物性凝集素，可以專一地辨識β-半乳糖 (β-galactosides)。 在免疫系統中，galctin-1 和 -3已經被報導可以調控各種不同的免疫細胞的免疫反應，然而galectin-1和 -3對於漿狀樹突細胞 (plasmacytoid dendritic cell, pDC) 之生長與功能的影響尚未釐清。 pDC在受到病毒感染時，能夠分泌大量的第一型干擾素 (type I Interferon, IFN-I) 並引發各種抗病毒的活性。因此，我們除了研究galectin-1和 -3對於pDC的生長影響外，也用類鐸受體7/9的配體，R848或CpG刺激pDC，以研究pDC產生IFN-I的能力是否會受到galectin-1和 -3的影響。在初步的實驗結果中，我們發現不論是活體外 (*in vitro)* 或是活體內 (*in vivo*) 的試驗，皆發現galectin-1和 -3對於pDC的生長沒有顯著的影響。此外，半乳糖凝集素-3基因剔除 (*lgals3*$^{-/-}$) pDC在CpG刺激後，其第一型干擾素IFN-I (*ifnα, ifnα4, ifnβ*) 和干擾素調控因子7 (Interferon regulatory factor 7, IRF7) 的基因表現量的增加顯著高於野生型 (wild type, WT) pDC。而且，*lgals3*$^{-/-}$ pDC在R848或CpG刺激後所產生的IFN-I也比WT pDC來的高。與*lgals3*$^{-/-}$ pDC相反的是*lgals1*$^{-/-}$ pDC，在R848或CpG刺激後，其IFN-I 基因表現量的增加顯著低於WT pDC，另外產生的IFN-I 也較WT pDC來的低。因此，我們推測galectin-3 在pDC中可能是扮演了負向調控其產生IFN-I 功能的角色，而galectin-1 則是扮演了正向調控的角色。然而，galectin-1和 -3是經由何種機轉調控IFN-I 的產生，仍然需要進一步的研究。

關鍵詞：漿狀樹突細胞、半乳糖凝集素-1、半乳糖凝集素-3、第一型干擾素、類鐸受體7/9

Abstract

Galectins are animal lectins that can bind to β-galactosides. In the immune system, galectin-1 and galectin-3 have been shown to modulate immune responses in various types of immune cell. However, the role of galectin-1 and galectin-3 in the development and the function of plasmacytoid dendritic cell (pDC) has not yet been studied. pDCs produce type-1-interferon (IFN-I) in response to viral infection and then enhance antiviral activities. Thus, we examined the effect of galectin-1 and galectin-3 on pDC development and IFN-I production by pDCs upon the stimulation of R848 or CpG through TLR7/9. In preliminary results, we show that galectin-1 and galectin-3 didn't influence the development of pDC *in vitro* or *in vivo*. In addition, we found that CpG or R848-induced mRNA levels of IFN-I *(ifnα, ifnα4, ifnβ*) and interferon regulatory factor (IRF7) were significantly higher in gal-3$^{-/-}$ Flt3L-BMDCs and in the contrast, significantly lower in gal-1$^{-/-}$ Flt3L-BMDCs when compared to that in WT Flt3L-BMDCs. Moreover, at the protein level, the production of IFNα by gal-3$^{-/-}$ Flt3L-BMDCs stimulated with CpG or R848 is higher than WT Flt3L-BMDCs. And, the production of IFNα by gal-3$^{-/-}$ Flt3L-BMDCs is lower than WT Flt3L-BMDCs. Our data suggested that galectin-3 may negatively regulate the function of pDCs, on the other hand that galectin-1 may positively regulate the function of pDC. However, the mechanism of how galectin-3 and galectin-1 involved in IFN-I production need to be further explored.

Key word: plasmacytoid dendritic cell, galectin-1, galectin-3, type-I-IFN, TLR7/9

Abbreviations

Allophycocyanin (APC)

Allophycocyanin-indotricarbocyanine (APC/Cy7)

Ammonium buffered chloride-potassium solution (ACK)

Bone marrow (BM)

Bone marrow stromal cell antigen (Bst2)

Conventional dendritic cell (cDC)

Dendritic cell (DC)

Fluorescein isothiocyanate (FITC)

Fms-like tyrosine 3 ligand (Flt3L)

Granulocyte-macrophage colony-stimulating factor (GM-CSF)

Type I interferon (IFN-I)

Interferon regulatory factor (IRF)

Interleukin (IL)

Interferon-sensitive response element (ISRE)

Phycoerythrin (PE)

Phycoerythrin-indotricarbocyanine (PE/Cy7)

Plasmacytoid dendritic cell (pDC)

Sialic acid binding immunogolobulin-like lectin H (siglecH)

Spleen (SP)

Toll-like receptor (TLR)

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Chapter I Introduction

1.1 The role of galectin-1 and galectin-3 in DC

Galectins are animal lectins that can bind to β-galctosides through its C-terminal carbohydrate-recognition domains (CRD) (Rabinovich & Toscano, 2009). Based on the CRD composition of galectins, they can be divided into 3 groups. Prototype with one CRD (galectin-1, -2, -5, -7, -10, -11, -13, -14 and -15) and are either monomers or noncovalent homodimers.; tandem repeat type consists of two CRDs (galectin-4, -6, -8, -9, -12) joined by a linker peptide, and galectin-3 is the only member that belongs to chimeric type which has an extended N-terminal region composed of tandem repeats of short amino acid segments connoted to carbohydrate recognition domain (CRD) (R. Y. Yang, Rabinovich, & Liu, 2008). It has been reported that galectin-1 and-3 expressed in many immune cells, such as T cells, B cells, neutrophils, macrophages and conventional dendritic cells (cDCs) (Cummings & Liu, 2009). Galectin-1 induce Th1 and Th17 cells apoptosis via extracellular regulation and galectin-1-deficient mice developed greater Th1 and Th17 responses (Toscano et al., 2007). Recombinant galectin-1 expands the IL-10 secreting regulatory T cell via induction of tolerogenic DC (Blois et al., 2007). Also, LPS stimulated BMDCs, which differentiate with GM-CSF in the presence of recombinant galectin-1, showed a lower IL-12 production and higher IL-10. Consistent with the finding that DC from galectin-1-deficient mice after LPS stimulation exhibited an augmented IL-12 production and impaired IL-10 production (Ilarregui et al., 2009). Together, galectin-1 may play a negative role in regulating immune response in T cells through transduce a tolerogenic signal by DCs. Galectin-3 plays an important role in diverse immune cell processes, such as pathogen recognition, cell migration, adhesion, activation and

apoptosis, and regulating the adaptive immune response and the inflammatory response(Sundblad, Croci, & Rabinovich, 2011). In cDC, galectin-3 negatively regulate the function and capable of interfering the adaptive immune response. The splenic $CD11c^+$ cDC from $gal3^{-/-}$ mice had a higher production of IL-12p40 when stimulated with LPS compared to WT cDC. The higher level of IL-12 produced by cDC will lead to the higher Th1 response developed by $gal3^{-/-}$ mice (Bernardes et al., 2006). Also, upon the simulation of *Histoplasma* infection, $gal3^{-/-}$ cDC produced higher amount of IL-23, TGF-β, and IL1-β than WT cDC. And after *Histoplasma* infection, $gal3^{-/-}$ mice exhibited higher production of IL-17A and higher percentages of Th17 cells. Together, galectin-3 negatively regulates the Th17 response via modulation the production of IL-23 by cDCs (S. Y. Wu, Yu, Liu, Miaw, & Wu-Hsieh, 2013). However, the role of galectin-1 and -3 in the development and the function of plasmacytoid dendritic cell(pDC) has not yet been determined.

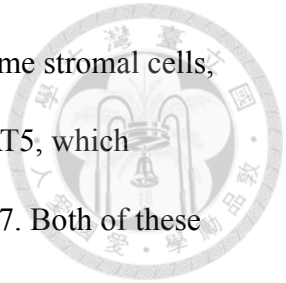
1.2 pDC

DCs are a heterogeneous population that comprises two major types: cDC and pDC. pDCs have a round-shape that with a plasma-cell morphology and well developed rough endoplasmic reticulum (Soumelis & Liu, 2006). pDC can be distinguished from cDC by several markers such as $CD11c^{int}$ $CD11b^-$ $B220^+$ Siglec H^+BST2^+ . Siglec H is a novel member of the sialic acid-binding immunoglobulin (Ig)-like lectin (Siglec) family that specific expressed on pDC . Siglec H associates with an ITAM-containing adaptor protein, DNAX adaptor protein 12 (DAP12), which can induce a negative signal to modulate the type I IFN response, reducing the production of type I IFN by pDC after the stimulation with TLR agonist (Blasius, Cella, Maldonado,

Takai, & Colonna, 2006). Moreover, pDC from DAP12-deficient mice produce higher levels of IFNα, IFNβ and IL-12 during viral infection (Sjolin et al., 2006). Bone marrow stromal cell antigen 2 (BST2) is predominantly expressed on pDC, and can be recognized by the Abs plasmacytoid dendritic cell antigen 1 (PDCA-1) and 120G8 in the naive mice. BST2 is an interferon stimulated gene (ISG), it will up-regulate on various cell types upon the stimulation that can trigger type I IFN response, including pDC (Blasius, Giurisato, et al., 2006). PDC-TREM, a member of the triggering receptor expressed on myeloid cells family (TREM) which expressed on activated pDC after TLR-stimulation. PDC-TREM directly associates with DAP12 and Plexin-A1. When the ligand of Plexin-A1, Sema6D bind to Plexin-A1, pDC will produce robust type I IFN which suggest that PDC-TREM/Plexin-A1/DAP12 complex mediates a positive signal for type I IFN response in pDC (Watarai et al., 2008).

1.3 Development of pDC

pDC can generated from both myeloid progenitor, or lymphoid progenitor. At the steady state, Fms-like tyrosine 3^+ ($Flt3^+$) common DC progenitors (CMP) or $Flt3^+$ common lymphoid progenitors (CLP) will develop to pDC in the presence of Flt3L (L. Wu & Liu, 2007). Flt3-Flt3L ligation will activates signal transducer and activator of transcription 3 (STAT3) and transduce a signal that is critical for the development of pDC in humans and mice (Liu, 2005). The pDC numbers are dramatically decreased in *flt3*$^{-/-}$ and *flt3l*$^{-/-}$ mice (Waskow et al., 2008)(Brawand et al., 2002). On the other hand, pDC numbers in Flt3L-transgenic mice are significant increased (Brawand et al., 2002). Under the inflammatory condition, granulocyte-



macrophage colony-stimulating factor (GM-CSF) is produced by some stromal cells, activated T and NK cells, and macrophages. GM-CSF activates STAT5, which directly or indirectly inhibits the transcription of Flt3, IRF8 and IRF7. Both of these transcription factors and the receptor of Flt3L are essential for the development of pDCs (Onai & Manz, 2008). Together, Flt3L activated STAT3 supports pDC differentiation,in the contrast, GM-CSF activated STAT5 inhibits pDC differentiation.

1.4 The main function of pDC

The expression of TLRs are different between cDC and pDC. In contrast to cDC, pDCs selectively express TLR7 and TLR9 (Kaisho, 2012). pDCs, also named type-1-interferon producing cells(IPCs), can produced large amounts of type I interferon (IFN-I) in response to viral infection through TLR7 or TLR9 (Gilliet, Cao, & Liu, 2008). TLR7 sense guanosine- or uridine-rich single-stranded RNAs (ssRNA) from viruses and synthetic imidazoquinoline compounds imiquimod and R-848 (Diebold, Kaisho, Hemmi, Akira, & Reis e Sousa, 2004). TLR9 sense unmethylated CpG motifs in ssDNA, respectively, motifs found in the viruses or bacteria (Hemmi et al., 2000). Synthetic ODNs containing CpG motif can directly interact with TLR9 through its ectodomain and induce downstream signaling, including the induction of the transcription of IFN-I and pro-inflammatory cytokine gene (Latz et al., 2007). The production of IFN-I can enhance maturation and activation of immature DC, induce IFNγ production by T_H1 and NK cells, promote plasma cell proliferation and class switching, and can induce over 400 ISGs that are antiviral, anti-proliferative, and immunomodulatory, to against the viral infection (Decker, Muller, & Stockinger, 2005). However, pDC can also sense self DNA when necrotic and apoptotic cell death

(Pisetsky & Fairhurst, 2007) and cause autoimmune disease, such as SLE (Blanco, Palucka, Gill, Pascual, & Banchereau, 2001), psoriasis (Lande et al., 2007), and type I diabetes (TID) (Li et al., 2008) when dysregulating the IFN-I response.

1.5 TLR7/9 dependent signaling pathway

After ligand engagement, activated TLR7/9 recruit the myeloid differentiation primary response gene 88 (MyD88) through the Toll/IL1 receptor (TIR) domain. MyD88, as a key adaptor protein, then recruit the IL-1 receptor associated kinase 4 (IRAK4) to TLR7/9 (Kaisho & Akira, 2006). In MyD88 or TLR9-deficiency mice,pDC completely lose their ability to produce IFNα when stimulated with the TLR9 ligand CpG ODNs (Hemmi, Kaisho, Takeda, & Akira, 2003). IRAK4 activates TNF receptor-associated factor 6 (TRAF6) and form the signal-transducing complex in the cytoplasm which comprise of IRAK4 (K. Yang et al., 2005), TRAF6 (Hacker et al., 2006) and Bruton's tyrosine kinase (BTK) (Pisitkun et al., 2006). The complex then activate IRF7 or transforming growth factor-beta-activated kinase-1 (TAK1) or IRF5, each leading to different signaling pathway. For type I IFN response, IRF7 is activated through the phosphorylation by IRAK4 (Kim et al., 2007) and ubiquitylation by TRAF6 (Kawai et al., 2004). Activated IRF7 interacts with TRAF3, IRAK1 (Uematsu et al., 2005), IκB kinase α (IKKα) (Hoshino et al., 2006), osteopontin (OPN) (Shinohara et al., 2006), phosphoinositide 3-kinase (PI3K) (Guiducci et al., 2008), and then translocate to nucleus to initiating type I IFN gene transcription. On the other hands, for the induction of transcription of pro-inflammatory cytokine and co-stimulatory molecules, TRAF6 in the signal-transducing complex ubiquitylates the TAK1, and activated TAK1 then transduce the

signal to activate NF-B and MAPKs (Osawa et al., 2006). IRF5 both involved in the signaling pathway of induction of type I IFN and pro-inflammatory cytokine and co-stimulatory molecules (Takaoka et al., 2005). By contrast, IRF4 inhibits the function of IRF5 through competition (Xu, Meyer, Ehlers, Blasnitz, & Zhang, 2011).

1.6 Specific aims

In our preliminary data, we show that galectin-1 and galectin-3 expressed in pDC and gal-3$^{-/-}$ Flt3L-BMDC treated with CpG had a greater IFN-I response and IL-12 production compared to WT Flt3L-BMDCs. Gal-1$^{-/-}$ Flt3L-BMDCs are opposite from gal-3$^{-/-}$ Flt3L-BMDC, which produced lower IFN-I in response to TLR7/9 agonists. Hence, we hypothesized that galectin-3 may play a negative regulatory role and galectin-1 play a positive role in the IFN-I response of pDCs.

Aim1: Establishing assay systems.

To study the development and function of pDC, we first establish *in vitro* pDC culture system. And for *ex vivo* functional assay, we use B16/Flt3L cell line to expand pDC percentage and numbers *in vivo*. Next, to detect the IFN-I production, we use a C4-ISRE-RFP reporter cell line which is provided by Dr. Chien-Kuo Lee. We also confirm the production of IFNα by intracellular staining and use a 293-IFNα cell line which overexpressed IFNα as a positive control for intracellular staining. For future studies on IFN-I signaling pathway, we will use human pDC cell line which is a gift from Dr. Masuhiro Takahashi. Before exploring the signaling pathway of INF-I, we will test the production of IFN-I after TLR agonists stimulation.

Aim2: Examining galectin expression profile.

After setting up the assay systems, we will first confirm the galectins expression in pDC. It has been reported that galectin-1 and galectin-3 is expressed in human pDC (Cabezon, Sintes, Llinas, & Benitez-Ribas, 2011), although the mRNA expression level of galectin-3 is quite lower than other galectins (Harman et al., 2013). However whether the galectin-1 and galectin-3 express in mouse pDC has not yet been determined. We also want to know the galectin expression patterns in PMDC05. Therefore, we screen the expression of galectins in mouse pDC and PMDC05 first. As expected, galectin-1 and galectin-3 are express in pDC. The expression level of galectin-3 is lower than cDC which is already known that cDC had a high level expression of galectin-3 (S. Y. Wu et al., 2013).

Aim3: To elucidate whether galectin-3 involve in development of pDC and function of producing type I IFN by pDC.

Next, we want to examine whether galectin-3 involved in the differentiation and development of pDC. It has been reported that galectin-3 has been reported that galectin-3 in DCs negatively regulates Th17 cell differentiation and development (Fermin Lee et al., 2013). However, there is little known about whether galectin-3 play a role in pDC development. Therefore, we hypothesized that galectin-3 may be potential to modulate the differentiation and development of pDC.

After we explore the influence of galectin-3 on pDC development, we next examined whether galectin-3 participate in IFN-I production by pDC after TLR7/9 agonists stimulation. Galectin-3 regulates the function of various type immune cells. It has a suppressive effect on human eosinophils by inhibiting the IL-5 production (Cortegano

et al., 1998). And, galectin-3 deficient mice exhibited a reduced IgE-mediates response of mast cells (Rabinovich, Liu, Hirashima, & Anderson, 2007). Also, as mentioned before, galectin-3 modulates Th17 responses by regulating dendritic cell cytokines (Fermin Lee et al., 2013) and a role in the regulation of Th1/Th2 differentiation by affecting IL-12 production in DCs (Saegusa et al., 2009), on the other hand, galectin-3 promote the Th2 response in a mouse model of atopic asthma (Zuberi et al., 2004). Together, galectin-3 deficiency mice revealed a promotion of inflammatory responses. Hence, we hypothesized that galectin-3 may be negatively regulates the production of IFN-I and pro-inflammatory cytokine by pDC.

Aim4: To exam the role of galectin-1 in pDC development and type I IFN production.

After we confirm the expression of galectin-1 in pDC. We want to exam the role of galectin-1 in pDC differentiation and development. It has been reported that exogenously added galectin-1 promotes proliferation of neural stem cells (Sakaguchi et al., 2006), and myoblasts from galectin-1 deficient mice exhibited delayed development at the neonatal stage (Georgiadis et al., 2007). Therefore, we hypothesized that galectin-1 may get involved in pDC development. Next, we interested in the role of galectin-1 in modulating the IFN-I response in pDC. Galectin-1 can selectively bind to Th1 and Th17 cells induce apoptosis, in contrast, Th2 cells are protected from galectin-1 induced cell death (Toscano et al., 2007). Consistent with this finding, galectin-1-deficient mice exhibited an enhanced Th1 and Th17 cell responses. Also, recombinant galectin-1 induce an expansion of IL-10-producing T cells (Ilarregui et al., 2009) and $CD4^{+}CD25^{+}$ Treg cells (Juszczynski et al., 2007).

Besides, LPS-treated BMDC from galectin-1-deficient mice showed a higher IL-12 production and lower IL-10 and IL-27 production. Together, galectin-1 may play a negative role in regulating Th1 and Th17 cell responses and delivering a tolerogenic signal via DC. Hence, we hypothesized that galectin-1 negatively regulating the IFN-I responses after TLR7/9 stimulation in pDC. Surprisedly, we found that galectin-1 positively regulating the IFN-I production by pDC.

Chapter II Materials and Methods

2.1 Mice

C57BL/6 wile-type were provided by Dr. Betty A. Wu-Hsieh. *Lgals1*$^{-/-}$ and *lgals3*$^{-/-}$ mice were provided by Dr. Fu-Tong Liu. All mice are bred at the Laboratory Animal Center of National Taiwan University, College of Medicine. All mice were maintained in specific pathogen-free conditions, and were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

2.2 *In vitro* pDC culture and DC subsets analysis

Bone marrows were isolated from femurs and tibiae and filter the suspension through a 70μm filter. After using ammonium buffered chloride-potassium solution (ACK) to lyse the red blood cells (RBCs). BM cells were seeded into 12-well coating plate in RPMI1640 medium with 10% fetal bovine serum (FBS) and penicillin-streptomycin containing 100 ng/ml or indicated concentration of mouse Flt3L (PeproTech Inc.) at a density of $4x10^6$ cells/ml or indicated density for 8 days or indicated days. Culture medium was replaced at d3 and d6 supplement with 100 ng/ml Flt3L. On day8, Flt3L-BMDC were harvested. After cell counting, Flt3L-BMDC were reseeded to 24-well plate or 96-well plate and stimulated with TLR7/9 agonists. On d8 or d9, DC subsets were analyzed by flow cytometry, DCs were stained with allophycocyanin (APC) anti-CD11c, fluorescein isothiocyanate (FITC) anti-CD11b, phycoerythrin-indotricarbocyanine (PE/Cy7)

anti-Bst2, phycoerythrin (PE) anti-siglecH, biotin-anti B220, streptavidin allophycocyanin-indotricarbocyanine (APC/Cy7) for secondary labeling, and analyzed by BD FACSCanto II. pDCs are defined as $CD11c^+$ $CD11b^-$ $Bst2^+$ $siglecH^+$ $B220^+$, respectively, cDCs are defined as $CD11c^+$ $CD11b^+$ $Bst2^-$ $siglecH^-$ $B220^-$.

2.3 *In vivo* expansion of pDCs

To expand the pDCs population, we use B16 melanoma cell line which stably transfected with mouse Flt3L. 3-4x10^6 cells were injected s.c. into back, and animals were sacrificed after 10-14 days. The percentage and number of splenic pDCs were significant enriched after tumor cell line injection. Enrichment of pDCs were confirmed by flow cytometry and cell counting.

2.4 Splenic DC isolation and DC subsets analysis

After spleen isolated from tumor cell line injected mice or control mice, spleen were grounded by grinder and lysing the RBCs by ACK. For splenic DC isolation, splenic cells were stained by APC-anti CD11c, FITC-anti CD11b and PE/cy7 anti-Bst2, and pDC were defined as $CD11c^{int}$ $CD11b^{-}$ $Bst2^{+}$, respectively, cDC were defined as $CD11c^{high}$ $CD11b^{+}$ $Bst2^{-}$. Before sorting, the suspensions need to filter through 40un filter. For DC subsets analysis, splenic cells were stained by APC-anti CD11c, FITC-anti CD11b, PE/cy7 anti-Bst2, PE-anti siglecH, biotin anti-B220 and streptavidin-APC/cy7.

2.5 Functional assays of pDCs

For *in vitro* Flt3L-BMDC functional assays, on d 8, Flt3L-BMDC or Flt3L-BMDC sorted by BD FACS Aria were seeded into 24-well plate with a density of 1x10^6 cells/ml and stimulated with 1000nM CpG1585, 1000nM CpG2336, 1000nM CpG1826, 0.5 μg/ml R-848, or 5μg/ml IMQ for 6, 16 h to perform qCPR. Or, seeded in 96-well plate with a density of 2x10^5 cells/200μl and stimulated with 1000nM CpG1585, 1000nM CpG2336, 1000nM CpG1826, 0.5μg/ml R-848, or 5μg/ml IMQ for 24 h, and added Brefeldin A (BFA) 9 h before harvest to perform intracellular staining

(ICS) of IFNα or IL-12. For ex *vitro* DC functional assay, splenic pDC and cDC were sorted by BD FACS Aria and stimulated with 1000nM CpG1585 or 0.5μg/ml R-848 in 96 well plate for 24 h, and BFA was added 9 h before harvested to perform ICS. Expression levels of cytokine and activation marker were analyzed by BD FACS Canto II. For all functional assay, supernatant was collected for the detection of IL-12 by ELISA and IFN-I by bioassay.

2.6 qPCR

qPCR was performed on cDNA samples. cDNA (4 μl) was mixed with 6 μl Fast SYBR Green Master Mix (ABI?) contained 0.2 μM forward and reverse primers. The reaction was performed on a PikoReal 96 Real-Time PCR system (Thermo Scientific), and the result was analyzed by PikoReal software 2.1. Each PCR reaction was performed in duplicate. The relative number of each gene is normalized to mGAPDH, and the relative fold change was calculated based on the fold induction of the non-treated sample.

2.7 Bioassay for IFN-I

IFN-I was detected by a reporter cell line which is a gift from Dr. Chien-Kuo Lee. The reporter cell line was stably transfected ISRE-RFP (interferon-sensitive response element-red fluorescent protein) a *Stat3*$^{-/-}$ MEF. The IFN-I conjugate to ISRE and drive RFP expression which can analyze by flow cytometry. Reported cells were seeded into 96-well flat-plate with a density $1.5x10^4$ at a total volume 50 μl for one day. Sample supernatant and IFNα4 standard were added for 24 h. Standard concentrations were added in two-fold serial dilution from 300 U/ml to 0.15 U/ml. RFP^+ cells were analyzed by BD FACS Canto II.

2.8 Assessment of cytokine production by ELISA

IFNα and IL-12p70 were quantified using an enzyme-linked immunoassay (ELISA:) , following the manufacturer's instruction.

2.9 Flow cytometry

For intracellular staining of IFNα, 293-IFNα cell line which over-express IFNα4 were used to as a positive control.

2.10 PMDC05 cell line

PMDC05 cell line is a human pDC cell line with is a gift from Dr. Masuhiro Takahashi. The human pDC cell line was cultured in Iscove's modified Dulbecco's media (IMDM, Invitrogen, Grand Island, NY, USA) with 10% FBS at first, and then switch to compete medium with RPMI 1640 contained 10% FBS.

2.11 Statistical analysis

Statistical analysis of experimental groups was performed by unpaired Student's t-tests using GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA). $P < 0.05$ was considered significant.

Chapter III Results

3.1 Establishing *in vitro* pDC culture system

First, we need to setup the *in vitro* pDC culture system to perform the following experiment. We have tested several culture conditions, including numbers of BM cell at initial seeding, size of culture plate, culture duration (Fig. 1a), medium ,cytokine concentration (Fig. 1b). And we find out the optimal culture condition is seeding BM cell into 12-well plate at a density $4x10^6$ in a total volume 2 ml medium which contain 10%FBS, 100x P/S, and 100 ng/ml Flt3L in RPMI 1640 for 9 d. At d3 and d6, 1 ml medium was discarded and replaced with 1 ml fresh medium containing 100 ng/ml Flt3L. At d8, the population of Flt3L-BMDC were harvested and reseeding to perform further functional assay. Also, analyzed by flow cytometry, and the Flt3L-BMDCs are comprised of about 30-40% pDC and 30-40% cDC and harvested about $1x10^6$ cells per well.

3.2 Expansion of pDC *in vivo*

For *ex vivo* functional test, we need to isolate pDC from spleen. However, the percentage and numbers of pDC are little in the spleen. Hence, we use B16 melanoma cell line which stably transfected with murine Flt3L to expand the pDC percentages and numbers *in vivo* (Bjorck, Leong, & Engleman, 2011; Mach et al., 2000). $5x10^6$ B16/Flt3L cells were injected to mouse s.c. and the spleen enlargement can be observed after 14 d (Fig. 2a). The percentage of pDC were enriched about 10 times and the numbers were enriched about 40 times after B16/Flt3L injection (Fig. 2b).

3.3 Positive control of IFNα intracellular staining

We use 293-IFNα cell line which can over express IFNα as a positive control of IFNα intracellular staining and use 293T as negative control (Fig. 3).

3.4 Galectins expression in pDC

After setting up the assay system, we further analyze the expression profile of galectin by qPCR in sorted splenic pDC. The mouse pDC expressed Gal-1, -3, -4, -8, -9. Galectin-1 has highest expression level and the second is galectin-3 (Fig. 4a), on the other hands, in cDC, galectin-3 has highest expression level, and galectin-1 is the second (Fig. 4b). Although the expression levels of galectin in pDC are different from human pDC, but the expression profile is similar (Harman et al., 2013). We also use flow cytometry to confirm the expression of Gal-3 in splenic pDC (Fig. 4c) and splenic cDC (Fig. 4d). The expression level of galectin-3 acquired by flow cytometry is consistent as we observed by qPCR. We also confirm the expression level of galectin-3 in Flt3L-BMDC (Fig. 5), and find out that Gal-3 are mainly expressed in the cytosol. There are no Gal-3 binding on the surface of mouse pDC. Because of Gal-1 and Gal-3 in deed expressed in mouse pDC, we hypothesized that Gal-1 and Gal-3 may be involved in the development of pDC.

3.5 Galectin expression profile in human pDC cell line, PMDC05

We also interested in the galectin expression level in human pDC cell line, and acquired the expression profile by qPCR. PMDC05 is a gift from Dr. Masuhiro Takahashi. The human pDC cell line was created from leukemia cells of pDC leukemia which express TLR4, TLR7 and TLR9. PMDC05 can produce IFNα and IL-12 after stimulation with CpG-A or LPS (Narita et al., 2009). PMDC05 expressed almost every galectin except gal-13 and gal-14 (Fig. 6), which expressed only in the placenta (Than et al., 2004). In the future, we can use PMDC05 to investigate the

mechanism of how galectin-1 or galectin-3 regulates the IFN-I response by knock down or over-expression galectins.

3.6 Galectin-3 doesn't get involved in mouse pDC development

It has been reported that gal-3 involved in T cell differentiation and development (Fermin Lee et al., 2013), however whether gal-3 expression affect the development of pDC is still unknown. To investigate the role of galectin-3 in pDC development, we first compare the pDC population and numbers of Flt3L-treated BM cell from WT and gal-$3^{-/-}$ mice (Fig. 7a and 7b). Second, we compare the population and numbers of splenic pDC from WT and gal-$3^{-/-}$ mice (Fig. 8a and 8b). There is no significant difference in the pDC population or numbers between WT and gal-$3^{-/-}$ mice *in vitro* or *in vivo*. Hence, we conclude that galectin-3 may not get involved in the development of mouse pDC.

3.7 Gal-$3^{-/-}$ Flt3L-BMDCs exhibit augmented IFN-I and IL-12 production in response to CpG or R848

Galectin-3 deficiency mice revealed a promotion of inflammatory responses. To investigate whether galectin-3 also play a negative regulatory role in pDC, we first use CpG or R848 stimulate Flt3L-BMDC from WT and gal-$3^{-/-}$ mice *in vitro*. Surprisingly, we found that after TLR7 or TLR9 stimulation, the induction of IFN-I mRNA,ifn*α, ifnα4,*and *ifnβ* (Fig. 9a and Fig. 11a) were significantly higher than WT Flt3L-BMDC. We further use a ISRE-RFP reporter cell line which can detect IFN-I to determine the production of IFN-I. At the protein level, the production and induction of IFN-I produced by gal-$3^{-/-}$ Flt3L-BMDC were higher than WT Flt3L-BMDC after CpG or R848 stimulation (Fig. 9b and Fig.11b). Because the reporter cell can only

sense IFN-I but can not distinguish difference expression level of IFNα or IFNβ. Hence, we use intracellular staining to confirm the expression of IFNα and found that the production of IFNα by gal-3$^{-/-}$ Flt3L-BMDC were higher than WT Flt3L-BMDC (Fig. 9c and Fig 11c). It is possible that the augmented production of IFN-I by gal-3$^{-/-}$ Flt3L-BMDC is due to the higher TLR7 or TLR9 expression. To clarify this possibility, we acquired the TLR7 or TLR9 expression level after stimulation and find out that the expression level of TLR7 or TLR9 are comparable between WT and gal-3$^{-/-}$ Flt3L-BMDC after stimulation (Fig. 9a and Fig. 11a). To gain more information about the mechanism that how galectin-3 regulate the production of IFN-I in pDC. We analyze the maturation status of WT and gal-3$^{-/-}$ Flt3L-BMDC to investigate that whether the different ability of IFN-I production is due to different maturation status between WT and gal-3$^{-/-}$ Flt3L-BMDC. After CpG stimulation, upregulation of CD40, CD80, CD86 can be observed (Fig. 10). However, there are no significant difference between WT and gal-3$^{-/-}$ Flt3L-BMDC. We also compare the expression of PDC-TREM, which is a specific maturation marker of pDC, and found that the expression level of PDC-TREM in WT and gal-3$^{-/-}$ Flt3L-BMDC are comparable (Fig. 10). These results suggest that galectin-3 doesn't affect the maturation status of Flt3L-BMDC *in vitro*. And the augmented production of IFN-I by gal-3$^{-/-}$ Flt3L-BMDC was not caused by the different maturation status. It has been reported that gal-3$^{-/-}$ CD11c^{+} DCs produce higher IL-12 in response to LPS stimulation (Bernardes et al., 2006). Similar to LPS stimulation, gal-3$^{-/-}$ Flt3L-BMDC treated with CpG or R848 had a higher induction of IL-12 gene and production of IL-12 than WT Flt3L-BMDC (Fig. 9d and Fig. 11d). These data suggest that galectin-3

may negatively regulate the IFN-I and production in Flt3L-BMDC upon the TLR9/7 stimulation.

3.8 Sorted Flt3L-BMpDC from Gal-3$^{-/-}$ mice had higher IFN-I production after stimulation with CpG or R848

The Flt3L-BMDC are comprised of about 30-40% pDC and 30-40% cDC (Fig. 7a and 7b), so we need to sort out the pure pDCs to exclude the possibility that the enhanced IFN-I production by gal-3$^{-/-}$ Flt3L-BMDC were actually mainly affected by cDC but not pDC or other influenced by cell-cell cross-talked. We sorted out the Flt3L-BMpDC and Flt3L-BMcDC as previously described, and define pDC as CD11c^{int}CD11b^{-}Bst2^{+}, respectively, cDC as CD11c^{+}CD11b^{+}Bst2^{-}. After CpG or R848 stimulation, Flt3L-BMpDC from gal-3$^{-/-}$ mice had enhanced IFN-I production compared to WT (Fig. 12b and 13b), although the induction of IFN-I gene is comparable (Fig. 12a and 13a). We also acquired the TLR7 and TLR9 expression level and found that the expression level of TLR7 and TLR9 after stimulation are similar between WT and gal-3$^{-/-}$ sorted Flt3L-BMpDC (Fig. 12a and 13a). Although the induction of IL-12 gene was comparable between WT and gal-3$^{-/-}$ sorted Flt3L-BMpDC after TLR9/7 stimulation. Sorted Flt3L-BMcDC from gal-3$^{-/-}$ mice had a higher production of IL-12 compare to Flt3L-BMcDC from WT after TLR7 or TLR9 stimulation (Fig. 12c and 13c). Our data suggest that galectin-3 may play a negative regulatory role in BMpDC *in vitro*. However, it still needs further examination whether galectin-3 really play a negative role *ex vivo or in vivo*.

3.9 Sorted splenic pDC from Gal-3$^{-/-}$ mice had higher IFN-I production after stimulation with R848

Beside sorted Flt3L-BMDC, we examine the pDC sorted from spleen to determine the role of galectin-3 *ex vivo*. Because of the pDC percentages and numbers are little in the spleen, we expand the pDC *in vivo* as previously described. After pDC sorted from spleen, we use CpG and R848 to stimulate pDC and perform further analysis. We found that upon CpG or R848 stimulation, splenic pDC from gal-3$^{-/-}$ mice produce more IFNα than splenic pDC form WT (Fig. 14a and 14c). And the IFNα production by gal-3$^{-/-}$ splenic pDC stimulated with R848 were higher than WT splenic pDC (Fig. 14e). Besides, the gal-3$^{-/-}$ splenic cDC treated with R848 produce more IL-12 compared to splenic cDC from WT (Fig. 14b and 14d). Our data suggest that galectin-3 may also play a negative regulatory role in regulating IFN-I responses in pDC *ex vivo*.

3.10 Galectin-1 doesn't get involved in mouse pDC development

It has been reported that galectin-1 participate in T cell differentiation and development (Fermin Lee et al., 2013), however whether gal-3 expression affect the development of pDC is still unknown. To investigate the role of galectin-3 in pDC development, we first compare the pDC population and numbers of Flt3L-treated BM cell from WT and gal-3$^{-/-}$ mice (Fig. 7a and 7b). Second, we compare the population and numbers of splenic pDC from WT and gal-3$^{-/-}$ mice (Fig. 8a and 8b). There is no significant difference in the pDC population or numbers between WT and gal-3$^{-/-}$ mice *in vitro* or *in vivo*. Hence, we conclude that galectin-3 may not get involved in the development of mouse pDC.

3.11 Gal-1$^{-/-}$ Flt3L-BMDCs exhibit an impaired IFN-I response after CpG or R848 stimulation

Not only galectin-3, we also explored the role of galectin-1 in pDC functions. After CpG or R848 stimulation, the induction of IFN-I genes (Fig. 17a and Fig. 19a) and the production of IFN-I (Fig. 17b and Fig. 19b) were significantly lower in gal-1$^{-/-}$ Flt3L-BMDC compared to WT Flt3L-BMDC. We also use intracellular staining to confirm the production of IFNα (Fig. 17c and 19c). Similar results can be seen in intracellular staining that gal-1$^{-/-}$Flt3L-BMDC exhibit an impaired IFN-I response after CpG or R848 stimulation. The lower induction and production of IFN-I by gal-1$^{-/-}$ Flt3L-BMDC are not due to the lower expression of TLR7 and TLR9. The expression level of TLR7 and TLR9 of gal-1$^{-/-}$ Flt3L-BMDC are compared to WT Flt3L-BMDC (Fig. 17a and Fig. 19a). Either, the lower production of IFN-I doesn't cause by different maturation status of gal-1$^{-/-}$ and WT Flt3L-BMDC (Fig. 18). The expression level of CD40, CD80, CD86 and PDC-TREM of gal-1$^{-/-}$ Flt3L-BMDC were comparable to WT Flt3L-BMDC. It has been reported that BMDC or Flt3L-DC from galectin-1-deficient mice produce higher IL-12 than BMDC or Flt3L-DC from WT mice after exposure to LPS (Ilarregui et al., 2009). Upon CpG or R848 stimulation, gal-1$^{-/-}$Flt3L-BMDC produce higher amount of IL-12 than WT Flt3L-BMDC (Fig. 17d and Fig. 19d). Together, our data suggest that galectin-1 may play a positive role in regulating IFN-I production in pDC which contract to galectin-3.

Chapter IV Discussion

4.1 Distinct roles of galectin-1 and -3 in regulating the production of IFN-I by pDC

Both gal-1$^{-/-}$ and gal-3$^{-/-}$ Flt3L-BMDC showed an augmented production of IL-12 after treated with CpG or R848 (Fig. 9d ,11d, 17d and 19d). Our data suggest that galectin-1 and -3 play a same role in negatively regulate the production of IL-12 after TLR9/7 stimulation which consistent with previously reported that gal-1$^{-/-}$ and gal-3$^{-/-}$ BMDC had a higher IL-12 production than WT BMDC (Ilarregui et al., 2009) (Saegusa et al., 2009). Surprisingly, we found that the role of galectin-1 and galectin-3 are distinct in regulating the production of IFN-I by pDC. Gal-3$^{-/-}$ Flt3L-BMDC showed an enhanced production of IFN-I (Fig. 9 and 11), in contrast, gal-1$^{-/-}$ Flt3L-BMDC exhibited a impaired production of IFN-I (Fig. 17 and 19). These data suggest that galectin-3 plays a negative role in IFN-I response, on the other hands, galectin-1 plays a positive role in regulating the IFN-I production by pDC. However, the mechanism of how galectin-1 and galectin-3 influence the production of IFN-I need to be further examined. We first demonstrated that the different ability to produce IFN-I between WT, gal-1$^{-/-}$ and gal-3$^{-/-}$ Flt3L-BMDC are not due to different expression of TLR7 or TLR9 (Fig. 9a ,11a, 17a and 19a). Also, it is not because the different maturation status between WT, gal-1$^{-/-}$ and gal-3$^{-/-}$ Flt3L-BMDC. All of WT, gal-1$^{-/-}$ and gal-3$^{-/-}$ Flt3L-BMDC had a comparable expression of CD40, CD80, CD86 and PDC-TREM after CpG or R8484 stimulation (Fig. 10 and 18). This finding consistent with previously reported that the expression level of CD80 and CD86 in human monocyte deviated DC didn't affect by galectin-1 or galectin-3 knock down (Mobergslien & Sioud, 2012). Interestingly, the pDC specific maturation marker,

PDC-TREM can only be induced by CpG but not R848, which means that the expression of PDC-TREM is TLR9-dependent. According to previously report, PDC-TREM could be induce by polyU (Watarai et al., 2008), however, we couldn't observed the expression of PDC-TREM after R848. Perhaps the induction of PDC-TREM is ligand specific.

When comparing the difference of induction and production of IFN-I by WT and gal-1$^{-/-}$ Flt3L-BMDC, we noticed that the magnitude of induction of IFN-I gene were more obvious than the production of IFN-I. Suggest that there were post-transcriptional regulation in modulating IFN-I signaling pathway.

We also acquired the expression level of IRF7, an important signal molecule that could be phosphorylated and translocated to nucleus to initiate the transcription of IFN-I gene, and found that the basal level of IRF7 are comparable between WT and gal-3$^{-/-}$ Flt3L-BMDC (Fig. 9a). This data suggest that the phosphorylation of IRF7 could be enhanced in gal-3$^{-/-}$ Flt3L-BMDC which leads to augmented induction and production of IFN-I.

It has been reported that galectin-1-deficient mice had lower survival rates compare to WT mice when influenza virus infection (M. L. Yang et al., 2011). IFN-I is an important cytokine to induce the antiviral ability against viral infection such as influenza virus infection (Pauli et al., 2008). Our data suggest that Flt3L-BMDCs from gal-1$^{-/-}$ mice are impaired in producing IFN-I could be an explanation that gal-1$^{-/-}$ mice had lower survival rates when influenza infection. To address this question, we could adoptive transfer pDC from WT, gal-1$^{-/-}$ and gal-3$^{-/-}$ mice to WT mice infected with influenza virus or other RNA or DNA virus. And evaluated the survival rate and

inflammation status to demonstrate the role of galectin-1 and galectin-3 in regulating the IFN-I response.

4.2 Galectin expression profile of human pDC cell line, PMDC05

The galectin expression profile in human pDC has been reported. The most higher expression level of galectin in pDC is galectin-10 and the second is galectin-4. Unexpectedly, the galectin expression profile in PMDC05 is quite different. The most higher expression level of galectin in PMDC05 is galectin-1 and the second is galectin-9. The expression level of galectin-10 and galectin-4 are lower than other galectins. However, we still could knock down or over express the galectins in PMDC05 to study the IFN-I signaling pathway.

4.3 Autophagosome involved in transferring the nucleic-acid to TLR

Recently, TLRs and autophagy had been thinking that they cooperate in the response to PAMPs (Arroyo et al., 2014). It has been reported that autophagy is involved in mediating type I IFN production in pDC in responses to RNA viruses. Autophagy facilitates the transferring viral RNA in the cytosol to endosome which containing of TLR7 (Lee, Lund, Ramanathan, Mizushima, & Iwasaki, 2007). Autophagy also enhances the delivering self DNA-containing immune complexes to TLR9 and induce augmented IFNα production (Henault et al., 2012). Besides, galectin-8 has been reported that involved in mediating autophagy. When *Salmonella entrica* infection, the expression level of galectin-8 up-regulated, and galectin-8 targets damaged vesicles for autophagy to defend cells against bacterial invasion (Thurston, Wandel, von Muhlinen, Foeglein, & Randow, 2012). Together, there are possibility that

galectin-1 or galectin-3 also involved in mediating the autophagy machinery and affect the nucleic acid transferring to TLR7 or TLR9 which will lead the different amount of IFN-I produce by pDC.

4.4 Functionless sorted splenic pDC

When we try to examine our finding that gal-1$^{-/-}$ and gal-3$^{-/-}$mice ex vivo, there was a strange problem came out. At first we use siglecH as marker to sort out the splenic pDC, but the pDC seems to be nonfunctional when stimulate with CpG. And because of that ligation of siglecH may induce a negative signal which lead to attenuate TLR9-induced type I IFN production (Blasius, Cella, et al., 2006). Probably that the Ab we used to recogniz pDC via siglecH inhibit the production of IFN-I, hence we use Ab recognize Bst2 instead of siglecH. However, the pDC sorted from the spleen still couldn't produce IFN-I when response to CpG. There is a study addressed this question at 2011. They consider that pDC can be divide into 2 subset by CD9 expression. CD9^{+} pDCs are able to produce large amount of IFN-I, after CD9^{+} pDCs migrate to peripheral tissues, they lose CD9 expression and the ability to produce IFNα. They also demonstrate that splenic pDC are mainly CD9^{-} (Bjorck et al., 2011). So, this is may be the reason that the pDCs sorted from spleen are always having lower ability to produce IFN-I. However, after using TLR7 agonist instead of TLR9 agonist, we can observed the IFNα production by intracellular staining.

4.5 phosphorylation of galectin

In many signaling pathway, phosphorylation is important to activate signal transducing molecules to transduce the signaling. Galectins are able to be phosphorylated, such as galectin-3 and galectin-4. Galectin-4 can be tyrosine

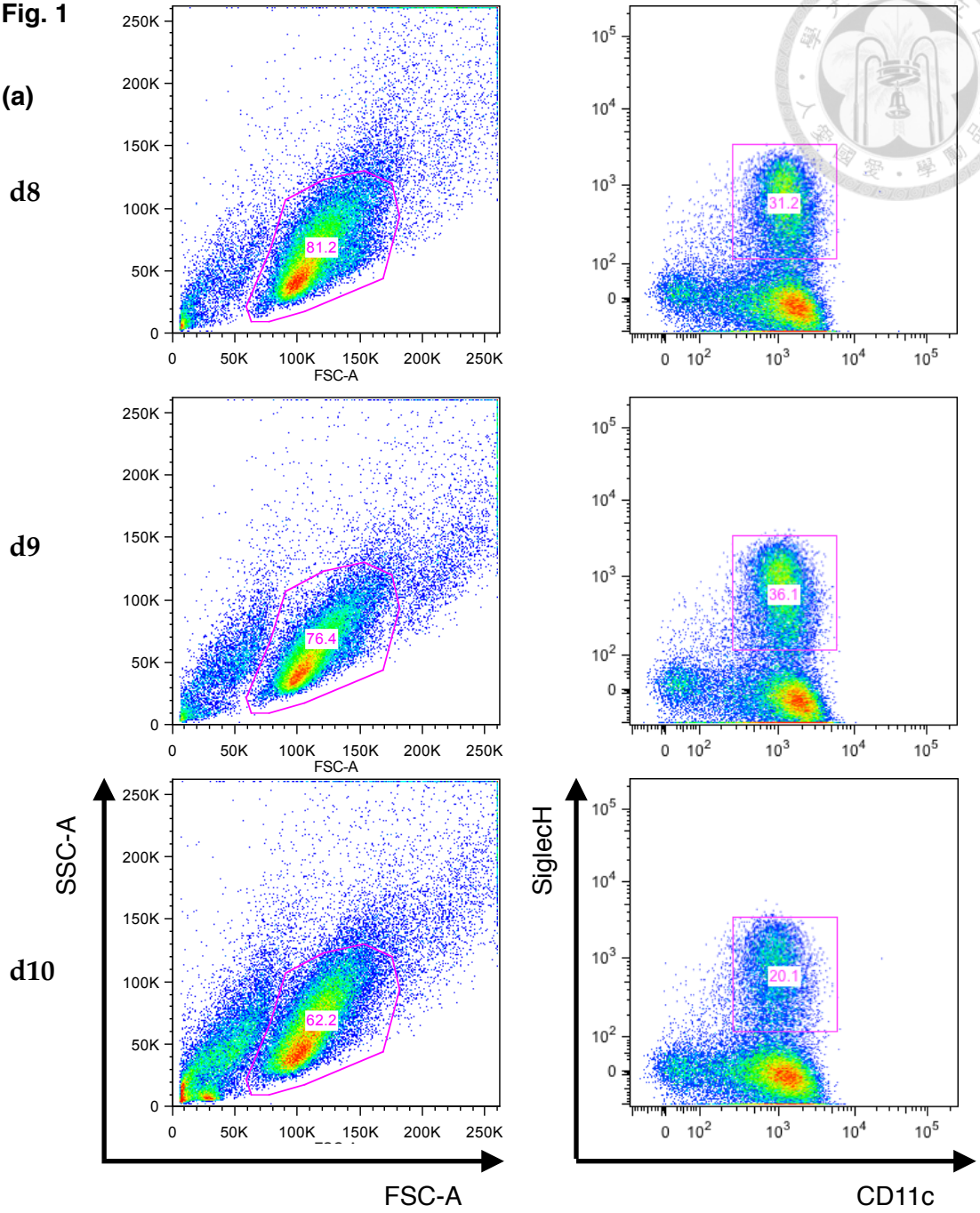
phosphorylated by members of the Src kinase family (Ideo, Hoshi, Yamashita, & Sakamoto, 2013) and galectin-3 could be phosphorylated at N-terminal Ser6(major) and Ser12 (minor) (Mazurek, Conklin, Byrd, Raz, & Bresalier, 2000). These study indicate that galectins may has the ability to interact with other signal transducing molecules. Perhaps, phosphorylation of galectin-3 involved in regulating IFN signaling in pDC and lead to augmented IFN-I production.

In sum, this study provides an evidence that galectin-1 and -3 get doesn't involved in pDC development and offers an insight into the distinct role of galectin-1 and galectin-3 in regulating IFN-I production by pDC.

Figures

Fig. 1

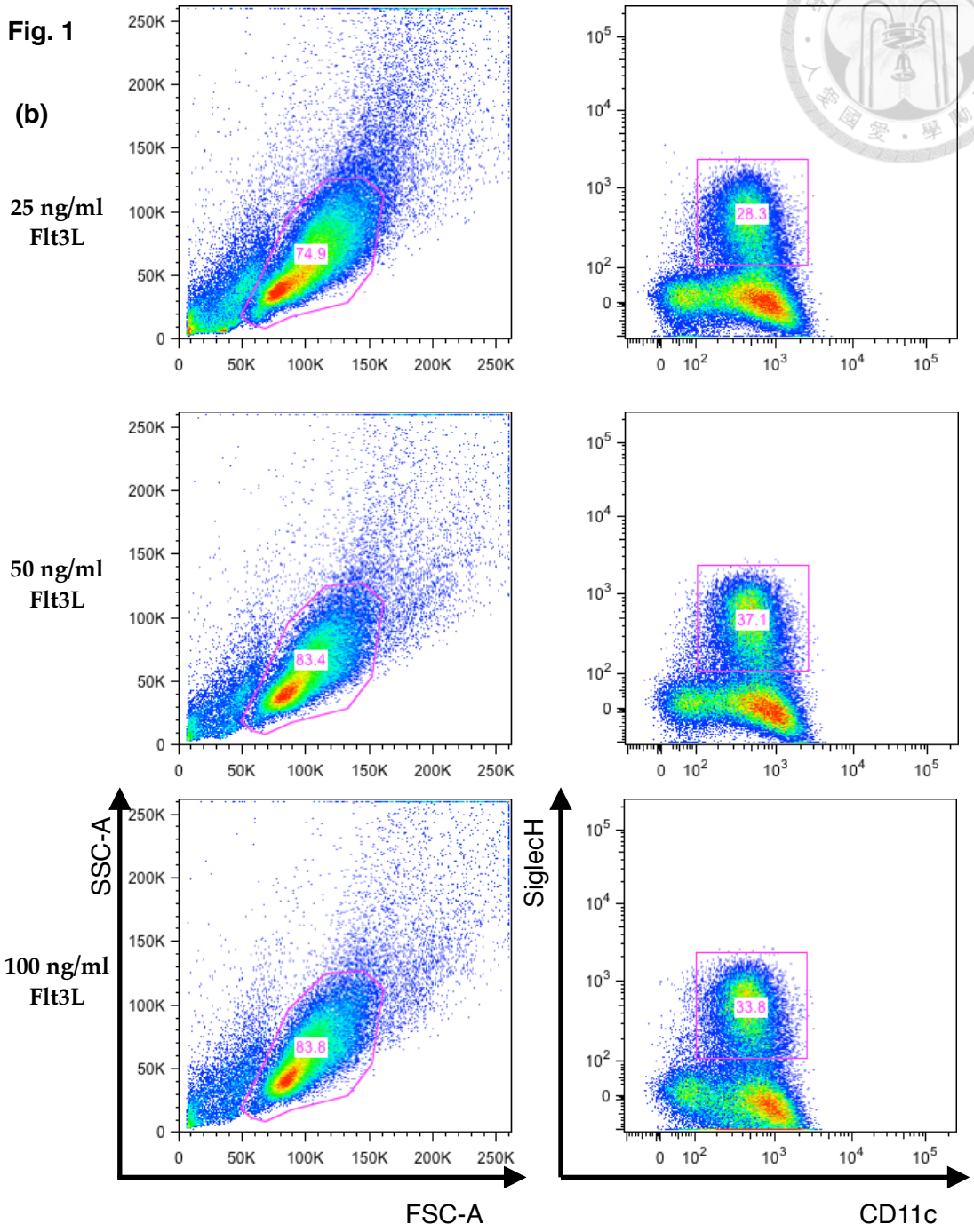
(a)



	harvested cell number	pDC%(CD11c siglecH	total pDC number
d8	4.3×10^{6}	25.3%	1.089×10^{6}
d9	4.0×10^{6}	27.6%	1.103×10^{6}
d10	1.3×10^{6}	12.5%	1.625×10^{5}

Fig. 1

(b)



	harvested cell number	pDC%(CD11c siglecH	total pDC number
25 ng/ml	1.4×10^{6}	21.2%	2.968×10^{5}
50 ng/ml	2.3×10^{6}	30.94%	7.117×10^{5}
100 ng/ml	3.1×10^{6}	28.32%	8.781×10^{5}

Figure 1. Establishing pDC in vitro culture system.

Different culture conditions such as culture plate and numbers of BM cell at initial seeding (a) culture days (b) concentration of Flt3L were tested. Finally, the optimal culture condition is seeding BM cell into 12-well plate at a density $4x10^6$ in a total volume 2 ml medium which contain 10%FBS, 100x P/S, and 100 ng/ml Flt3L in RPMI 1640 for 9 d. At d3 and d6, 1 ml medium was discarded and replaced with 1 ml fresh medium containing 100 ng/ml Flt3L.

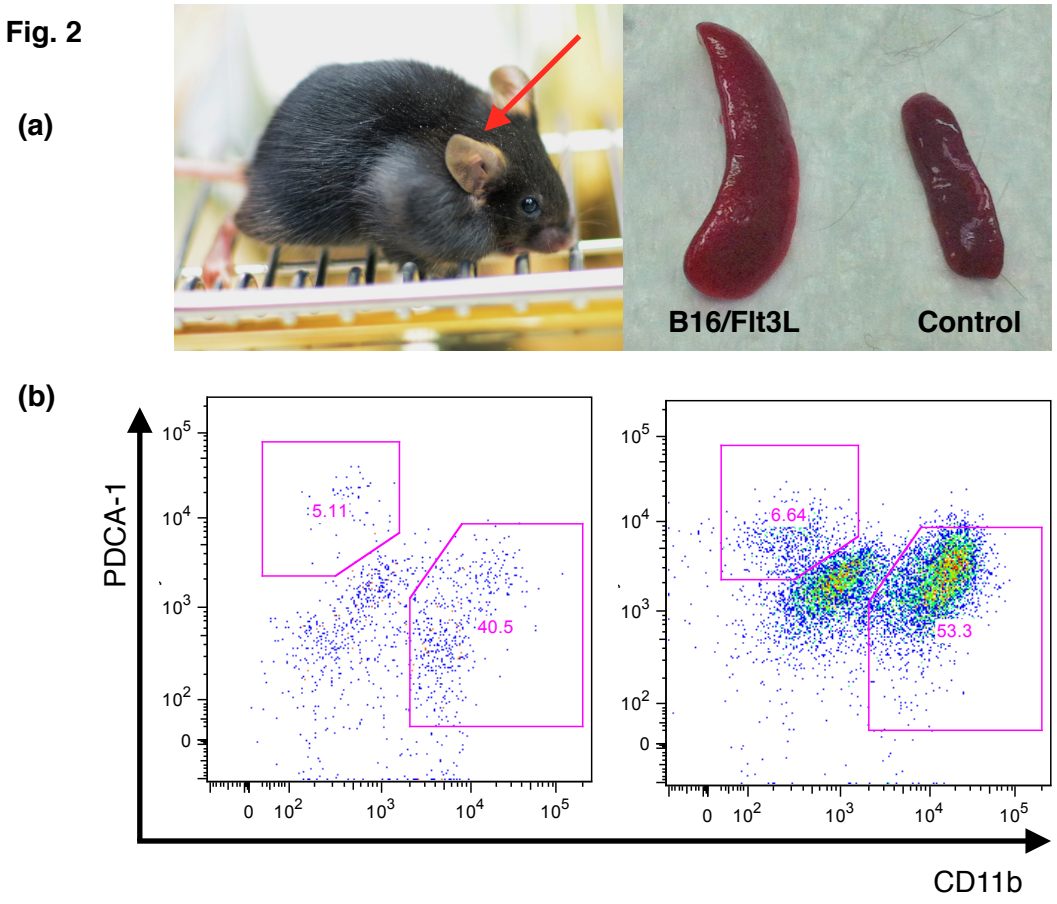


Figure 2. pDC expansion *in vivo*.

(a) The size of spleen was enlarged after $5x10^6$ B16/Flt3L cells injected to mouse s.c. for 14 d. (b) The percentage and numbers of pDC and cDC are enriched by B16/Flt3L injection. The arrow indicates the injection site.

Fig.3

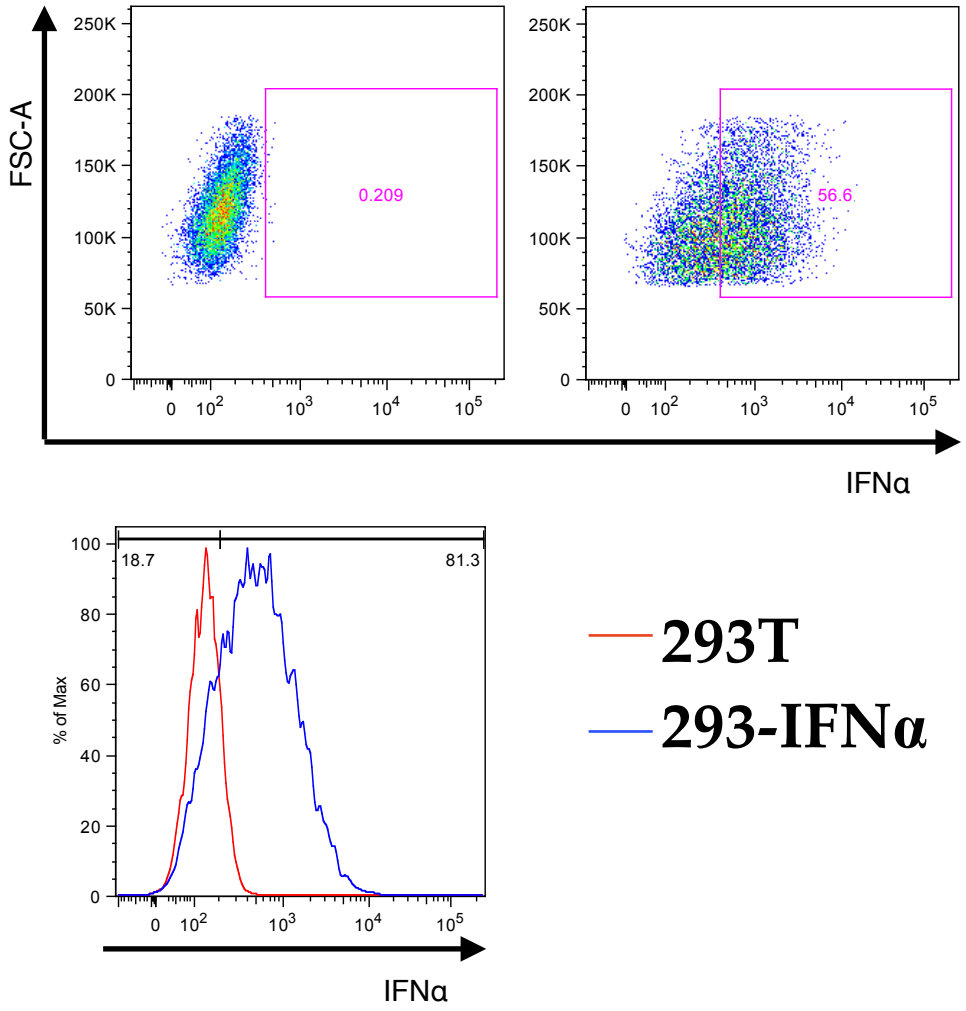


Figure 3. Positive control of IFNα intracellular staining

We use 293-IFNα cell line which can over express IFNα as a positive control of IFNα intracellular staining.

Fig. 4

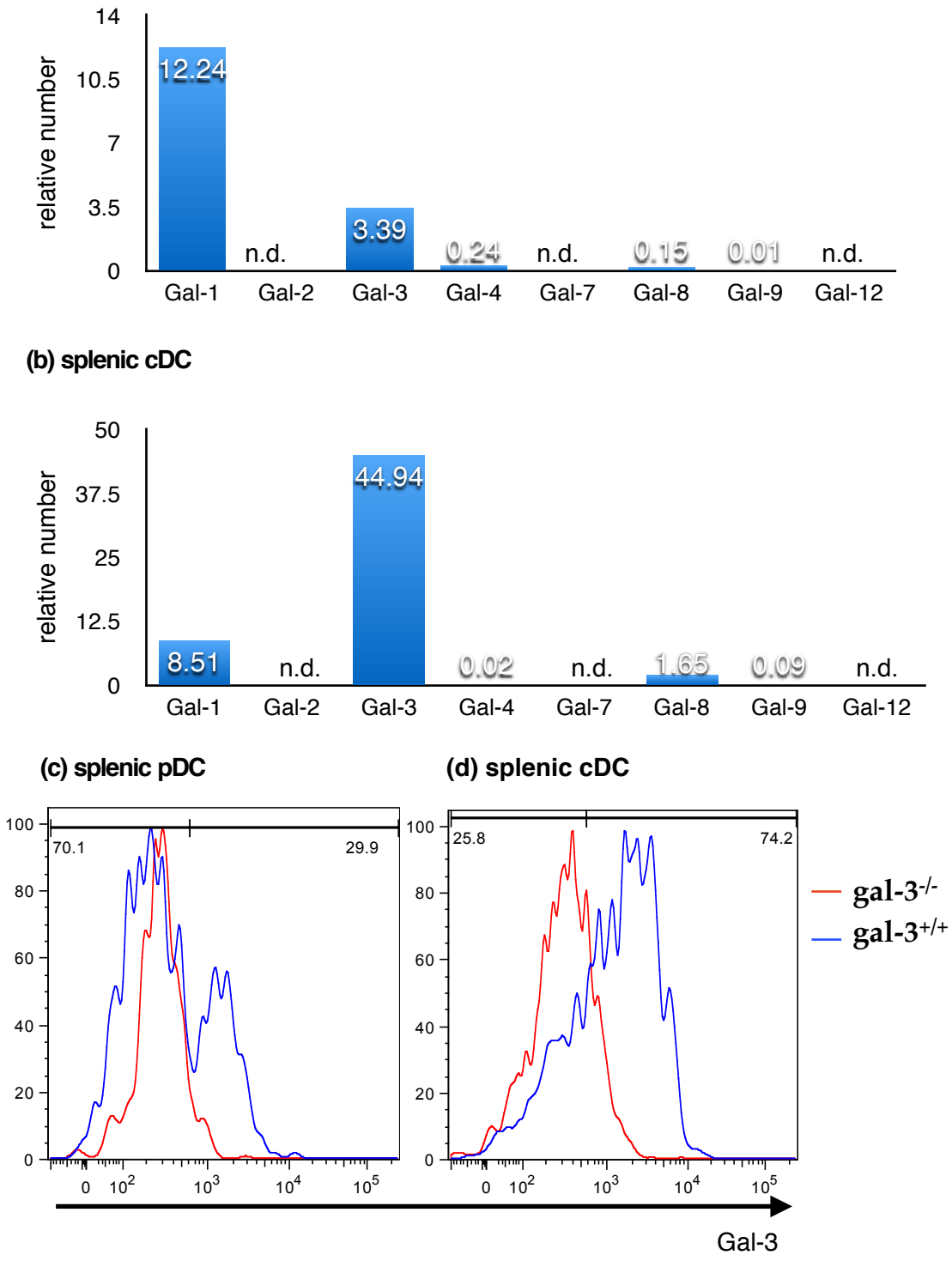

(a) splenic pDC
relative number
14
10.5
7
3.5
0
12.24
n.d.
3.39
0.24
n.d.
0.15
0.01
n.d.
Gal-1
Gal-2
Gal-3
Gal-4
Gal-7
Gal-8
Gal-9
Gal-12
(b) splenic cDC
relative number
50
37.5
25
12.5
0
8.51
n.d.
44.94
0.02
n.d.
1.65
0.09
n.d.
Gal-1
Gal-2
Gal-3
Gal-4
Gal-7
Gal-8
Gal-9
Gal-12
(c) splenic pDC
(d) splenic cDC
70.1
29.9
25.8
74.2
gal-3-/-
gal-3+/+
Gal-3

Figure 4. Galectin expression in splenic pDC and cDC.

Mouse splenic pDC and cDC were sorted by FACS Aria and galectin expression profile in mouse splenic (a) pDC and (b) cDC were acquired by qPCR. Relative number of galectin mRNA expression levels were normalized to mGAPDH. Galectin-3 expression in mouse splenic (c) pDC and (d) cDC were confirmed by intracellular staining. Splenic pDC was defined as CD11c^{int} CD11b^{-} siglecH^{+}, respectively, cDC was defined as CD11c^{high} CD11b^{+} siglecH^{-}.

Fig. 5

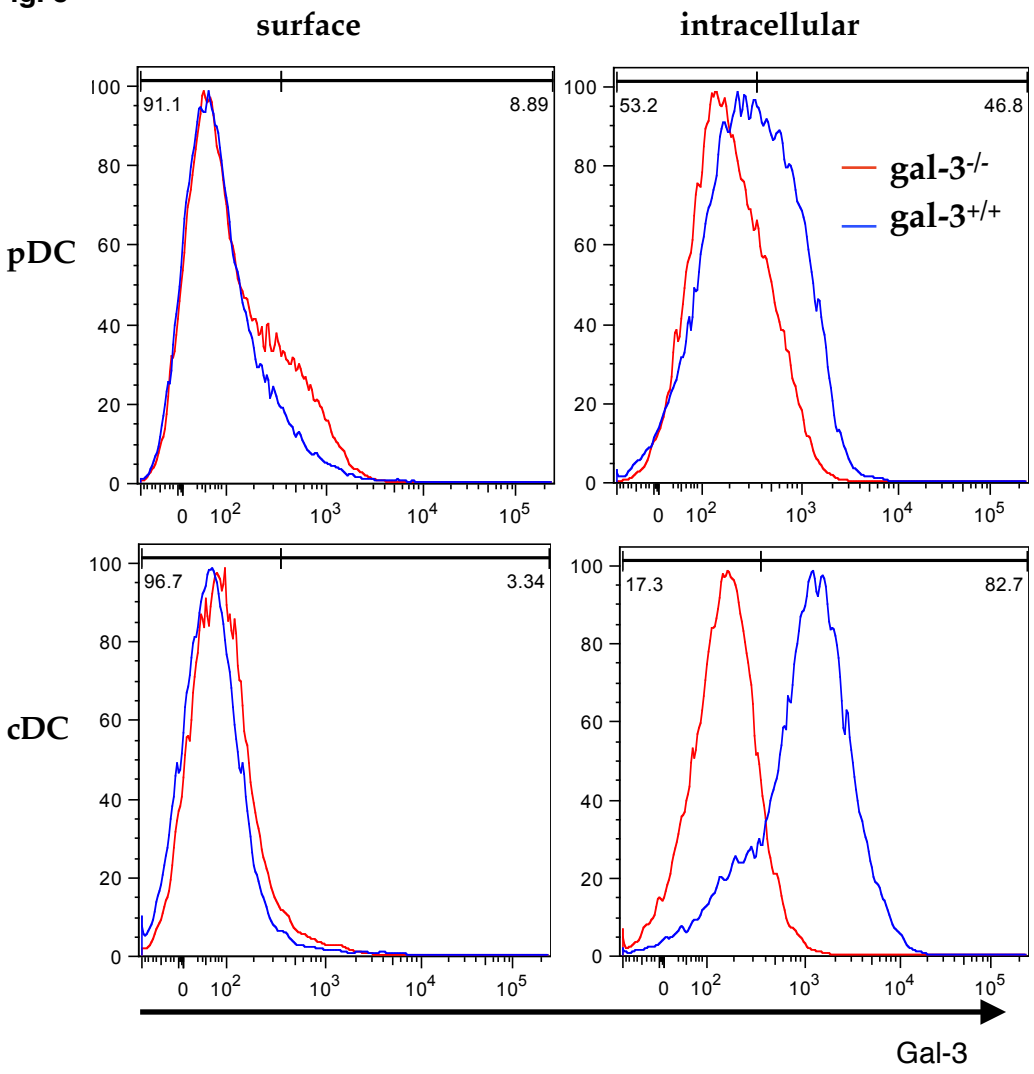


Figure 5. Galectin-3 expression in Flt3L-BMDC.

Galectin-3 expression in Flt3L-BMDC was examined by flow cytometry. pDC and cDC were defined as previously mentioned. Galectin-3 expression are not on the surface but mainly express in cytosol.

Fig. 6

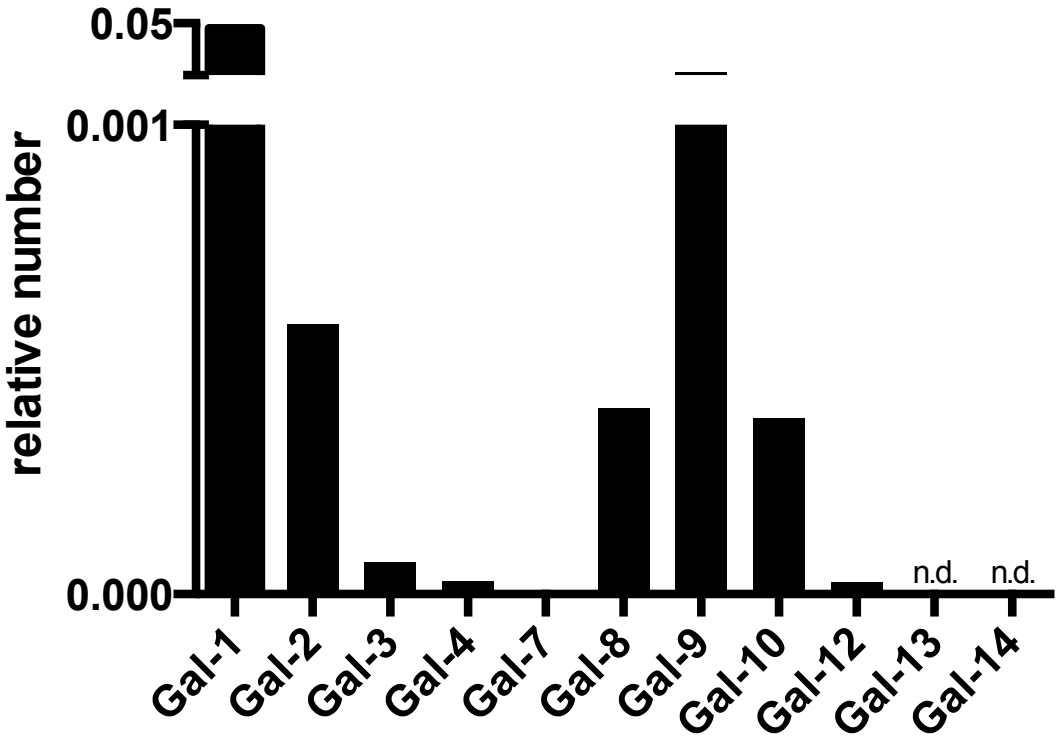


Figure 6. Galectin expression profile in PMDC05.

Galectins expression profile in human pDC cell line was acquired by qPCR. The relative number of mRNA expression level of each sample was normalized to human actin expression.

Fig. 7

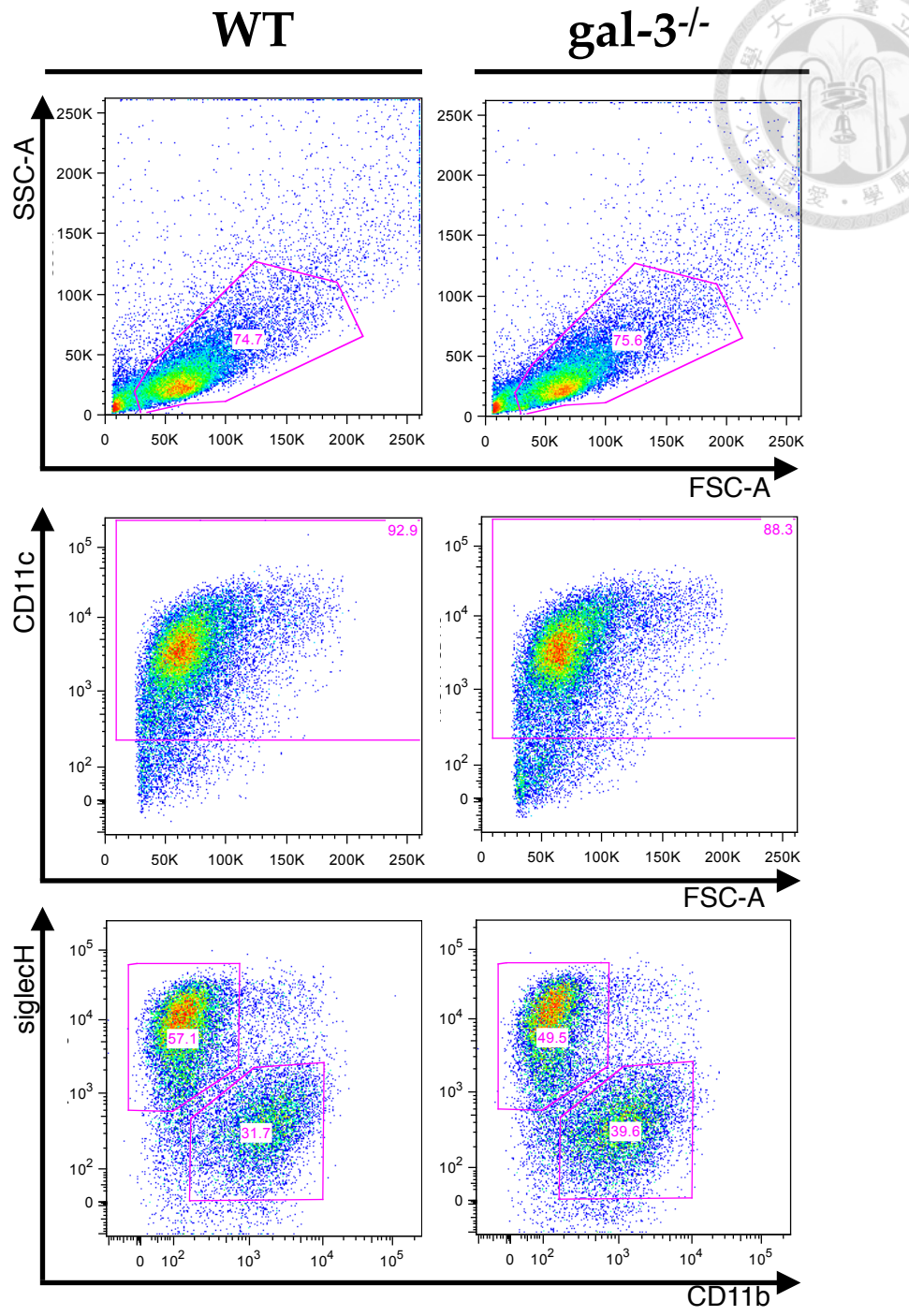
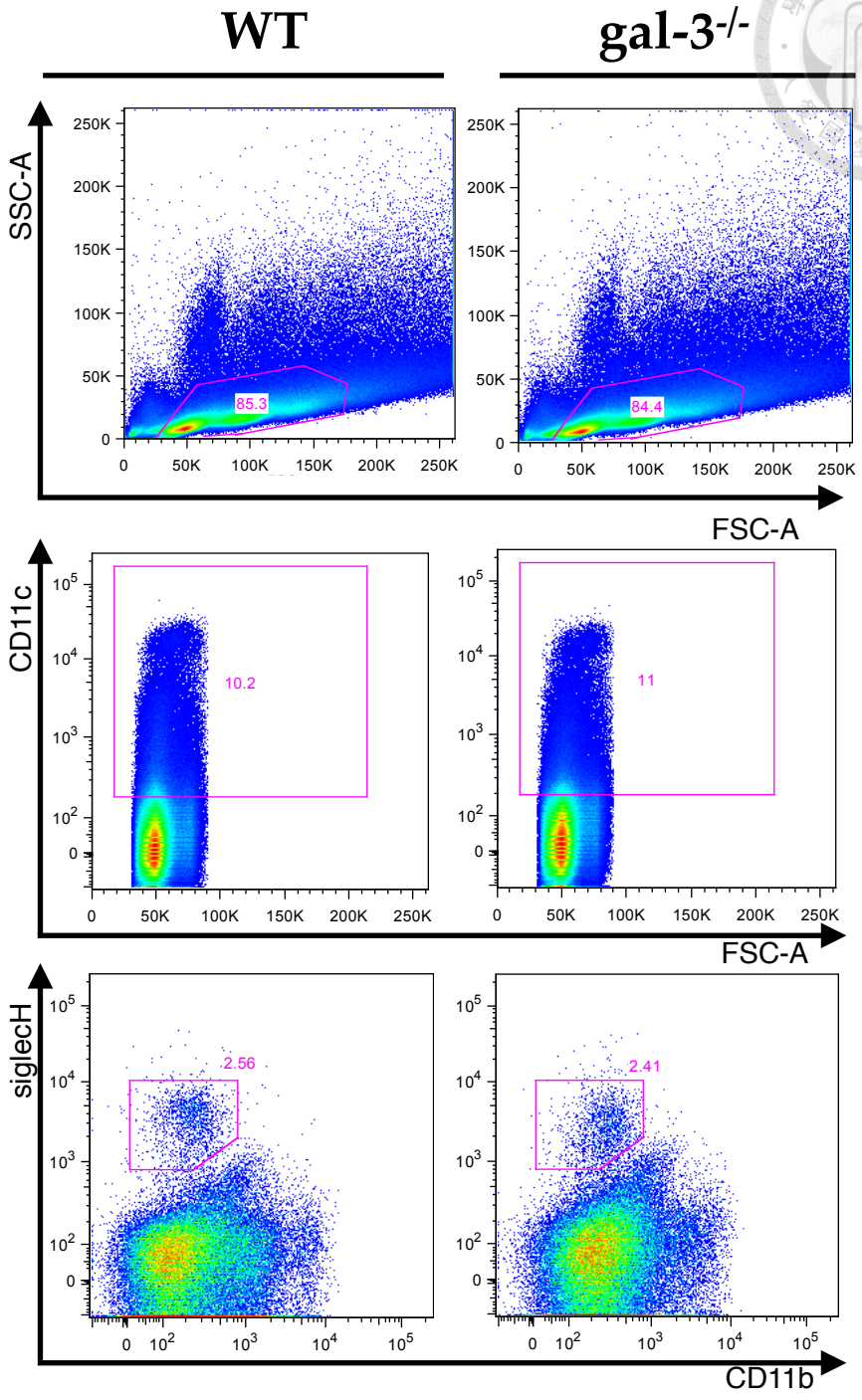

WT
gal-3-/-
(a)
SSC-A
FSC-A
74.7
75.6
CD11c
92.9
88.3
siglecH
CD11b
57.1
31.7
49.5
39.6
(b)
Flt3L-BMDC
Flt3L-BMDC
n.s.
n.s.
percentage %
cell numbers (x10^4)
WT
Gal-3-/-

Figure 7. Compare percentage and numbers of Flt3L-BMDC from WT and gal-3$^{-/-}$ mice *in vitro*.

(a,b) BM cell from WT and gal-3$^{-/-}$ mice were cultured with 100 ng/ml mFlt3L for 8 d. The percentage and number of Flt3L-BMDC from WT and gal-3$^{-/-}$ mice were analyzed by flow cytometry. pDC was defined as CD11c^{+}CD11b^{-}siglecH^{+}. The percentage and cell numbers of Flt3L-BMDC from WT and gal-3$^{-/-}$ mice are comparable.

Fig. 8

(a)


WT
gal-3-/-
SSC-A
FSC-A
85.3
84.4
CD11c
FSC-A
10.2
11
siglecH
CD11b
2.56
2.41

(b)

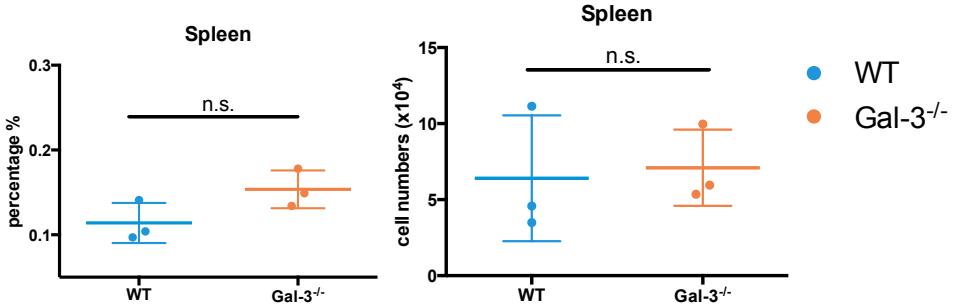
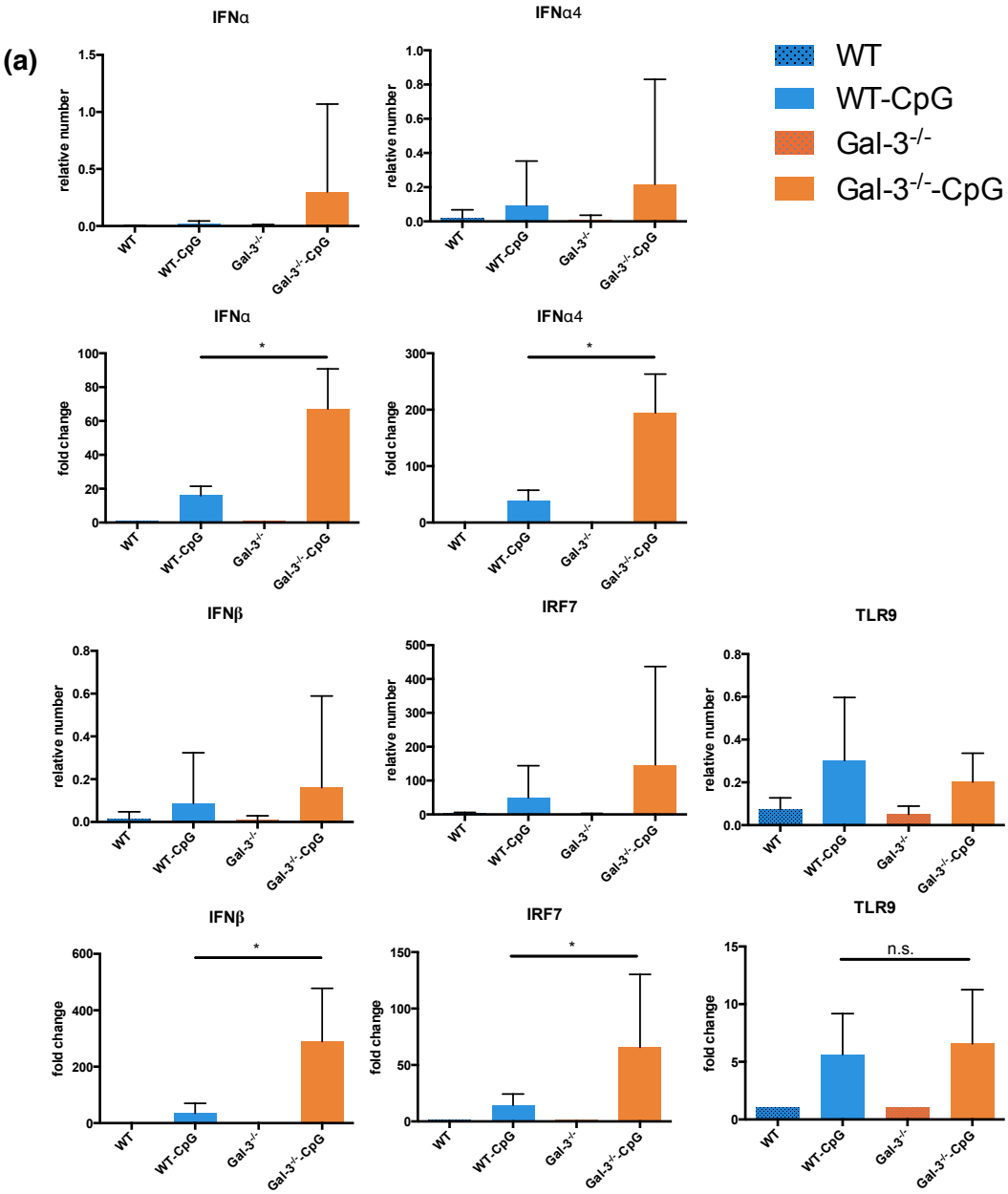
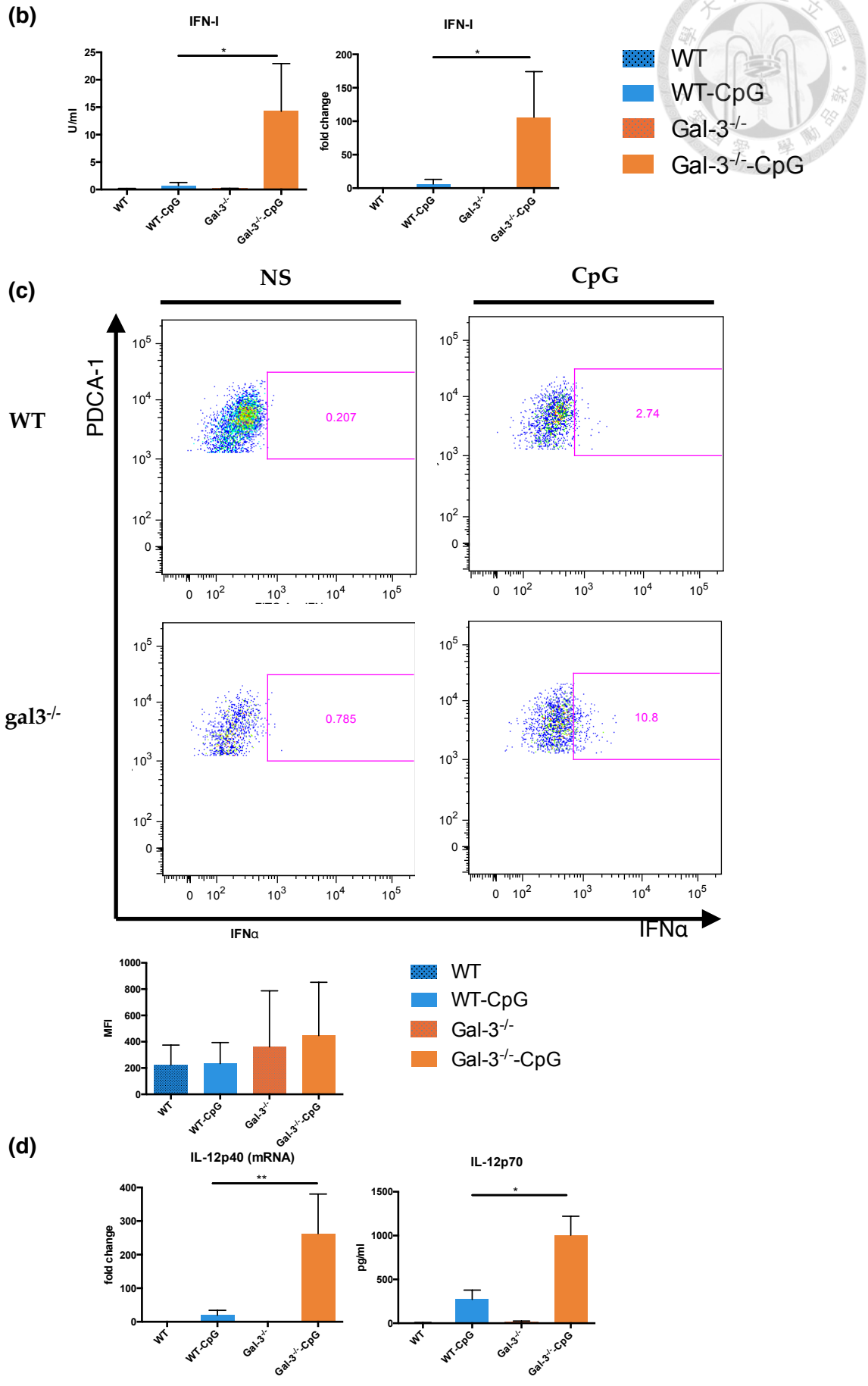

Spleen
n.s.
percentage %
WT
Gal-3-/-
Spleen
n.s.
cell numbers (x10⁴)
WT
Gal-3-/-

Figure 8. Compare percentage and numbers of splenic pDC from WT and gal-3$^{-/-}$ mice *ex vivo*.

(a,b) The population and number of splenic pDC from WT and gal-3$^{-/-}$ mice were analyzed by flow cytometry. pDC was defined as CD11c^{+}CD11b^{-}siglecH^{+}. The percentage and cell numbers of splenic pDC from WT, gal-1$^{-/-}$ and gal-3$^{-/-}$ mice are comparable.

Fig. 9




(b)
IFN-I
U/ml
fold change
WT
WT-CpG
Gal-3-/-
Gal-3-/--CpG
(c)
NS
CpG
WT
gal3-/-
PDCA-1
IFNα
0.207
2.74
0.785
10.8
MFI
(d)
IL-12p40 (mRNA)
IL-12p70
pg/ml

(c)

Figure 9. Examine the role of galectin-3 in Flt3L-BMDC response to CpG *in vitro*.

The mRNA expression level of IFN-I, IRF7, TLR9 (a) and IL-12p40 (d) in Flt3L-BMDC induced by 1000nM CpG-A for 16 h. The relative number of mRNA expression level of each sample was normalized to mouse GAPDH expression. The fold change was calculated by basing on the fold induction of nontreated samples. To analysis the production of IFNα (a) and IL-12p70 (d), the supernatants of Flt3L-BMDC were harvested after 1000nM CpG-A stimulation for 24 h. (c) Flt3L-BMDC were stimulated with 1000nM CpG-A for 24 h, and BFA were added for 9 h before harvested. Cells were gated on $CD11c^{+}CD11b^{-}Bst2^{+}$ and analyzed IFNα production by ICS.

Fig. 10

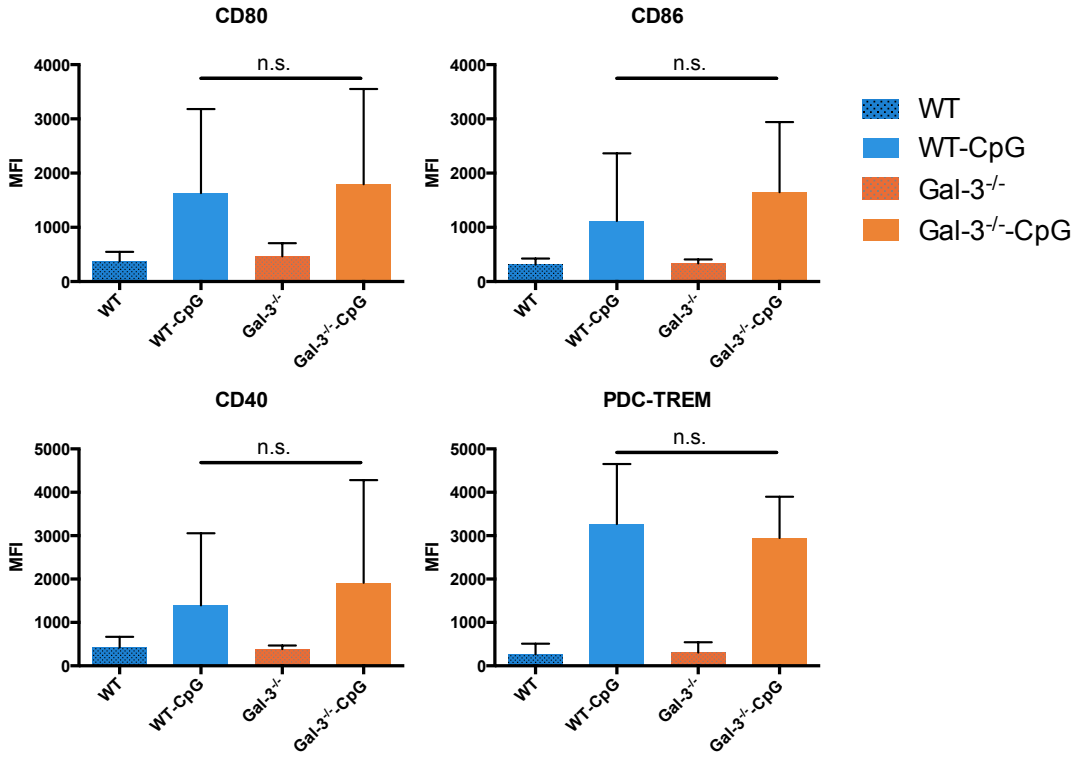
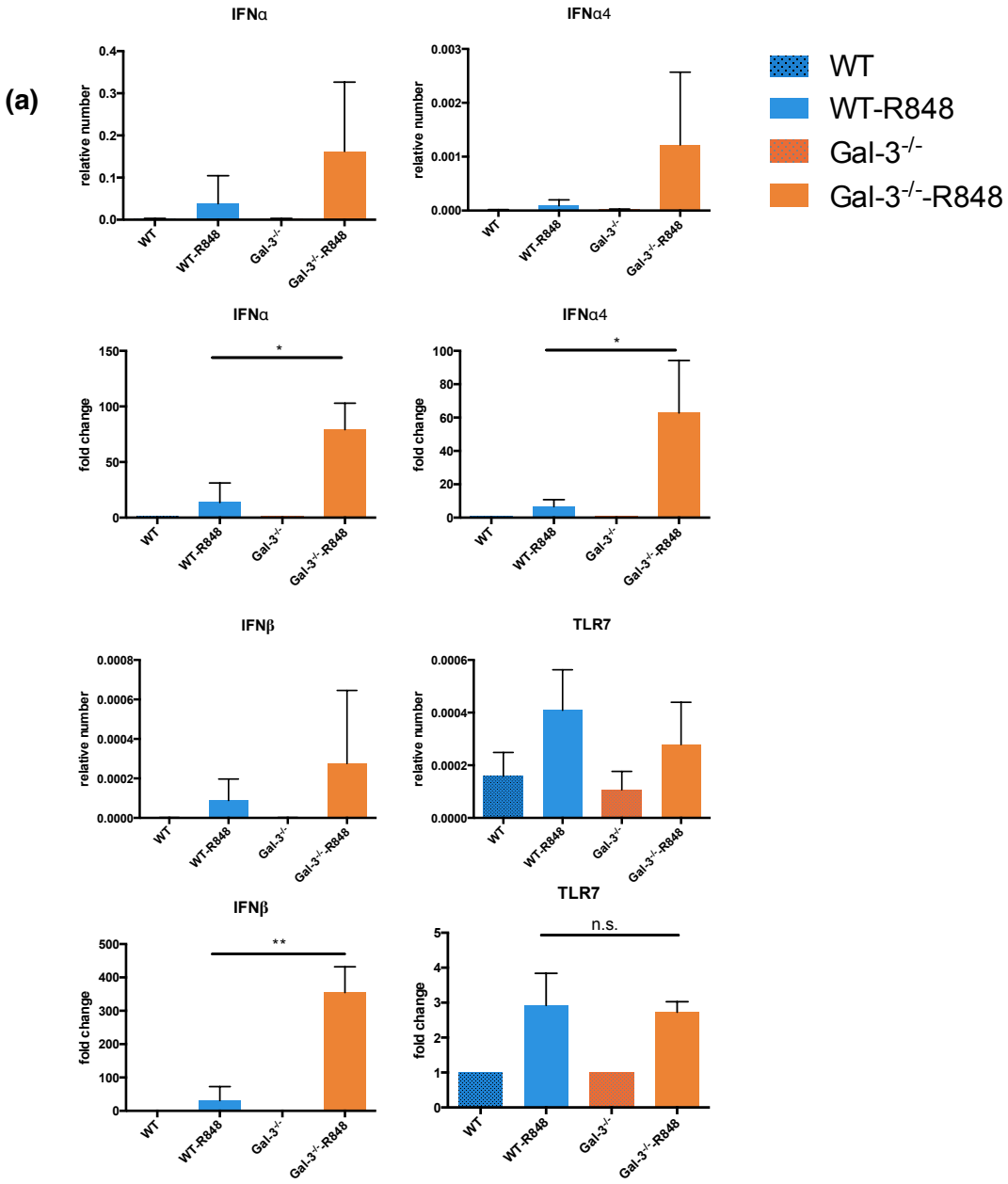


Figure 10. Examine the maturation status of WT and gal-3$^{-/-}$ Flt3L-BMDC response to CpG *in vitro*.

Flt3L-BMDC were stimulated with 1000nM CpG for 16 h and analyzed the expression level of maturation markers, CD80, CD86, CD40 and PDC-TREM. Cells were gated on CD11c^{+}CD11b^{-}siglecH^{+} and analyzed various maturation markers.

Fig. 11



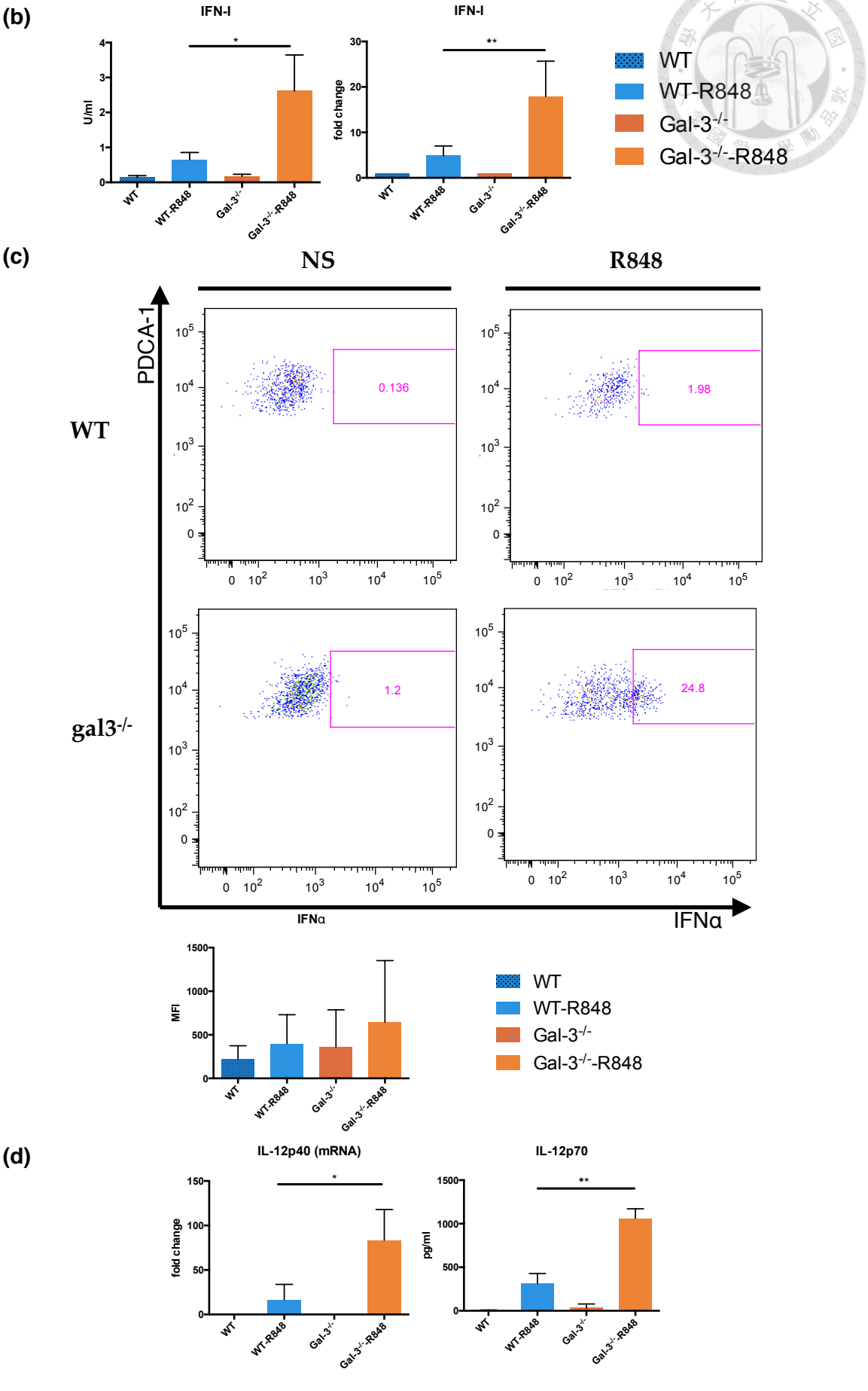
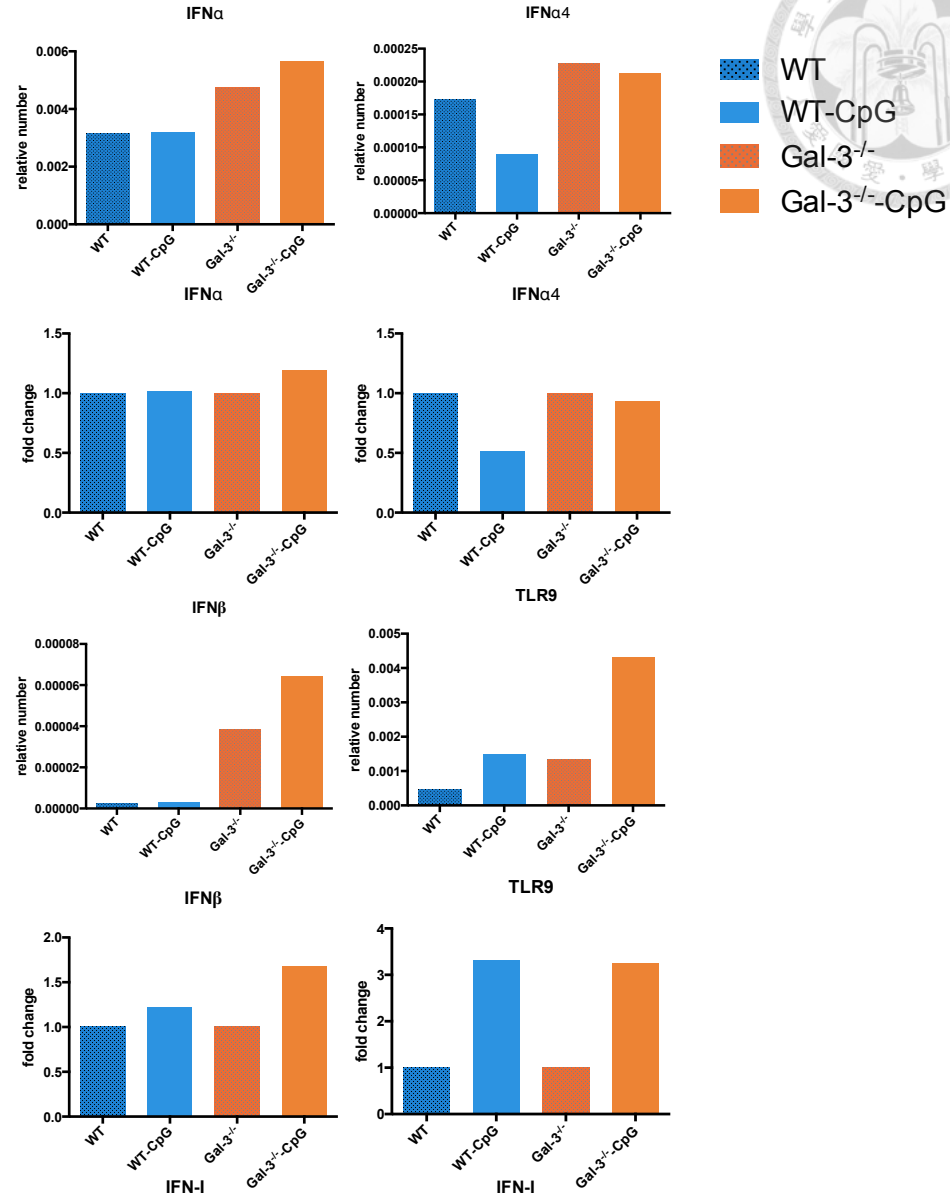

(b)
IFN-I
U/ml
*
WT
WT-R848
Gal-3-/-
Gal-3-/--R848
IFN-I
fold change
**
WT
WT-R848
Gal-3-/-
Gal-3-/--R848
WT
WT-R848
Gal-3-/-
Gal-3-/--R848
(c)
NS
R848
PDCA-1
WT
gal3-/-
0.136
1.98
1.2
24.8
IFNα
IFNα
MFI
WT
WT-R848
Gal-3-/-
Gal-3-/--R848
(d)
IL-12p40 (mRNA)
fold change
*
WT
WT-R848
Gal-3-/-
Gal-3-/--R848
IL-12p70
pg/ml
**
WT
WT-R848
Gal-3-/-
Gal-3-/--R848

Figure 11. Examine the role of galectin-3 in Flt3L-BMDC response to R848 *in vitro*.

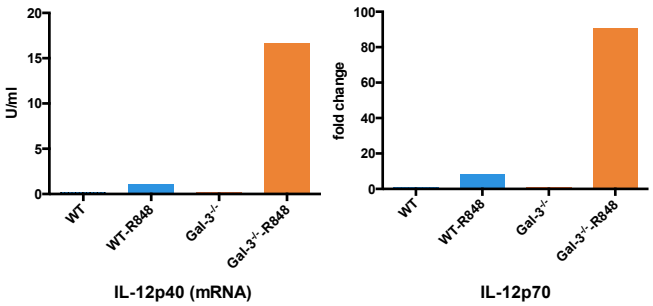
The mRNA expression level of IFN-I, IRF7, TLR9 (a) and IL-12p40 (d) in Flt3L-BMDC induced by 0.5 μg/ml R848 for 16 h. The relative number of mRNA expression level of each sample was normalized to mouse GAPDH expression. The fold change was calculated by basing on the fold induction of nontreated samples. To analysis the production of IFNα (a) and IL-12p70 (d), the supernatants of Flt3L-BMDC were harvested after 0.5 μg/ml R848 stimulation for 24 h. (c) Flt3L-BMDC were stimulated with 0.5 μg/ml R848 for 24 h, and BFA were added for 9 h before harvested. Cells were gated on $CD11c^{+}CD11b^{-}Bst2^{+}$ and analyzed IFNα production by ICS.

Fig.12

(a)


IFNα
IFNα4
WT
WT-CpG
Gal-3-/-
Gal-3-/--CpG
relative number
fold change
IFNβ
TLR9
IFN-I

(b)


U/ml
fold change
WT
WT-R848
Gal-3-/-
Gal-3-/--R848
IL-12p40 (mRNA)
IL-12p70

(c)

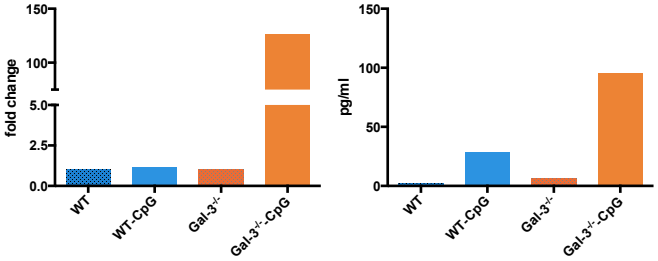
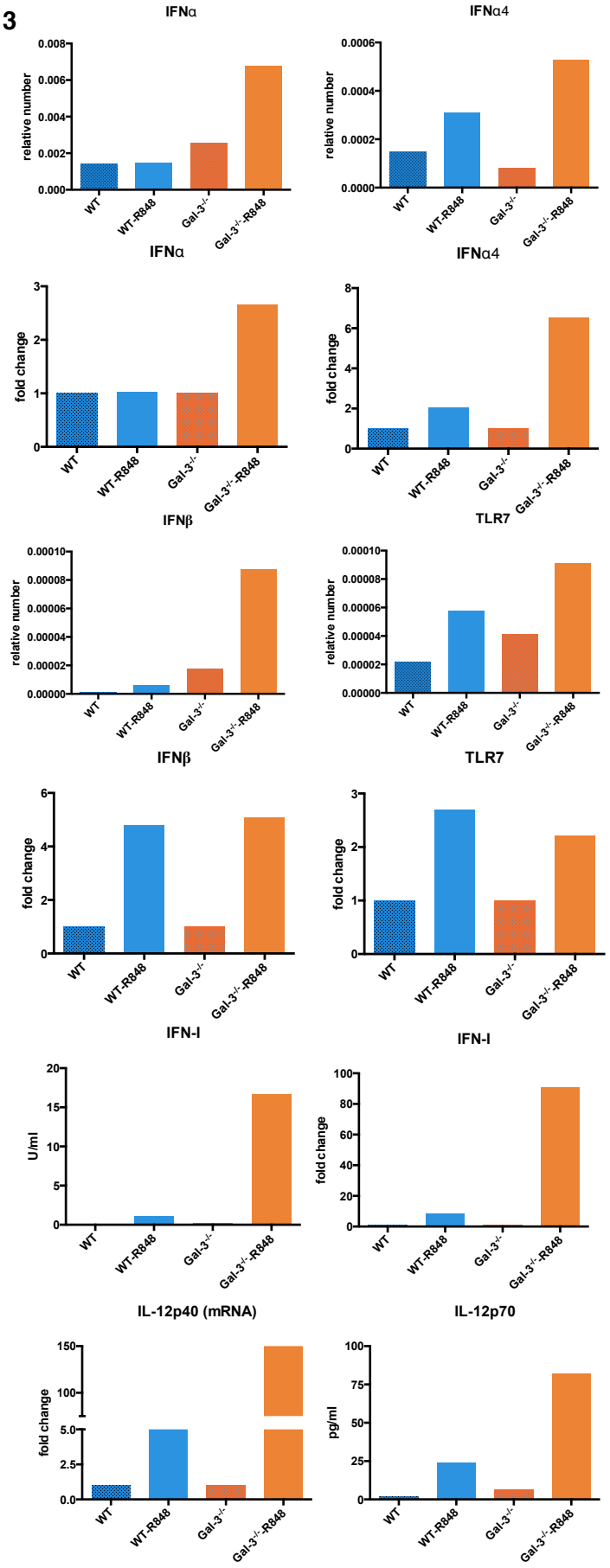

fold change
pg/ml
WT
WT-CpG
Gal-3-/-
Gal-3-/--CpG

Figure 12. Examine the role of galectin-3 in sorted Flt3L-BMDC response to CpG *in vitro*.

The mRNA expression level of IFN-I, TLR9 in sorted Flt3L-BMpDC (a) and IL12 in BMcDC (c) induced by 1000nM CpG-A for 16 h. The relative number of mRNA expression level of each sample was normalized to mouse GAPDH expression. The fold change was calculated by basing on the fold induction of nontreated samples. (b,c) The supernatants of sorted Flt3L-BMpDC and BMcDC were harvested after 1000nM CpG-A stimulation for 24 h. Supernatants of BMpDC were collected and analyzed the IFN-I production, respectively, supernatants of BMcDC were collected to perform ELISA for IL-12.

Fig.13

(a)


IFNα
IFNα4
WT
WT-R848
Gal-3-/-
Gal-3-/--R848
relative number
fold change
IFNβ
TLR7
IFN-I

(b)

U/ml
fold change
IL-12p40 (mRNA)
IL-12p70

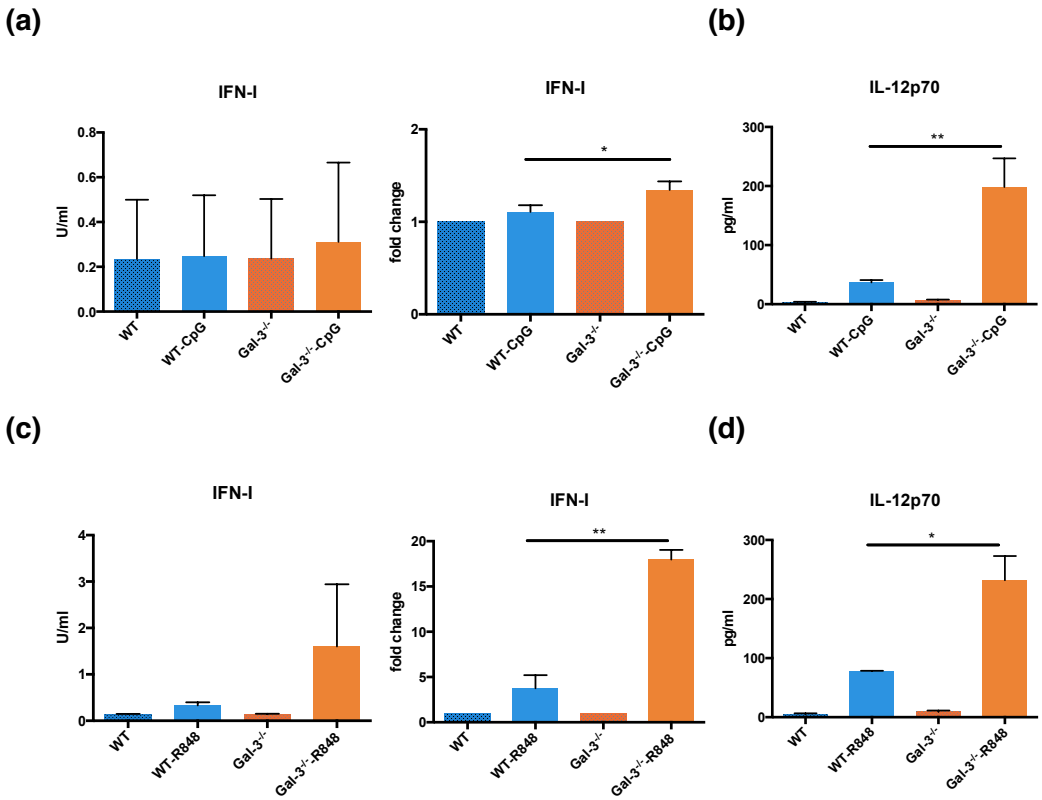
(c)

fold change
pg/ml

Figure 13. Examine the role of galectin-3 in sorted Flt3L-BMDC response to R848 *in vitro*.

The mRNA expression level of IFN-I, TLR9 in sorted Flt3L-BMpDC (a) and IL12 in BMcDC (c) induced by 0.5μg/ml R848 for 16 h. The relative number of mRNA expression level of each sample was normalized to mouse GAPDH expression. The fold change was calculated by basing on the fold induction of nontreated samples. The supernatants of sorted Flt3L-BMpDC (b) and BMcDC (c) were harvested after 0.5μg/ml R848 stimulation for 24 h. Supernatants of BMpDC were collected and analyzed the IFN-I production, respectively, supernatants of BMcDC were collected to perform ELISA for IL-12.

Fig.14



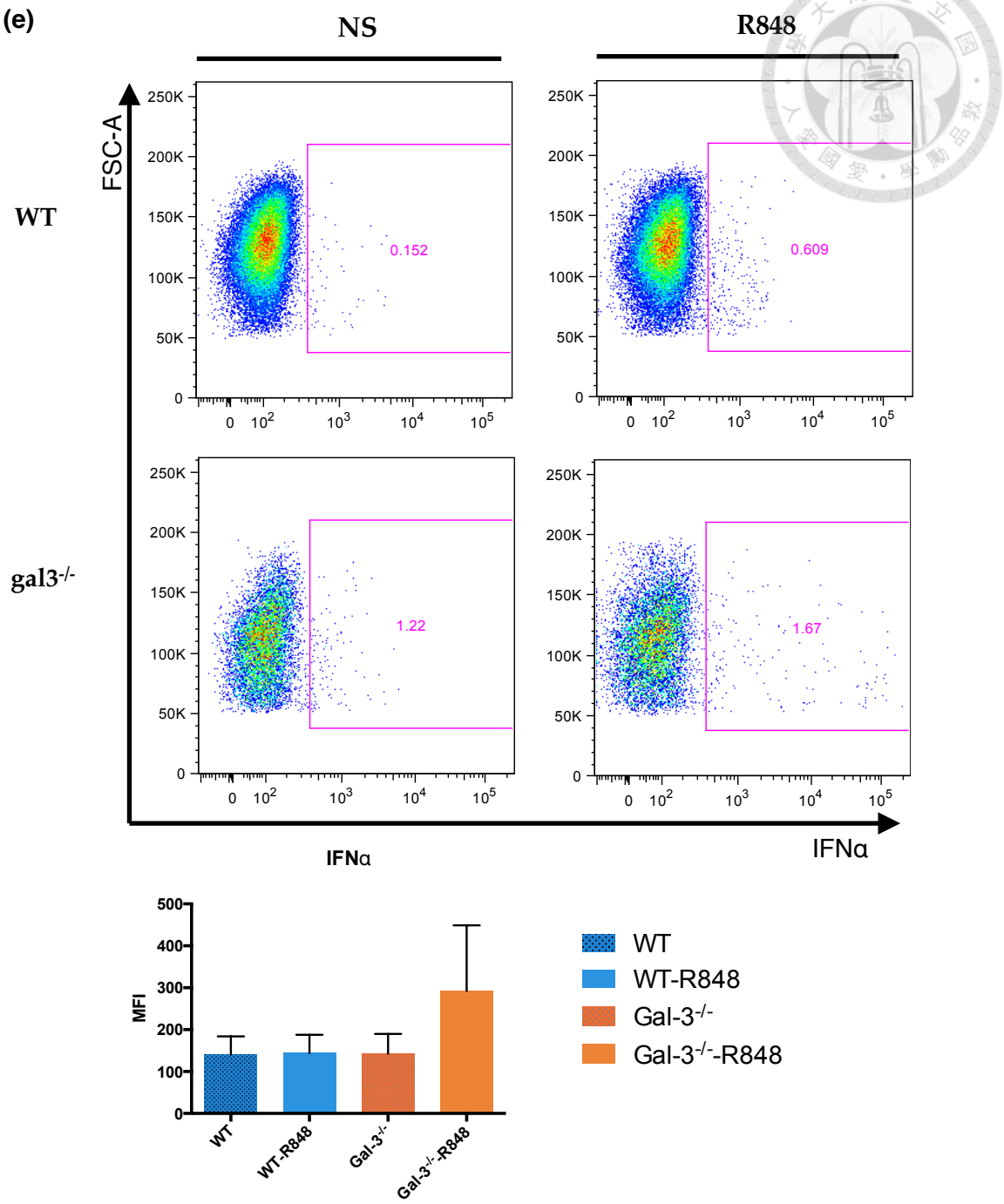

(e)
NS
R848
WT
gal3-/-
FSC-A
IFNα
0.152
0.609
1.22
1.67
MFI
WT
WT-R848
Gal-3-/-
Gal-3-/--R848

Figure 14. Examine the role of galectin-3 in sorted splenic DC response to CpG ex *vivo*.

Sorted splenic pDC (a, c) and cDC (b, d) from B16/Flt3L tumor cell injected mice were stimulated with 1000 nM CpG (a, b) or 0.5 μg/ml R848 (c, d) for 24 h. The supernatant were collect to analysis the production of IFN-I and IL-12. (e) To analysis the IFNα production by ICS. Sorted splenic pDC were stimulate with 0.5 μg/ml R848 for 24 h, BFA were added for 9 h before harvested. Cells were gated on $CD11c^{+}CD11b^{-}Bst2^{+}$ and analyzed IFNα production.

Fig. 15

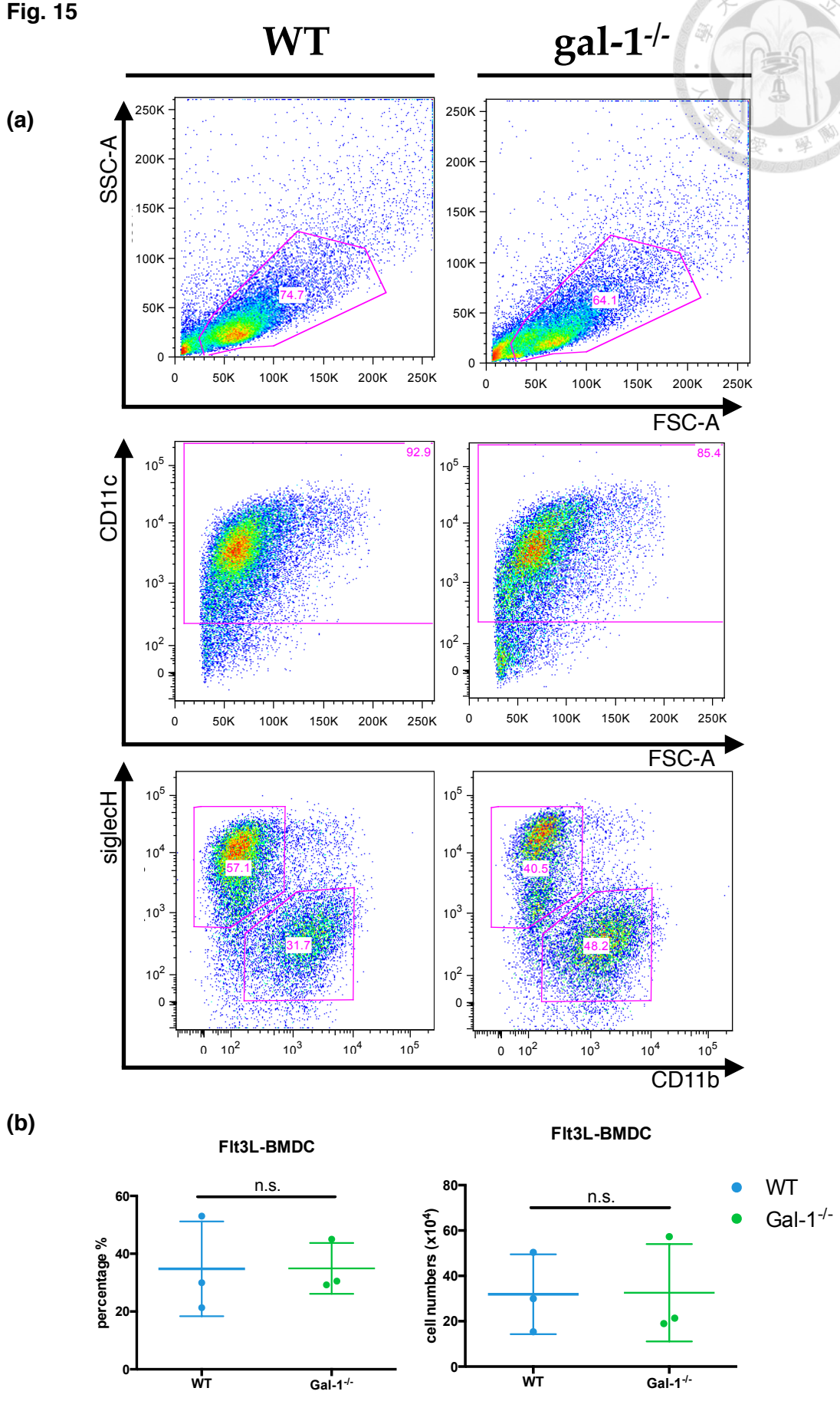
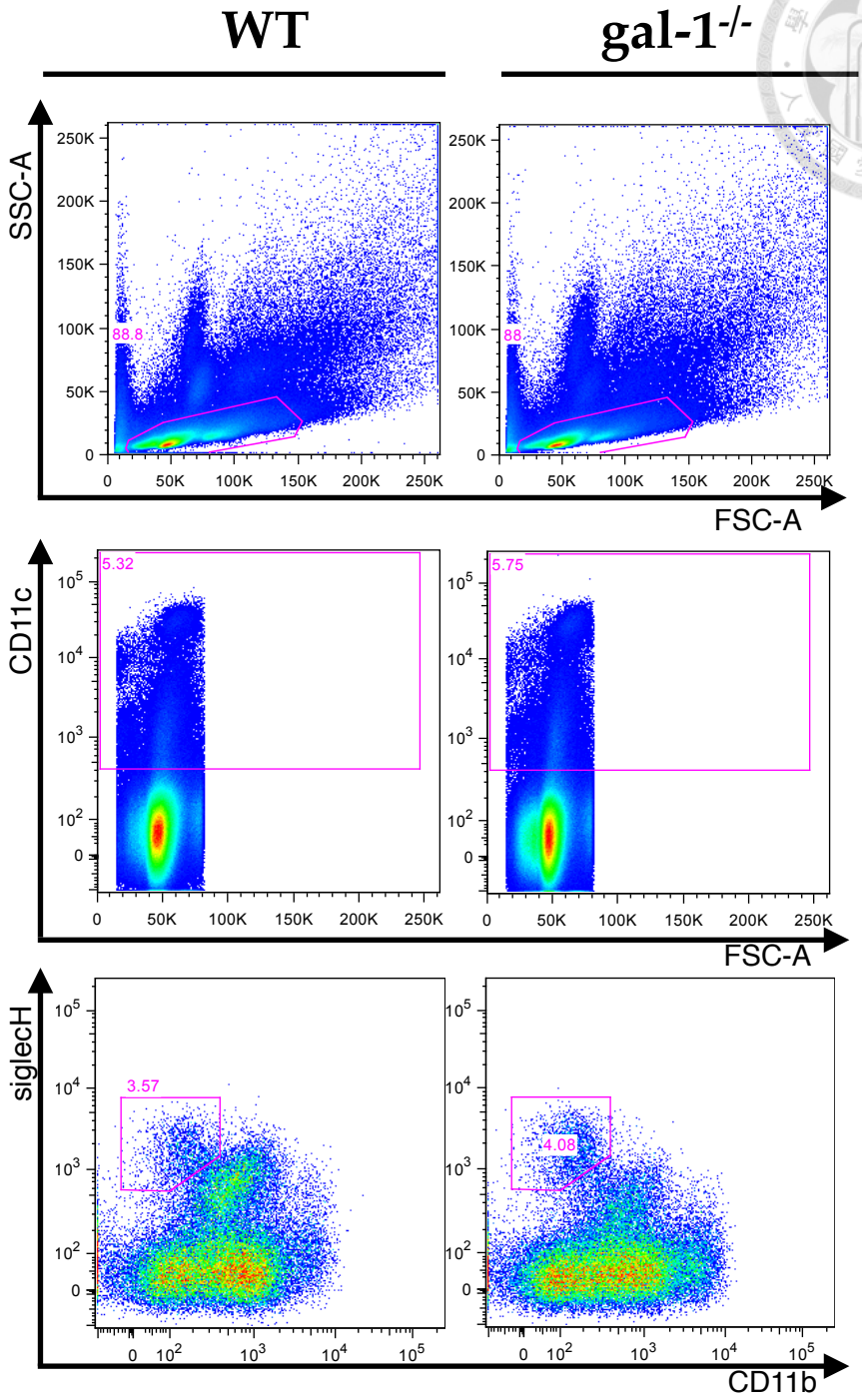

WT
gal-1-/-
(a)
SSC-A
FSC-A
74.7
64.1
CD11c
92.9
85.4
siglecH
CD11b
57.1
31.7
40.5
48.2
(b)
Flt3L-BMDC
n.s.
percentage %
WT
Gal-1-/-
Flt3L-BMDC
n.s.
cell numbers (x10^4)
WT
Gal-1-/-

Figure 15. Compare percentage and numbers of Flt3L-BMDC from WT and gal-1$^{-/-}$ mice *in vitro*.

(a,b) BM cell from WT and gal-1$^{-/-}$ mice were cultured with 100 ng/ml mFlt3L for 8 d. The percentage and number of Flt3L-BMDC from WT and gal-1$^{-/-}$ mice were analyzed by flow cytometry. pDC was defined as CD11c^{+}CD11b^{-}siglecH^{+}. The percentage and cell numbers of Flt3L-BMDC from WT and gal-1$^{-/-}$ mice are comparable.

Fig. 16

(a)


WT
gal-1-/-
SSC-A
FSC-A
88.8
88.
CD11c
5.32
5.75
siglecH
CD11b
3.57
4.08

(b)

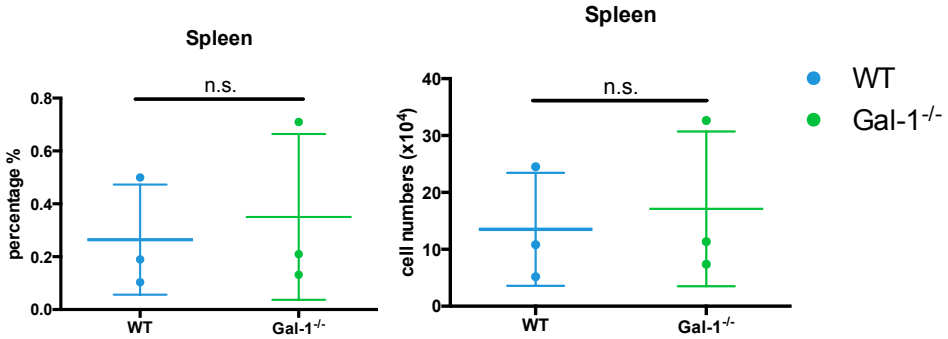
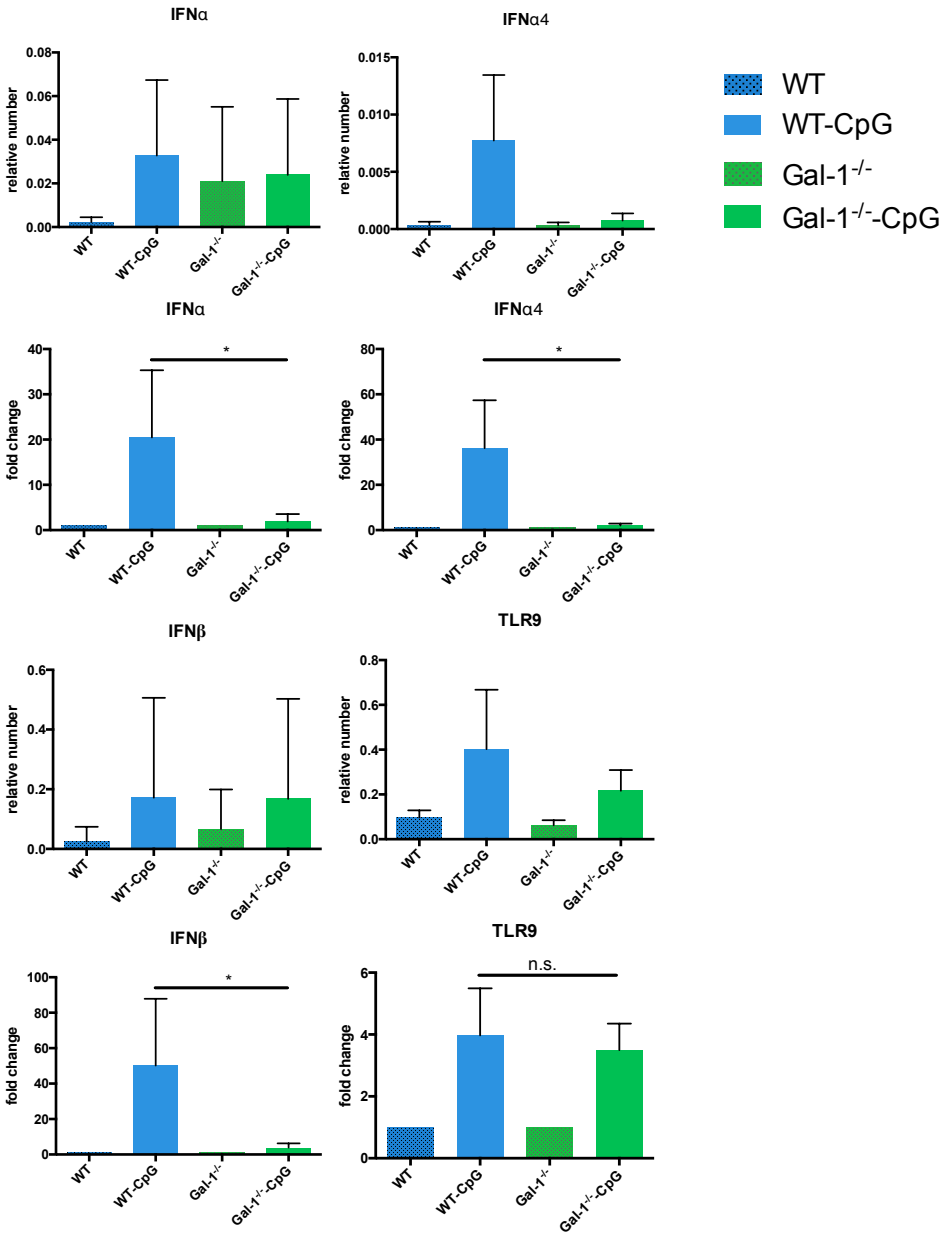

Spleen
n.s.
percentage %
WT
Gal-1-/-
Spleen
n.s.
cell numbers (x10⁴)
WT
Gal-1-/-

Figure 16. Compare percentage and numbers of splenic pDC from WT and gal-1$^{-/-}$ mice *ex vivo*.

(a,b) The population and number of splenic pDC from WT and gal-1$^{-/-}$ mice were analyzed by flow cytometry. pDC was defined as CD11c^{+}CD11b^{-}siglecH^{+}. The percentage and cell numbers of splenic pDC from WT and gal-1$^{-/-}$ mice are comparable.

Fig. 17

(a)



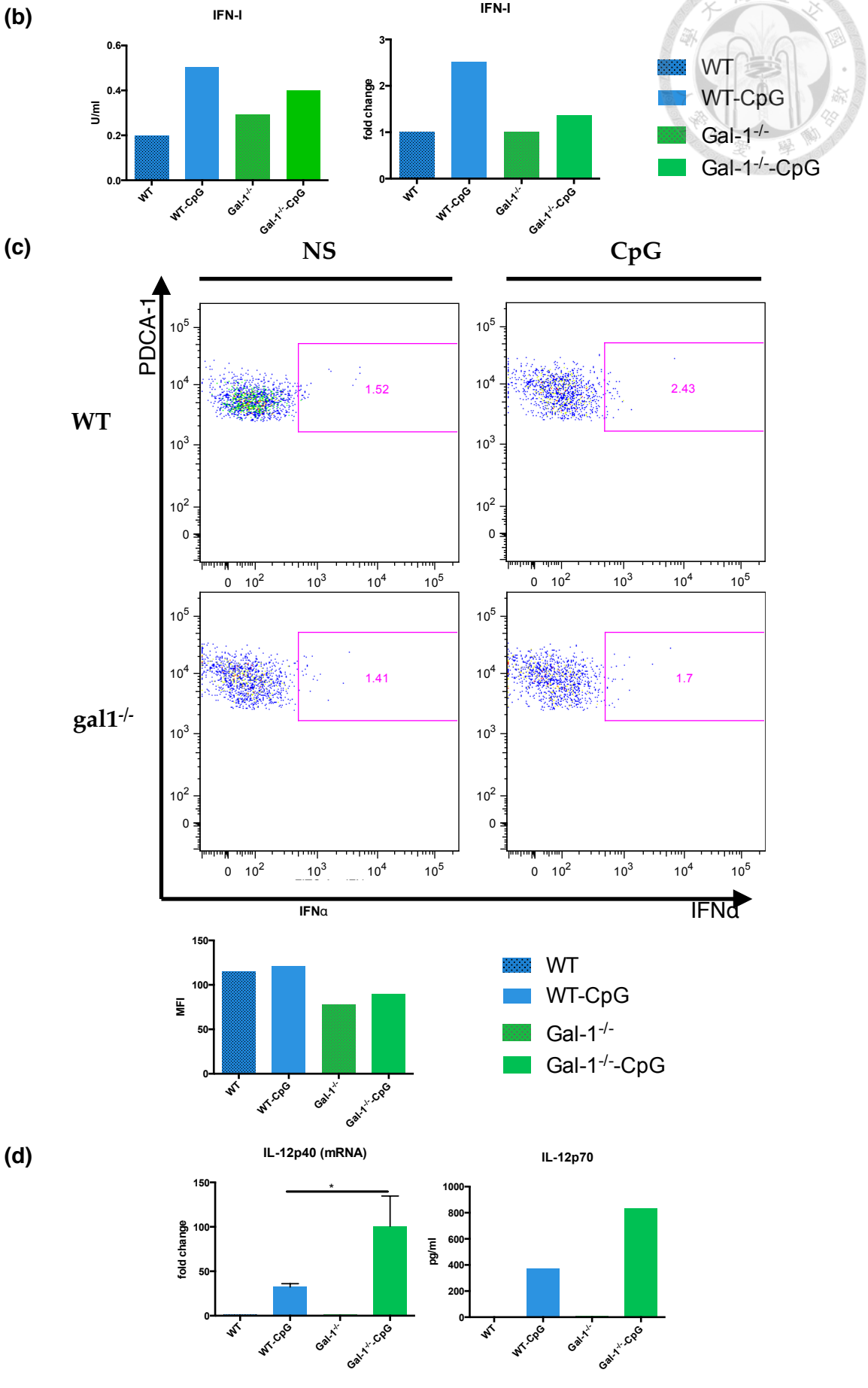

(b)
IFN-I
U/ml
0.6
0.4
0.2
0.0
WT
WT-CpG
Gal-1-/-
Gal-1-/--CpG
IFN-I
fold change
3
2
1
0
WT
WT-CpG
Gal-1-/-
Gal-1-/--CpG
WT
WT-CpG
Gal-1-/-
Gal-1-/--CpG
(c)
NS
CpG
PDCA-1
WT
gal1-/-
1.52
2.43
1.41
1.7
IFNα
IFNα
MFI
150
100
50
0
WT
WT-CpG
Gal-1-/-
Gal-1-/--CpG
WT
WT-CpG
Gal-1-/-
Gal-1-/--CpG
(d)
IL-12p40 (mRNA)
fold change
150
100
50
0
*
WT
WT-CpG
Gal-1-/-
Gal-1-/--CpG
IL-12p70
pg/ml
1000
800
600
400
200
0
WT
WT-CpG
Gal-1-/-
Gal-1-/--CpG

Figure 17. Examine the role of galectin-1 in Flt3L-BMDC response to CpG *in vitro*.

The mRNA expression level of IFN-I, TLR9 (a) and IL-12p40 (d) in Flt3L-BMDC induced by 1000nM CpG-A for 16 h. The relative number of mRNA expression level of each sample was normalized to mouse GAPDH expression. The fold change was calculated by basing on the fold induction of nontreated samples. To analysis the production of IFNα (a) and IL-12p70 (d), the supernatants of Flt3L-BMDC were harvested after 1000nM CpG-A stimulation for 24 h. (c) Flt3L-BMDC were stimulated with 1000nM CpG-A for 24 h, and BFA were added for 9 h before harvested. Cells were gated on $CD11c^{+}CD11b^{-}Bst2^{+}$ and analyzed IFNα production by ICS.

Fig. 18

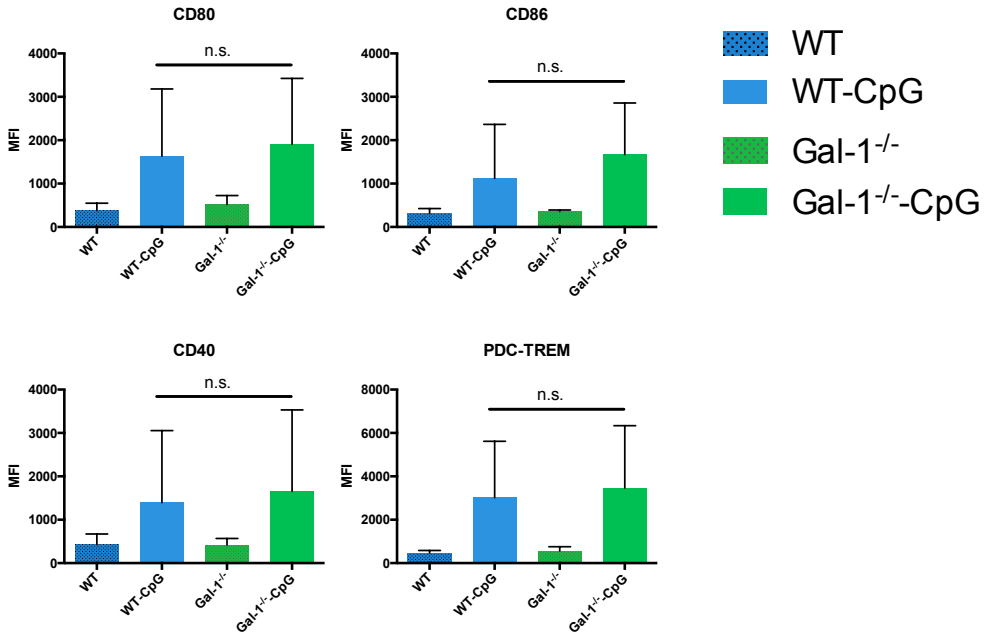
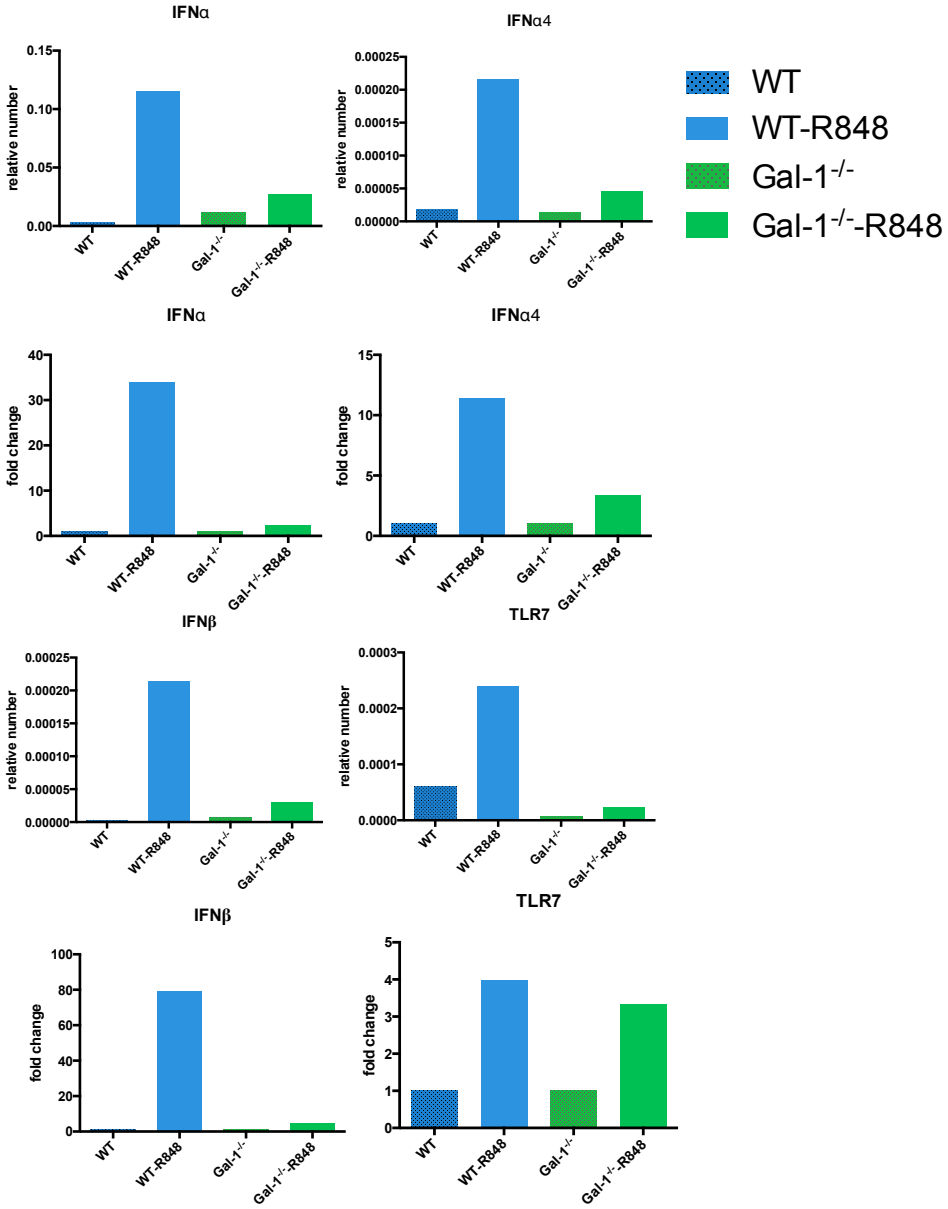


Figure 18. Examine the maturation status of WT and gal-1$^{-/-}$ Flt3L-BMDC response to CpG *in vitro*.

Flt3L-BMDC were stimulated with 1000nM CpG for 16 h and analyzed the expression level of maturation markers, CD80, CD86, CD40 and PDC-TREM. Cells were gated on CD11c^{+}CD11b^{-}siglecH^{+} and analyzed various maturation markers.

Fig. 19

(a)



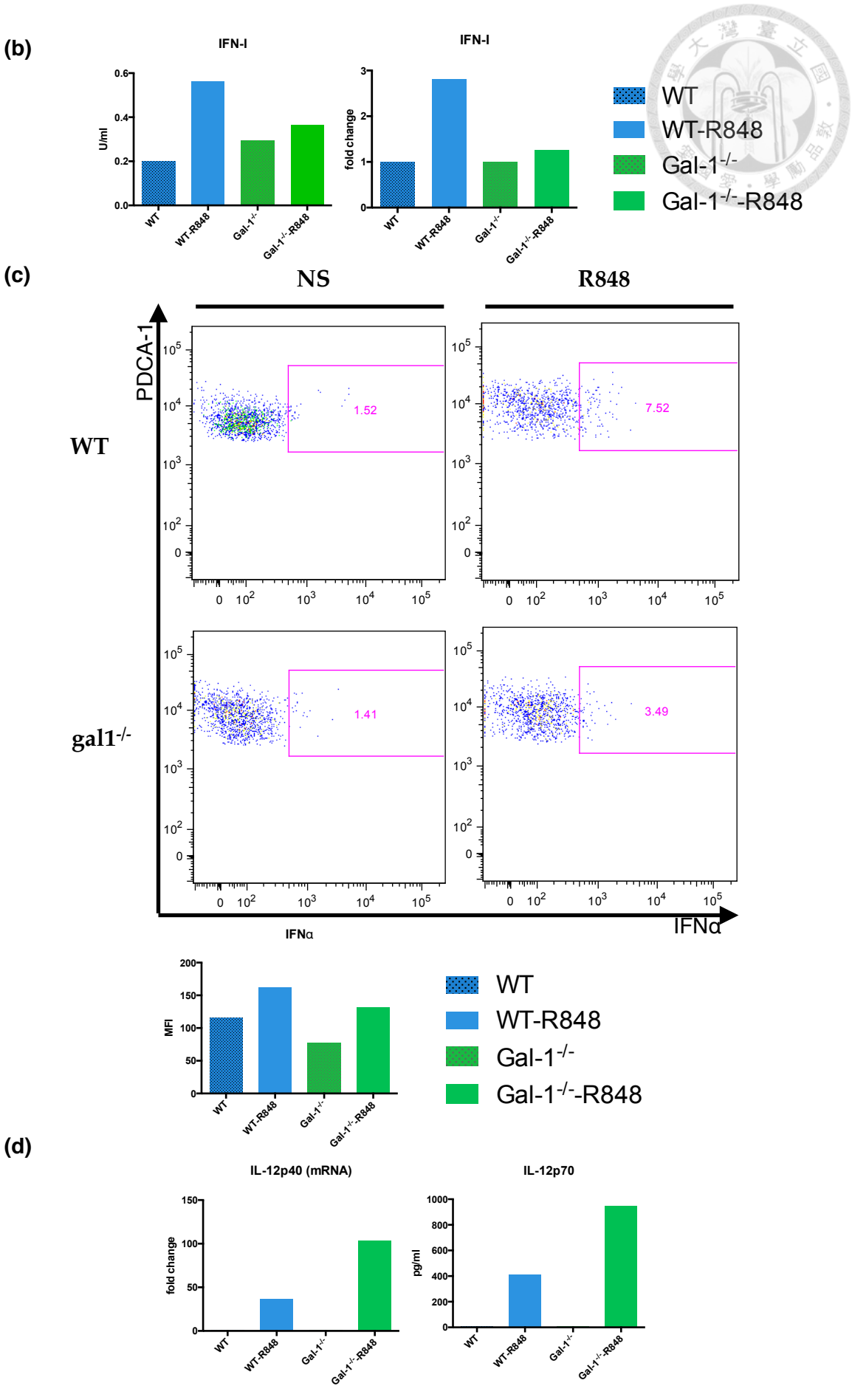

(b)
IFN-I
U/ml
0.6
0.4
0.2
0.0
WT
WT-R848
Gal-1-/-
Gal-1-/--R848
IFN-I
fold change
3
2
1
0
WT
WT-R848
Gal-1-/-
Gal-1-/--R848
WT
WT-R848
Gal-1-/-
Gal-1-/--R848
(c)
NS
R848
PDCA-1
WT
gal1-/-
1.52
7.52
1.41
3.49
IFNα
IFNα
MFI
200
150
100
50
0
WT
WT-R848
Gal-1-/-
Gal-1-/--R848
WT
WT-R848
Gal-1-/-
Gal-1-/--R848
(d)
IL-12p40 (mRNA)
fold change
150
100
50
0
WT
WT-R848
Gal-1-/-
Gal-1-/--R848
IL-12p70
pg/ml
1000
800
600
400
200
0
WT
WT-R848
Gal-1-/-
Gal-1-/--R848

Figure 19. Examine the role of galectin-3 in Flt3L-BMDC response to R848 *in vitro*.

The mRNA expression level of IFN-I, IRF7, TLR9 (a) and IL-12p40 (d) in Flt3L-BMDC induced by 0.5 μg/ml R848 for 16 h. The relative number of mRNA expression level of each sample was normalized to mouse GAPDH expression. The fold change was calculated by basing on the fold induction of nontreated samples. To analysis the production of IFNα (a) and IL-12p70 (d), the supernatants of Flt3L-BMDC were harvested after 0.5 μg/ml R848 stimulation for 24 h. (c) Flt3L-BMDC were stimulated with 0.5 μg/ml R848 for 24 h, and BFA were added for 9 h before harvested. Cells were gated on $CD11c^{+}CD11b^{-}Bst2^{+}$ and analyzed IFNα production by ICS.

Fig. 20

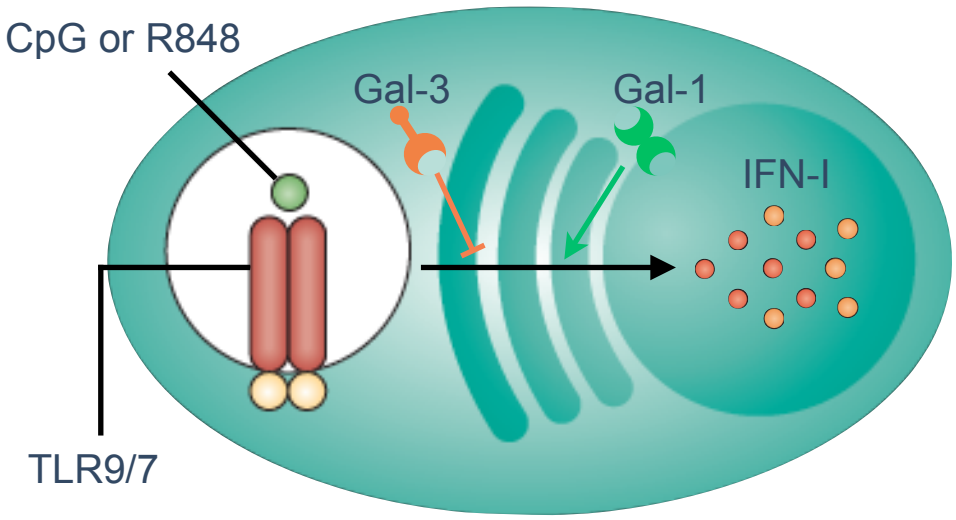


Figure 20. A model of the role of galectin-1 and -3 in regulating IFN-I response after TLR9/7 stimulation.

Galectin-3 may play a negative role in regulating IFN-I production by pDC, in contrast, galectin-1 may play a positive role to regulate the IFN-I response.

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