Effects of Different Surface Expression of the CD40 Co-stimulatory Molecules on Dendritic Cell Functions

by

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Abstract

Dendritic cell is one of the professional antigen presenting cells, and it bridges innate immunity and adaptive immunity. To fully activate naïve T cells, it requires DC to provide at least two signals, the interaction between T cell receptor and the MHC class II molecule loaded with antigen processed by DC, and the co-stimulatory signals provided by the co-stimulatory molecules expressed on DC. The identification of more and more co-stimulatory molecules expressed on DC and the studies on their functions highlight the importance of co-stimulatory molecules on the regulation of DC functions. We here hypothesized that different expression levels of co-stimulatory molecules expressed on DC is pivotal of directing DC function towards immunity, tolerance and polarization of Th1/Th2 immune response. Using CD40 as the model molecule to study the effect of its expression levels on DC functions, we found that no/low expression level of CD40 on DC induced antigen-specific immunological tolerance was due to the induction of CD4+CD25+Foxp3+ regulatory T cells, while the polarization of Th2 immune response induced by DC with medium expression level of CD40 was partially due to the impaired IL-12 production by DC during CD40 crosslinking. Our findings that different levels of co-stimulatory molecules have different regulations on DC functions has the significance in DC based immunotherapy for GVHD as well as the Th1 diseases.

List of abbreviations

AADC	alternatively activated DC
APC	antigen presenting cell
CD	cluster of differentiation molecules
CTL	cytotoxic T cell
CXCR	Chemokine, CXC Motif, Receptor
DC	dendritic cell
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
FBS	fetal bovine serum
Flt-3L	fms-like tyrosome kinase-3 ligand
GM-CSF	granulocyte-macrophage colony stimulating factor
IFN	interferon
Ig	immunoglobulin
IL	interlukin
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
JNK	Jun N-terminal Kinase
LAMP	lysosome associated membrane protein
LCMV	Lymphocytic Choriomeningitis Virus
LOX-1	Oxidized low-density lipoprotein receptor 1
LPS	lipopolysaccharide
mDC	myeloid dendritic cell
MAPK	Mitogen-Activated Protein Kinase
MIP	macrophage inflammatory protein
MHC	major histocompatibility complex
MyD88	myeloid differentiation factor
OVA	ovalbumin
pDC	plasmacytoid dendritic cell
PAMPs	pathogen-associated molecular patterns
PRR	pattern recognition receptors
RANTES	Chemokine (C-C motif) ligand 5
SDF-1	stromal cell-derived factor 1
TAP	transporters for antigen presentation
TGF	transforming growth factor
Th	T helper cell
TLR	toll-like receptor
TNF	Tumor Necrosis Factor
Tr1	regulatory T cell type 1
TRAIL	TNF related apoptosis inducing Ligand
TRIF	TIR-domain-containing adapter-inducing interferon-β

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Chapter 1 Introduction

Dendritic cells (DC) were first identified by Steinmen R. M. & Cohn Z.A. in 1973 based on their distinct cytologic features and the missing of lymphocytes and macrophage markers [1-3]. Under immunoelectron microscopy, the morphology of DC varies. The most recognized DC morphology is irregular surface with numerous projections and cytoplasmic vacuoles, which results in a very large surface of contact with their surroundings[4]. DC are bone marrow derived, lineage-negative, MHC class II positive mononuclear cells that distribute widely in tissues throughout the body, especially those sites such as skin and gastrointestinal tract that form the first line of body defence against the invasion of microorganisms[5].

DC are highly specialized antigen presenting cells (APC) that direct immune response to immunity, tolerance or Th1/Th2/Th17 polarization[6-8]. At immune steady state, immature DC reside in a wide range of body organ tissues where they example antigens and serve as sentinels of the immune system. Once they encounter antigen, immature DC uptake and process antigens in association with MHC class II molecules, and become mature DC. Upon maturation, DC secrets cytokines and chemokines and migrate to lymph node, where they present the complex of MHC class II molecule and processed antigen to naïve T cells to initiate immune response.

1.1 Heterogenecity of DC

DCs is a heterogenous group of cells containing multiple cells subsets that present differences in the phenotypes of cells as well as functions and localizations of distinct anatomical sites. They originate from $CD34^+$ hemotopoietic precursors in bone marrow. Based on the surface expression of particular markers and tissue distribution, DC subsets can be characterized as immature DC, mature DC, Langerhans cells, interstitial or mucosal DCs, myeloid DC (mDC) and plasmacytoid DC (pDC) [9-13].

DCs developed from myeloid or lymphoid lineage are distinct from each other both phenotypically and functionally. In human peripheral blood, mDCs are $CD11c^+CD123^{lo}$ and have a monocytoid appearance, while pDC is $CD11c^-CD123^{hi}$ and have similar morphology features to plasma cells. in vitro studies have revealed that CD14-derived DC favours Th1 response in a IL-12 dependent manner during T cell priming[14, 15]. CD14 but not CD1a-derived mDC also has the ability to activate naïve B cells to produce IgM in the presence of CD40L and IL-2 [16]. On the contrary, human $CD123^+$ pDC favours Th2 response. These pDCs are important in innate anti-viral immunity as well as autoimmunity. They also are the major IFN-α producing cells inducing anti-viral and anti-tumor immune responses [17]. Moreover, mDCs from myeloid lineage home to peripheral tissues, where they uptake exogenous antigens and migrate to secondary lymphoid tissue. pDCs from lymphoid lineage are involved in the maintenance of central tolerance and elimination of autoreactive T cells.

Immature DCs are defined by the expression of CD11c, low level expression of costimulatory molecules CD80, CD86, and CD40, and intermediate expression level of MHC class II molecules. Whereas, mature DCs express high level of CD80 CD86 CD40 and MHC class II molecules [18]. It is worthy of noting that DCs also express T cell co-receptors, CD4 and CD8, but as homodimer instead of heterodimer as T cell does. Using CD11b, CD4, and CD8, five distinct DC subsets have been identified [19]. Three of them, $CD4^{+}CD8^{-}$, $CD4^{-}CD8^{+}$, and $CD4^{-}CD8^{-}$ have been shown to reside in spleen. Where $CD8^{+}$ localized at marginal zone while $CD8^{-}$ localized at T cell area[12]. $CD11b^{+}CD4^{-}CD8^{-}$ $DEC205^{int}$ were found in all lymph nodes [19].

Due to the multifunctional properties of DC, there are two models that have been proposed for the differentiation of DC from bone marrow hemotopoietic progenitor cells. One suggested that DC has a single committed lineage that has functional plasticity, while the other suggested different DC lineages that renders DCs different functions [12]. In either model, the DC differentiation can be defined by three stages, which are DC precursor, immature DC and mature DC characterized by surface expression of particular makers.

DC differentiation requires certain cytokines. For instance, the growth of human monocyte can differentiate into immature nonproliferating DCs expressing low levels of costimulatory molecules in response to granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4.[20] Another cytokine, fms-like tyrosome kinase-3 ligand (Flt-3L) is pivotal for the differentiation of pDCs. [17, 21, 22]

1.2 Functions of DC

DCs are one of the three professional APCs identified so far. They are highly specialized in antigen uptake, process and presentation. The functional outcome of the antigen presentation by DC varies. It can be immunity, central tolearance, peripheral tolerance or the induction of different subsets of T helper cells based on the maturation status, DC subsets and the type of maturation stimuli. Besides the initiation of adaptive immunity, DC is also important component of innate immunity. It exerts its functions through effector mechanisms of innate immunity such as phagocytosis, pinocytosis, endocytosis, and NK DC crosstalk. The importance of DCs in both innate and adaptive immunity bridges the two parts of immune system.

1.2.1 Antigen capture, processing and presenting

DCs recognize antigen through pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs) shown on pathogens. They capture recognized antigen phagocytosis, pinocytosis, and endocytosis in association with receptors expressed on the cell surface, such as Fc receptors,[23] integrins, C-type lectins, and scavenger receptors CD91 and LOX-1.[24-27] In addition to antigen capture, these receptors also function through initiation of intracellular signalling or mediation of cell-cell interaction.

Captured antigens are processed by DC into peptides and are loaded onto MHC class I and II molecules in order to be transported to the cell surface and form the complex of antigen and MHC molecules for the recognition of T cells specific for those antigens.

DCs process and present different forms of antigen in different pathways. Endogenous antigens are degraded into peptide by the proteasome in the cytosol before they are transported via transporters for antigen presentation (TAP) molecules into the endoplasmic reticulum (ER). Peptides are loaded onto MHC class I molecules and are transported to the cell surface via trans-Golgi network for the presentation to $CD8^+$ T cells. On the other hand, exogenous antigens were engulfed and processed in endosome. Protein degradation mediated by proteases occurs in lysosome which is fused with endosome. The peptides were loaded onto MHC class II molecules before they are transported to the cell surface via specialized vesicle for the presentation to $CD4^+$ T cells.[28]

Interestingly, exogenous antigens sometimes can be presented through MHC class I pathway and can be presented to $CD8^+$ T cells. This phenomenon, also called cross-presentation, allows exogenous antigens to induce $CD8^+$ T cell immune response as well $CD4^+$ T cell immune response. [24, 29, 30] Cross-presentation requires specialized, self-sufficient ER-phagaosome derived comparments, TAP, Sec61 protein, calreticulin & calnexin, and endosomal signalling motifs on the cytoplamic tail of MHC class I molecule,[31] and for some antigens, the efficiency of cross-presentation are remarkably impaired due to possible degradation of antigen or less efficient access to the endogenous pathway. [32]

1.2.2 DC migration and induction of T cell immunity

Upon activation, DCs migrate from tissues to the T cell zone of the secondary lymphoid organs where they become further mature and present antigen to naïve T cells. Chemokines play an important role in regulating DCs migration. Different immature DC population displays a unique spectrum of chemokine receptors. Chemokines to which they respond include many CC and CXC chemokines such as MIP-1a, MIP-1b, MIP-3a, MIP-5, MCP-3, MCP-4, RANTES, TECK, and SDF-1. Mature DCs, on the other hand, lost their responsiveness to inflammatory chemokines but respond to ECL/MIP-3b and SLC/6C resulting from the gradual upregulation of CCR7 receptor during DC activation. [33]

It has been shown that DCs were predominantly distributed throughout the T cell zone one day after they reached secondary lymphoid organs. [34] The interaction between DC and T cells over time has been studied using intravital multiphoton microscopy.[35] T cells scan many different DCs and establish short interactions that only last a few minutes in the first 8 hours after they enter lymph nodes. After 6 to8 hours, the motility of T cells decrease and they form synapse that lasts longer time. Long lasting interaction between DC and T cells occurs one day after the T cells enter lymph nodes and the synapse lasts till the primed T cells start proliferating.[34] Stable interaction between DCs and T cells is pivotal for the induction of T cell immune response.[36]

In adaptive immunity, DCs play the important role of directing the consequence of T cells immune response depending upon a combination of properties that DC obtained

during maturation instead of one certain molecule, including efficient antigen processing and presenting, upregulated cositmulatory molecules, and cytokine secreted. The requirement of DC for $CD8^+$ T cell activation has been demonstrated in vivo. Depletion of $CD11c^+$ DC in mice led to the severe impairment of the ability of these mice to mount antigen-specific $CD8^+$ T cell immune response against infections with intracellular bacterium *Listeria monocytogenes*, the parasite *Plasmodium yoelii*, LCMV, or antigen immunization.[37, 38]

After being primed and $CD8^+$ T cell expansion, $CD4^+$ T cells might differentiate towards T helper 1 (Th1) cells that produce IFN-γ and support cytotoxic T cell (CTL) response, or towards helper 2 (Th2) cells that produce IL-4, IL-5 and IL-13, supporting humoral immunity and downregulate Th1 response, or towards Th17 that mainly express IL-17 in inflammation responses. The $CD4^+$ T cell polarization is dependent upon the subset of DC and their maturation stimuli. [39] Different DC subsets, different micro environments, different sites of distribution and different stimuli are responsible for the different cytokine profile that DC might generate, and it is the cytokine profile of DC produced that determines the outcome of polarization.[40] It has been shown by far that IL-12, IL-18 and IL-27 skew the immune response towards Th1, CCL17, CCL22, and the absence of IL-12 are known for the polarization of Th2, while IL-6, IL-21 and IL-23 have been reported to induce Th17 cells.

Several pathways that are involved in the Th polarization have been described. By differentially modulating MAP kinase signalling through different TLR ligands, the

human mDCs are able to distinct Th immune response.[41] Ligand for TLR4 and TLR5 are able to induce DC to phosphorylate p38 and JNK1/2 kinase that induce Th1 response by the production of IL-12. On the other hand, agonist of TLR2 and a classic Th2 stimulus, schistosome egg antigen, stimulate ERK1/2 phosphorylation, leading to the stabilization of the transcriptional factor responsible for IL-12 synthesis, thus results in Th2 polarization. Other transcriptional factors, such as T-bet that induces the production of IFN-γ in DC, are also involved in the Th polarization.[42]

1.2.3 DC NK crosstalk

DCs play important roles in bridging both adaptive and innate immunity. However, the most important innate function of DCs is their interaction with natural killer (NK) cells. Studies focused on the functions of DCs during the early phase of immune response have revealed a predominant role of DC in the activation of NK.[43-46] NK cells constitute 15% of the circulating lymphocytes and are the major lymphocyte from innate immunity that are crucial for both immune surveillance and controlling of infectioncontribute to the eradication of infection.[47, 48] The activation of NK cells is regulated by the balance of activating or inhibitory receptors expressed on them. Other than surface receptors, cell-cell contact as well as soluble mediators such as IL-2, IL-12, IL-18, and type I IFN have been shown to be involved in NK cell activation.[49] The first evidence of NK-DC crosstalk was reported in 1999 by Fernandez group that demonstrated the activation of NK-mediated anti-tumor effects by DC.[44] It was shown more recently that the NK-DC interaction is important for the optimal immune cell activation and expansion during viral infection in vivo.[50]

Current data suggest that the interaction between DC and NK cells requires cell-cell contact,[44-46, 51, 52] which leaves few options for the common meeting ground of these two cell types. The first place that NK-DC interaction occurs could be the particular site of infection or inflammation, where the resident DC and other cell types on site secret cytokines and chemokines to recruit more DC and NK cells. Indeed, there is evidence showing the accumulation of NK cells in number at the skin of patients during yeast *Malassezia* infection.[53]

Activated DC migrated to draining lymph nodes via the upregulation of the expression of CC-chemokine receptor, CCR7.[54] It has been found that DCs matured by microbial stimuli, such as LPS and CPG, are able to produce large amount of CXCR3 ligands, CXCR9 and CXCR10, which partially regulate the recruitment of NK cells to draining lymph nodes.[55] Moreover, NK cells were found accumulating at draining lymph nodes[55] and colocalizing with DCs.[56] It was also reported that a NK subpopulation was able to respond to DC-derived stimuli, such as IL-12, and produce IFN-γ and membrane-bound IL-15, initiating NK proliferation.[56]

The interaction between NK and DC is not univocal but reciprocal. Several studies have shown that activated NK can mature monocyte-derived immature DC at low ratio.[46] Similarly, IL-2 activated NK cells are able to induce maturation of blood pDC and mDC, while synergize with microbial stimuli to enhance the production of IFN-α and TNF by pDC.[57] On the other hand, autologous NK cells lyse immature DCs at higher NK DC ratio. The NK cell-mediated lysis of immature DCs has also been observed in several

mouse models in vivo, which is denpendent on the TNF related apoptosis inducing Ligand (TRAIL).[58]Although the mechanism underlying this phenomenon is not well understood, it has been speculated that this is the mechanism that NK cells maintain the homeostasis of immature DCs. Also, the lysis of immature DCs that either do not present antigen efficiently or potentially induce tolerance will lead the most optimal immunity.

1.2.4 DC and the induction of central tolerance

In order to limit self-tissue damage and to maintain immune homeostasis and the large T repertoire required for the adaptive immunity, the immune system developed mechanisms for eliminating autoreactive T cells that respond to self antigens.

Since the thymus is the place where negative and positive selections occur, the cell types that reside in the thymus might contribute to the induction of self-tolerance.[59] There are three types of stromal cells, cortical epithelial cells, medullary epithelial cells and bone marrow-derived cells including DCs, macrophages, and B cells, presenting in the thymus.[60-62] Studies on these cells using mouse models in which the expression of MHC-peptide complex was restricted to particular thymic cell type revealed that thymic DC is responsible for the induction of central tolerance.[60, 61]

The role of DC in central tolerance was further demonstrated by a system in which the tolerance in APCs depleted thymus was restored by the reconstitution of spleen DC.[63] Similar results were reported using bone marrow chimera and transgenic models.[64, 65]

Moreover, in an in vivo model where the MHC I was exclusively expressed by DCs, efficient negative selection of $CD8^+$ T cells has been demonstrated.[66] Taken together, thymic DCs cannot positively select either $CD4^+$ or $CD8^+$ T cells and are specialized in tolerance induction.

1.2.5 DC and the induction of peripheral tolerance

Besides the central tolerance occurred in the intrathymic environment during the process of T cell development, there is tolerance effects that are mediated by DC reported in peripheral environment.[67-69] It has been shown that autoreactive T cells that can recognize self antigen undergo anergy and deletion as well as downregulation of T cell receptors and co-receptors.[70, 71]

Under immune steady state, immature DCs constantly present self-antigen to autoreactive T cells. According to the Danger model,[72] the fully activation of naïve T cells requires signal one and two provided by APC. In the presence of danger signals, DCs start maturation process and provide cositmulatory signals during their contact with T cells. In the absence of danger signals, DCs loaded with self-antigen will either undergo programmed cell death for lacking of survival signals or induce T cells tolerance due to the lack of cositmulatory molecules. Indeed, T cells tolerization was observed when they encounter immature DC loaded with antigen.[73, 74]

There is increasing data that suggests naturally occurring regulatory T cells are responsible for the maintenance of peripheral tolerance.[75, 76] And DCs were

implicated in the induction of regulatory T cells. It has been reported that the generation of regulatory T cells requires IL-10 production by DCs.[77] Similar results have also been described in human studies where the injection of immature DCs exposed to influenza peptide induced antigen-specific IL-10 secreting regulatory T cells and the clearance of the antigen-specific $CD8^+$ effector cells. And this result could be reversed by the matured DCs pulsed with peptide.[78]

It has also been reported that immature DCs are able to induce $CD8^+$ IL-10 producing regulatory T cells.[76, 79, 80] Moreover, mature DCs in the absence of exogenous antigen can induce a fraction of $CD4^+$ T cells with some regulatory properties observed in regulatory T cells.[81]

The alternatively activated DC (AADC) generated in the presence of IL-10 and TGF-β expressed low level of CD80/86 and CD40, and was able to suppress allogeneic immune response.[82] Moreover, the absence of CD40 alone is enough for the induction of T cell tolerance. Antigen-pulsed DC with no CD40 expression was reported to prevent T cells priming and successfully suppressed a primed immune response in an antigen-specific manner. The mechanism underlying these results is the induction of IL-10 producing Tr1 cells.[83]

pDCs are also reported to have toleragenic property. It has been demonstrated that pDCs can induce CD8+ regulatory T cells in vitro.[84] And, the ligation of specific receptor, BDCA-2, renders inhibition of T cell activation and proliferation.[40]

1.3 DCs maturation

Resting DCs or immature DCs initiate maturation process after they encounter stimuli or so called danger signals such as tissue injury and presence of antigen.[85, 86] DC maturation is a complicate process accompanied by the upregulated cositmulatory molecules such as CD80/83, CD86, and CD40, upregulated MHC class II molecule, reduced phagocytosis capacity, acquisition of motility and migration to secondary lymphoid tissues, cytoskeletal modification and the development of unique cytoplasmic extensions or dendrites.[87]

DCs maturation can be induced by a wide range of resources, including danger signals derived from microorganisms and damaged tissues that can be recognized by PRRs, host-derived inflammatory molecules such as TNF-α, IL-1, IFN-α, IL-6, and CD40 ligand. The lymphocytes from innate immune system, B cells and natural IgG antibodies are also involved in the modulation of DCs maturation.[88-91]

After maturation, DCs become activated with highly enhanced ability of antigen processing and presentation. There is accumulated data suggesting the maturation states of DCs play a pivotal role in determine the functional outcome of T cells activation to immunity or tolerance. One model is that DCs with immature phenotype induce tolerance when mature DCs are immunogeneic.[92, 93]

1.3.1 Immature DCs versus mature DCs

Immature DCs and mature DCs are two stages of DCs maturation process defined by the surface phenotype and different specialization in antigen capture, processing and presentation. Immature DCs are commonly referred to the resting DCs under immune steady state that function as immunological surveillance. They have strong endocytic capacity, and are able to engulf antigens by phagocytosis, capture fluid-phase antigen through macropinocytosis, and uptake protein antigen or immune complex by endocytosis mediated through Fc receptors.[94, 95] Immature DCs express low level of MHC class molecules and cositmulatory molecules such as CD40, CD80 and CD86.[96, 97] In addition, recent studies on pDCs have shown that under steady state pDCs present an immature phenotype with fairly weak endocytic ability.[98, 99] Immature DCs are demonstrated to be the key player in the induction and maintenance of central and peripheral tolerance.[73, 76, 100]

Mature DCs, on the contrary, are featured with impaired antigen capture ability due to downregulated phagocytosis and macropinocytosis, as well as the loss the antigen receptors.[24, 101] Meanwhile, the antigen processing efficiency and the half-life of MHC-peptide on DCs surface are increased, which make the antigen presentation by matured DCs more efficiently.[96] In addition, the surface expression of cositmulatory molecules including CD40, CD80, CD83 and CD86, as well as DC-specific lysosomal protein, DC-LAMP is upregulated.[102, 103] This transformation of DCs in phenotype alters DCs function and makes them more potent APC for T cells stimulation.

DCs maturation is followed by the activation process that is accompanied with the higher level of the expression of MHC class II and the production of cytokines required for T cells activation, such as IL-12 producion by myeloid DCs and IFN-α produced by pDCs.[104-107] It has also been reported that mature antigen-presenting DCs mediate T cells tolerance through the induction of regulatory T cells.[81, 105]

1.3.2 Toll like receptor dependent (TLR) maturation

TLRs are membrane-bound receptors that can be activated by the binding of molecular structures conserved among microbes. Different TLRs have different expression patterns and bind to different ligands.[108] Human mDCs are reported to express TLR1 through 5, TLR7 and TLR8 depending upon subsets, while pDCs express TLR1, 7 and 9.[108-110] Though all TLRs are transmembrane receptors, the function of TLR is not always exerted at cell surface. For instance, TLR9 localized in the ER of resting pDCS and TLR7 and 8 are found in the endosome.[111, 112]

The binding of TLRs and their ligands lead to the initiation of complex signalling cascade or intracellular events.[113] TLR signalling is mediated mainly through the adaptor protein myeloid differentiation factor (MyD88) that trigger the activation of transcription factors, such as NF-κB essential for the expression of proinflammantory genes.[114, 115] In addition, the ligation of TLR7 or 9 leads to the production of type I interferons regulated by the transcription factor IFN regulatory factor 7 (IRF-7).[114, 116, 117] Also, the Toll/IL-1 receptor domain-containing adaptor protein-inducing interferon-β (TRIF) mediates the production of type I IFNs through TLR3 ligation.[118, 119]

The common and pathogen-specific genes have been found through the microarray analysis of the transcription profiles of DCs stimulated by different class of pathegens.[120] Common genes regulate the synthesis of proteins respond against all microbes while distinct set of genes are expressed in a pathogen-specific manner. Further studies have shown that despite of the similar phenotype changes on DCs followed by the different TLR stimulation, DCs produce different pattern of cytokines, which leads to the Th1/Th2 polarization appropriate for the pathogen. For instance, DCs stimulated by TLR4 ligand LPS or TLR2 ligand PGN comparable levels of maturation markers but different cytokine and chemokine profiles. DCs produce Il-12p35 in response to LPS stimulation, while IL-8 and IFN-inducible protein 10 (IP-10) were produced in response to PGN stimulation.[121]

1.3.3 Innate lymphocyte induced DCs maturation

DCs maturation can be induced after NK cell recognize the downregulation of MHC class I molecule,[122] or after activation of phosphoantigen-specific CD1c-restricted γδ T cells,[123, 124] or after the NKT stimulation by the glycolipid α-galactosylceramide (α-GalCer) presented on DCs CD1d molecules.[125, 126] The DCs maturation induced by activated innate lymphocyte is identified by the changes in phenotype and cytokine production including upregulation of cositmulatory molecules on DC, the IL-12 production and the priming of T cells immune response.[45, 123, 124, 127] TNF-α is identified as the major inducer in this innate lymphocytes-mediated DC maturation.[45, 123, 124]

The interaction between DCs and innate lymphocytes is reciprocal. After maturation, DCs also stimulate NK, NKT and γδ T cells by secreting IL-12, IL-15, IFN-α and IFN-β to sustain the innate immunity mediated by these cells.[44, 56, 124, 125, 128] The interaction between DCs and innate lymphocytes in vivo mainly leads to the Th1 phenotype of the primary immune response due to the production of IFN-γ by these lymphocytes.[129]

Innate lymphocytes can also provide danger signals to DCs and induce DC maturation. Innate lymphocytes serve as effctor cells, and will generate fragments of the infected or tumor cells during the process of killing their targets cells. These fragments can alter DCs the invasion of pathogen and induce DCs maturation.[122]

1.3.4 DC maturation induced by B cells

Accumulated data suggests that in addition to environmental signals, crosstalk with innate lymphocytes and T cell-derived signals, B cells play a profound role in modulating DCs maturation and function. Studies using B cell deprived animal have shown that the ability of DCs to induce antigen-specific IL-4 producing CD4+ T cells is impaired in the absence of B cells.[130, 131] B cells regulate the maturation and functions of DCs either by FcR or by natural IgG.[88]

Both human and murine DCs express various types of FcR including FcγR, FcαR and FcεR. Human monocyte-derived DCs express FcγRII, FcγRI and transferrin receptor,[132-136] Langerhans cells express FcγRII and FcεR,[137-140] and DCs in

blood stream express FcγRII, FcγRI, and FcεRI.[141-144] On the other hand, mouse BMDC, splenic DCs and Langerhans cells express FcγRI, FcγRIII and FcγRIIB.[145-149] These FcR expressed on DCs can be classified into activating receptors and inhibitory receptors based on the presence of cytoplasmic motif. Activating receptors refer to those with the presence of immunoreceptor tyrosine-based activation motif (ITAM), whereas inhibitory receptors refer to those with immunoreceptor tyrosine-based inhibitory motif (ITIM).[149]

Recent data has shown that the balance between activating receptors and inhibitory receptors is pivotal in the induction of DCs maturation.[147, 150] Interfering with the inhibitory signals delivered by FcγRIIB enhances the ability of immunocomplex (IC) formed by antibodies to induce DCs maturation.[147] Indeed, selective blockade of inhibitory FcγR led to the maturation of human monocyte-derived DC.[150] Moreover, cross-linking of FcγR on murine BMDC induces phosphorylation of the tyrosine kinase Syk and ERK, which are critical for IC-induced DC maturation.[151, 152] Similarly, engagement of FcγRII with immobilized IgG or engagement of FcεRI on human LC results in the activation of NF-κB pathway, thus lead to the maturation of DCs.[153]

In addition, B cells also regulate DCs maturation through natural antibodies in a FcR independent manner. Natural antibodies refer to those circulating in the body under normal conditions. They are generated during germ-line Ig gene expression in the positively selected B cells.[154] The majority of natural antibodies are autoreactive and are involved in the maintenance of immune homeostasis.[155]

The importance of natural antibodies has been elaborated by the examination of DCs status in patients with primary immunodeficiencies such as common variable immunodeficiency (CVID) and X-linked agammaglobulinemia (XLA) where the patients have low level of circulating natural antibodies.[156, 157] The differentiation of DCs in these patients is severely impaired. However, this impairment in DCs differentiation could be partially reversed by reconstituting patient plasma with intravenous immunoglobulins, suggesting the deficieny in DCs differentiation was caused by the low level of circulating natural antibodies.

In addition, CD40 signal has been reported involved in the natural antibodies-mediated DC maturation.[156] Unlike CD40-CD40L-mediated DC maturation by T cells, CD40-reactive natural antibody induced mature DCs produced increased IL-10 and decresed IL-12. Moreover, the natural IgM antibodies B7-DC expressed on DC and enhance the ability of BMDC polarizes T cell towards Th1 phenotype through STAT4-depent pathway.

1.4 Costimulatory molecules and DC

The two-signal model for lymphocyte activation was first proposed in 1970.[158] New version of this model based on current data suggests that the activation of naïve T cells requires two distinct signals from APCs, one is from antigen-specific TCR while the other one is the cositmulatory signals provided through the interaction between the cositmulatory molecules expressed on APCs and their ligands expressed on the T cells. The cositmulatory molecules expressed on DC can be divided into two groups, which are B7 family and tumour necrosis receptor family (TNFR), based on their respective homology to the founding member. B7 family includes CD80 and CD86, PD-L1, PD-L2, B7-H1, B7-H2, B7-H3, B7-H4. TNFR includes CD40, TNFR superfamily member 4 (OX40) ligand, ICOS Ligand, TNFR superfamily member 13c (BAFF-R) Ligand, TNFR superfamily member 17 (BCMA) Ligand, and TNFR superfamily member 11a (RANK) Ligand, and 4-1BB Ligand/TNFSF9. So far, the ligands for these cositmulatory molecules were found on T cells or B cells, either naïve ones or activated ones.

The function of cositmulatory molecules was originally describe as providing signal 2 required for T cell activation. However, recent studies have shown that some of the cositmulatory molecules actually provide inhibitory signals in order to regulate T cells activation. Moreover, some of the members of B7 family have been found regulating the activation of both APCs and T cells.

Increased numbers of cositmulatory molecules identified on DC revealed a crital role of them in DC function. Understanding of these pathways will benefit the development of

new therapeutic strategy which targets the cositmulatory pathway to regulate T cells activation, tolerance and polarization in respond to different disease development.

1.4.1 CD40 and DC function

CD40 is a 48KDa type I transmembrance protein that contains a 193 amino acid (aa) extracellular domain, a 21 aa leader sequence, a 22 aa transmembrance domain, and a 62 aa intracellular domain in human. CD40 shares a homology of 22 cysteine residuals with other members of TNFR superfamily.[159] CD40 can be found on the surface of a wide range of cells, including B cells, T cells, DCs, monocytes, platelets, macrophages, epithelial cells, endothelial cells, and fibroblasts. Also, it can be found on the surface of B lymphoma and carcinoma.[160-162]

The Ligand of CD40, also known as CD154 or CD40L, is a type II transmembrance protein that has a variable molecular weight due to post-translation modification.[159] CD40L belongs to TNF superfamily with a characteristic sandwich structure that is composed by a β–sheet, α-helix loop, and a β-sheet.[163] It can be found expressing on the surface of activated T cells, B cells and platelets. It can also be induced on NK cells, monocytes, mast cells and basophil during inflammation.[164]

The interaction between CD40 and CD40L has profound effects on DCs. The engagement of CD40 by CD40L increases the clustering of CD40, which in turn recruits TNFR-associated factors (TRAFs) to the cytoplasmic domain of CD40.[165] The TRAFs proteins then activate several different signalling pathways including carnonical and non-

carnonical NF-κB pathway, mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K), and phospholipse Cγ(PLCγ) pathway.[165] These complex pathways induce important signals that are mediated through CD40 in order to regulate different cellular and immune responses, such as survival, maturation, proliferation, expression of cositmulatory molecules, expression of inflammatory cytokines as well as variant B cell functions and development.

CD40 was found to be expressed on activated T cells as well as APCs.[166] Likewise, CD154 was found to be expressed on activated human and murine DCs as well as activated T cells.[167, 168] Therefore, there is a bi-directional cross-talk between DCs and T cells, and the interaction between CD40 and CD154 has a reciprocal effect on the regulation of DCs and T cells.[169, 170]

DCs in both peripheral and lymph nodes express no or low level of CD40. It has been shown that the induction of CD40 expression is dependent on danger signal through PPRs, particular TLRs.[171] CD8α+ DCs produce high level of IL-12 in response to signals generated by CD40L. And other cytokines such as IL-10, IL-1β, and IL-4 can be produced depending on different TLR signals, which regulates T cell functions through polarization of different Th subtypes. CD8α- DCs, on the contrary, do not produce high level of IL-12 upon CD40L signal.[172, 173]

CD40 has been shown expressing on activated $CD4^+$ and $CD8^+$ T cells, and is important in the functions of these T cells. [161, 174] It has been recognized that cytotoxic T

lymphocytes (CTLs) is induced by CD40-mediated conditioning of DCs after the administration of agonist anti-CD40 mAb.[175] However, there is evidence showing that the generation of CD8+ memory T cells in vivo requires CD40 ligation to the CD40 expressed on $CD8^+$ T cells directly.[161] In addition, the 4-1BB deficient mice has Therefore, CD40 plays a critical role in CTL induction and function.

CD40 was also found to be expressed on thymocytes and up to 50% of peripheral T cells .[176] Subsequent functional analysis of the CD40 expression using nonobese diabetic (NOD) mice model of human type I diabetes mellitus showed clones that are diabetogenic express CD40 whether they are skewed to Th1 or Th2.[174] CD40 was also found on the majority of splenic and pancreatic $CD4^+$ T cells in diabetic NOD mice as well as its derivates. These findings suggest that CD40 expressed on $CD4^+$ T cells might be the marker for autoimmunity.

The epithelial cells function as immune effector cells in a wide variety of tissues by expressing inflammatory mediators and immune-related molecules. The expression of the CD40 receptor on these cells contributes this role. Engagement of CD40 activates epithelial cells and results in their release of pro- and anti-inflammatory mediators as well as pro-fibrotic molecules.[177]

Studies using parasites infection disease models have shed light on the importance of CD40 in the regulation of cell-mediated immunity as well as the possible explanations for the susceptibility to opportunistic infections observed in patients. For instance, in *T.*

gondii infection, CD40 synergizes with TNF-α and mediate autophagic killingof *T. gondii* in a TRAF6 dependent manner.[178, 179] However, in the infection of *L. major,* the parasites skew CD40 signaling toward ERK-1/2 and IL-10 production in macrophages from BALB/c mice, which in turn inhibits the activation of p38 MAPK, and the expression of NOS2 and of IL-12 production.[180]

Previous approaches employed to achieve peripheral tolerance include using immature DCs to present antigen to naïve T cells with the absence of cositmulatory signals, generation of alternatively activated DC (AADC) using IL-10 and TGF-β, blockade of B7-CD28 pathway or CD40 pathway. However, none of these approaches clearly clarify the fact that CD40 key determinant in the induction of peripheral tolerance. It has been shown that blocking CD40 is enough to prevent priming of immunity and to suppress a previously primed immune response.[83] CD40-/- DC induced $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells that functions on a IL-10 dependent manner.

Because of its prominent role in orchestrating humoral and cellular immune responses, CD40 and CD40L signalling pathway has become the major target of immunotherapy in many aspects. Anti-CD40 monoclonal antibody with 7E1-G2b isoform (IgG2b isotype) was shown to resemble anti-CD154 when administered in vivo, and synergized with with CTLA-4-Ig in promoting both allogeneic bone marrow chimerism and skin graft survival.[181]

1.4.2 B-7 family pathways: positive and negative

B7-1 and B7-2 are the first identified members of B7 family, which do not share high homology but have highly similar conformation with two extracellular domains coupled with transmembrance and short cytoplasmic domains. There are highly conserved residuals that define the immunoglobulin (Ig) Vregion like members of the Ig gene superfamily (IgSF) in the penultimate domains of B7-1 and B7-2.[182] Currently identified members of B7 family include B7-1, B7-2, B7-H1, B7-H2, B7-H3, and B7-H4.[183]

The B7-1/B7-2-CD28/CTLA-4 pathway was well studied for their function of T cell activation and regulation. B7-1 is constitutively expressed on DCs whereas B7-2 expressed at low level in immature DCs but upregulated after DCs activation,[184, 185] Similarly, CD28 is constitutively expressed on T cells while CTLA-4 expression is rapidly upregulated after T cell activation.[186, 187] B7-1 and B7-2 provide important cositmulatory signals to augment and sustain T cell response through the interaction with CD28.[188] CD28 signals synergized with the TCR signals to augment T cell activation. It is then speculated that B7-1/B7-2CD28 signalling regulates the threshold for T cells activation as it decreases the TCR engagements required to activate T cells. CTLA-4 engagement with B7-1/B7-2, on the other hand, inhibits TCR and CD28-mediated signal transduction. CTLA-4 inhinits IL-2 synthesis and progression through cell cycle and terminates T cells response. Therefore, CTLA-4 is the negative feedback control to overcome uncontrolled T cells activation by CD28 engagement.[189-192] CTLA-4 is also involved in peripheral T cell tolerance, which is induced as the consequence of B7-

1/B7-2 and CTLA-4 interaction instead of the absence of B7-1/B7-2 signals.[193, 194] Other pathways mediated by B7 superfamily members that confer positive cositmulatory regulation include B7-H2, also known as ICOS Ligand, and B7-H3. Costimulation of B7-H2 led to the production of IL-10, IL-4 and IFN-γ.[195-197] More Th2 cells were differentiated than Th1 cells in TCR-transgenic mice.[198] And, costimulation by B7-H3 resulted in a selective increase of IFN-γ production in allogenic responses.[199]

B7-H1 and B7-DC are the ligands of programmed death-1 (PD-1) that expresses majorly on the surface of activated T cells and B cells.[200] Binding of B7-H1 or B7-DC to PD-1 resulted in the inhibition of proliferation of pre-activated human and murine T cells to suboptimal CD3 stimulation.[201] B7-H1 was also suggested to be involved in the tumor evasion mechanism, where tumor-associated B7-H1 binds PD-1 on T cells to induce T cell apoptosis.[202] However, there are controversy data showing that B7-H1 and B7-DC have cositmulatory function by increasing T cell proliferation.[203] The reason for this discrepancy is not clear. However, it has been proposed that B7-H1 and B7-DC has other ligand(s) expressed on T cells. Both B7-H1 and B7-DC mutants with defect in PD-1 binding induced proliferation and cytokine production of T cells from normal and PD-1 deficient mice.[204]

B7-H4 is the most recent identified member of B7 superfamily, which has been shown to negatively regulate T cell response.[205, 206] B7-H4 inhibited T cell proliferation, suppressed IL-2, Il-4, IL-10 and IFN-γ production during CD3 stimulation and B7-1 costimulation.[205] Blockade of B7-H4 led to the aggravation of EAE.[206]

1.5 Lentiviral vector transduced DC mediated immunotherapy

Unlike other viral vectors that have been employed during the development of gene therapy, lentiviral vectors have the advantage of capable of transducing a wide rage of cell types including dividing and non-dividing or quiescent cells without inducing immunity against the vectors. In addition, lentiviral vectors have high transduction efficiency and are able to transduce cell type that is known hard to be transduced. Like retroviral vectors, the gene of interest carried by lentiviral vectors has the ability to integrate into host genome to achieve long-term stable gene transfer. Because of these advantages they possess, lentiviral vectors become the most efficient vehicle for gene delivery into different types of cells.

The first successful transduction on DC by lentiviral vector was reported in 1999. Since then, different groups have demonstrated long-term stable transduction on human monocyte-derived DC, human CD34+-derived DC, and mouse BMC. After that, several immunotherapy strategies combining the forces of lentiviral vectors and DCs have been developed to fight against cancer, viral infection, and autoimmune disease.

1.5.1 Lentiviral vector-mediated DC based cancer immunotherapy

Several genes encoding tumor associated antigen (TAA), such as TRP-2, MAGE-3, Melan/MART-1, tyrosinase, and the surrogate TAA ovalbumin (OVA), have been inserted into lentiviral vectors. DCs transduced with these lentiviral vectors have been shown to effectively process and present lentiviral vector-derived transgene and activate established T cell lines or clones specific for the epitopes derived from these TAA.

Furthermore, studies using DCs transduced with lentiviral vectors encoding one or several TAA genes in mouse models have shown positive results. BMDC transduced with lentiviral vectors carrying OVA gene induced OVA-specific CTL and OVA-expressing tumor cells in vitro or OVA-expressing autologous cells in vivo. Moreover, lentiviral transduced DCs demonstrated their ability of inducing protective immunity by protecting animals from a lethal dose of challenge using OVA-expressing B16 melanoma cells and slowing down the growth of pre-existing tumor.

The approach of immunizing animals with lentiviral vector directly has also been tested. Although the biodistribution of lentiviral vector is not well known, it has been shown that resting DCs at injection site has been selectively transduced by injected viral particles and homed to draining lymph nodes and spleen where they primed antigen-specific T cells. Compared with DC vaccines transduced with lentiviral vectors ex vivo, direct administration of lentiviral vectors encoding TAA genes induced more potent TAA-specific immune responses, including more number of IFN-γ producing CTL as well as stronger lytic capacity. Furthermore, direct administration of lentiviral vectors overexpress TAA genes showed more potent protective immunity and prolonged survival time.

Although lentiviral vectors encoding TAA genes induced potent CTL response, the anti-tumor induced is till weak in some cases, partially due to the tolerogenic mechanisms and active suppression employed by tumor cells to evade immune system. Current data suggests that a successful vaccine strategy requires not only potent tumor antigen-specific

immune responses, but a mechanism to break tolerance or overcome suppressive mechanisms. Several efforts have been made so far in order to overcome tolerance mediated by tumor cells. Lentiviral vector-mediated transgene delivery to DC progenitors including bone marrow cells and hematopoietic stem cells was capable of generating large number of tumor antigen-presenting DCs sufficient to overcome tumor-induced tolerogenic environment in an aggressive epithelia tumor bearing animal model. Improving antigen presenting ability by transducing calnexin, a protein important for proper protein folding and antigen presenting, has been shown to overcome the immune suppression and multiple myeloma-specific CD4 and CD8 T cell responses. Another strategy has been employed by strongly stimulating innate immunity to produce inflammatory cytokines as signal 3 for T cell activation. Lentiviral vector transduced DCs overexpressing TLR or TLR adaptor proteins including MyD88 and TRIF/TICAM-1 were able to enhance tumor-specific lysis and to slow down the growth of pre-existing tumor.

1.5.2 Lentiviral vector-mediated DC based anti-viral immunotherapy

It has been shown that wild type HIV-1 from induced both cellular and humoral immune responses in human patients.[207, 208] In addition, wild type HIV-1 was demonstrated to stimulate pDC through TLR 7 that recognize ssRNA in vitro.[209, 210] These data, therefore, makes lentiviral vectors that derived from HIV-1 good vaccine candidates for anti-HIV immunotherapy, and the potential candidates for vaccination against other viral infections.

Cellular immune response plays a key role in the control of HIV-1 infection. The onset CTLs controls viral load during acute infection while HIV-specific CD4+ T cells and CTL help control viremia.[211, 212] In two anti-HIV studies, direct injection of HIV-1 polyepitopes-encoding lentiviral vectors into HLA-A2 or HLA-B7 transgenic mice induced broad CTL response against all 13 epitopes in the HLA-A2 transgenic and 8 out of 12 in the HLA-B7 transgenic mice along with augmented number of IFN-γ producing T cells,[213] and DCs transduced with lentiviral vector overexpressing a codon-optimized simian immunodeficiency virus gag sequence induced expansion of gag-specific T cells in vitro.[214]

Moreover, lentiviral vectors have been employed for the immunotheray studies targeting other types of viral infection. Immunizing mice with lentiviral vector transduced DC protected immunized mice from a lethal dose of lymphocytic choriomeningitis virus challenge,[215] and single step immunization with lentiviral vectors encoding the secreted soluble form of the envelope E-glycoprotein from the highly virulent IS-98-ST1 strain of West Nile virus was efficient to elicite a long-lasting, protective and sterilizing humoral immunity.[216, 217]

In addition to anti-tumor and anti-viral immunotherapy, lentiviral vectors were also employed to control autoimmune diseases. Injection of DCs transduced with lentiviral vectors encoding shRNA against RelB gene suppressed a pre-exist experimental autoimmune myasthenia gravis (EAMG) immune response along with suppressed IFN-

gamma production and increased IL-10 and IL-4 production in vitro and in vivo as well as decreased anti-AChR IgG, IgG1, IgG2b Ab levels in serum.[218]

Other approaches employing lentiviral vectors in immunotherapy include improving antigen processing ability of DCs by transducing lentiviral vector overexpressing supraphysiological level of calnexin,[219] reducing apoptosis of activated immune cells by transuding DCs with lentiviral vectors encoding shRNA against MINOR to prolong their life span,[220, 221] and augmenting co-stimulatory signals on DCs using lentiviral vectors expressing gp34/OX40.[221]

Global Hypothesis and objectives

We hypothesize that different expression levels of CD40 on DC play a pivotal role in determining DC functions towards immunity, tolerance and Th1/Th2 polarization.

Objective 1: Develop a system that allows for quantitively expression of certain molecule

Objective 2: Use CD40 as model molecule in the established system to study the effect of different expression levels of CD40 on DC functions.

Chapter 2 Material and Methods

2.1 Cell lines and animals

Human embryonic kidney cell line, 293T, was cultured in IMDM medium supplemented with 10% fetal bovine serum (FBS) and 1% PSG. OVA MHC class I restricted cell line RF33.70 was cultured in RPMI medium containing 10% FBS, 1%PSG, and 1% non-essential amino acid. OVA MHC class II restricted cell line BO97.10 was cultured in MEM medium containing 10% FBS, 1%PSG, 1% non-essential amino acid and tumor cell cocktail. DC2.4 cell line was cultured in RPMI medium containing 10% FBS, 1%PSG, and 1% non-essential amino acid.

Female C57/B6, BALB/C, and CD40 KO mice (4-6 week old) were purchased from Jackson Laboratory, and were maintained at the central animal facility. OVA transgenic mice, DO11.10, were generously provided by Dr. Xi Yang. All experiments were conducted in accordance with the regulation of the animal facility in the University of Manitoba.

2.2 Bone marrow derived dendritic cell (BMDC) culture

The murine BMDC were generated from bone marrow precursors as previously described.[222] Briefly, bone marrow cells of C57/B6 or Balb/c mice were extracted from the femura and tibiae of these mice. The extracted bone marrow cells were filtered through a cell strainer placed on a petridish, and was collected into a 15ml conical tube. Next, the bone marrow cells were centrifuged at 1200rpm for 5 minutes and were resuspended in 10ml complete RPMI medium supplemented with 1% PSG (Invitrogen),

2-ME, 10% FBS (HyClone). 100ul of bone marrow cells was taken out and the red blood cells were lysed by ACK lysis buffer. Cell counts was performed using trypan blue (Invitrogen) under microscope. The appropriate amount of bone marrow cells were then centrifuged and were resuspened in complete RPMI 1640 (HyClone) medium containing 20ng/ml GM-CSF (PetroTech) at a final concentration of 1x10^6 cells/ml. 500ul/well of bone marrow cells was plated on 24-well plate. Culture medium was changed every 2 days. By D9, above 80% of the cells is DC. In some experiments, LPS (Sigma) was added to the culture at 1μg/ml 18 hours before cells were harvested. For puromycin selection of LKO vectors transduced DC, 4μg/ml of puromycin (Sigma) was added to the tranduced cells and one well of mock transduced cells on D6, and the puromycin containing media was then washed off 24 or 48 hours after selection, depending on the killing of mock tranduced cells.

2.3 Animal immunization and rechallenge

BMDC were pulsed on D7 with OVA or KLH protein at final concentration of 400μg/ml, and were stimulated with 1ug/ml LPS on D8. These DC were then harvested on D9, and were washed twice with PBS and was suspened in 200μl of PBS before injection. For the T cell priming experiments, 1X10^6 BMDC were injected into the footpads of mice, and T cells from immunized animals were collected 7 days after immunization for further experiments. For rechallenge experiments, 1X10^6 OVA protein or KLH protein pulsed LPS matured wide type BMDC were injected into immunized animals via i.v. 28 days after immunization. T cells from rechallenged animals were collected 7 days after rechallenge.

2.4 Lentiviral vector production, concentration and tritration

VSV-G pseudotyped lentiviral vectors were generated through 3-plasmid transfection on 293T cells as previously described.172 Briefly, 17x10^6 293T cells in 20ml of complete IMDM medium were plated on a T175 flask (BD Falcon) on the day before transfection. On the day of transfection, 5ml of complete IMDM medium containing 100ul of 10mM chloroquine (Sigma) was added to the flask before DNA plasmid mix was added to prevent the possible degradation of DNA plasmids in cell lysosome. To make plasmids mix, 12.5ug of 8.2Δvpr, 5ug of pVSV-G, and 12.5ug of gene therapy vector were added to a 50ml conical tube (BD Falcon). Cell culture grade water (HyClone) was added to bring the final volume to 977ul. The mixture was shaken vigorously and was incubated on ice for 10 minutes. Next, 133ul of 2M calcium chloride (Sigmal) was added to the tube drop by drop while the tube was shaken vigorously. The tube was incubated on ice for 5 minutes. Next, 1110ul of HEPES buffered saline containing 1% HEPES w/v, 1.6% NaCl w/v, 0.72% 0.25M Na2HPO4 v/v and 1% 1M KCl v/v (Sigma) was added to the tube drop wisely while the tube was shaken vigorously. The tube was incubated on ice for 20 minutes. After that, the plasmid DNA mixture was added to the roof of T175 flask and was mixed well by the culture medium. The flask was then incubated at CO2 incubator for 8 hours. Next, the plasmid DNA mixture was aspirated out and 40ml of IMDM medium supplemented with 10% calf serum and 1% PSG was added to the flask. 3 days after transfection, the supernatant in the T175 flask was collected into a 50ml conical tube and was centrifuged at 1200rpm for 5 minutes to get rid of cell debris. Next, the supernatant was filtered through using Nelgene filters (Nalge Nunc International).

To concentrate lentiviral vector, the viral supernatant was centrifuged at 17,000rpm for 1.5 hours at 4^{o}C. The supernatant was then decanted and 100ul of IMDM medium containing 10% FBS 1% PSG was added to the pallet of lentiviral vector. The pallet was left at 4^{o}C overnight for it to dissolve. Next day, the pallet was resuspended using the medium left in the ultracentrifuge tube. The lentiviral vector was then aloquated into small volume and was frozen down at -80oC to store.

To titrate the lentiviral vector, 5x10^4 293T cells per well was plated onto a 24-well plate on the day before titration. On the day of titration, the lentiviral vectors were diluted to 1X or 1/10X, respectively using complete IMDM medium. Polybrene (Sigma) was added to the diluted viral vectors to make a final concentration of 8ug/ml. The medium in the wells on titration plate was aspirated, and 250ul of the viral vectors mix was then added to the well. After that, the plate was incubated in CO2 incubator for 2 hours before the medium was changed by removing viral vectors containing medium and replacing with 1ml of fresh complete IMDM medium. 3 days after transduction, the 293T cells were harvested and the expression of EGFP was measured by flow cytometry. The titer was calculated according to the following formula,

Titer = (percentage of $EGFP^{+}$) X 4 X 10^{8}

For LKO vectors, puromycin was added to the transduced 293T cells 1 day after transduction. The transduced cells were harvest 3 days after transduction. Viable cell count and dead cell count were performed by staining cells with trypan blue. The formula of titer is as following,

Titer = (alive cell number/total cell number) X 4 X 10^8

2.5 BMDC transduction

BMDC was transduced by lentiviral vectors at MOI: 10 on D2 culture. Briefly, the old medium along with the floating cells was removed by aspiration. 280ul of the mixture of 100X lentiviral vector (with calculated volume according to the formula below), serum-free medium (plain RPMI medium containing 1% PSG and 2-ME), and polybrene (at final concentration of 8ug/ml) was added to the well before the plate was put back to incubator. 3 hours after transduction, 240ul of the lentiviral mixture was removed from the well, and 460ul of complete medium containing 20ng/ml GM-CSF was added to the well. For transduction using LKO vectors, puromycin was added to the cell culture at a final concentration of 8ug/ml on Day 7 to select transduced cells. On Day 8, the culture medium containing puromycin was centrifuged and the cell pallet was resuspended using fresh cytokine medium. One of the mock transduced well was selected by puromycin as quality control.

2.6 T lymphocyte functional assays

2.6.1 Tritium-based T lymphocyte proliferation assay

T cells were purified using CD90 (Thy1.2) MicroBeads (Miltenyi Biotec). Next, the T cells were washed once and were resuspended using complete RPMI medium. The cell concentration was adjusted to 1X10^6 cells/ml. 1X10^6 or 1X10^5 T cells were then plated on a 24-well plate or 96-well round bottom plate. BMDC stimulator cells were pulsed with chicken ovalbumin (OVA) (Sigma) protein and were stimulated by 1ug/ml

lipidpolysacharide (LPS) (Sigma) on Day 8 culture. These dendritic cells were harvested on D9 and were then centrifuged to wash off cytokine medium before they were resuspended in the complete RPMI medium to make a final concentration of 1X10^5 per ml. The dendritic cells were co-cultured with T cells at a ratio of 1:10 for 3 to 4 days. 16 hours before finishing the co-culture, tritium was added to the DC and T cell co-culture to label the cells.

2.6.2 Caboxyfluorescein Succinimidyl Ester (CFSE)-based T cell proliferation assay

T cells from spleen or draining lymph nodes were purified using CD90 (Thy1.2) MicroBeads (Miltenyi Biotec). These T cells were then centrifuged, and up to 20X10^6 T cells were resuspended in 5ml of PBS. 5mM CFSE was quickly added to the T cells while they were vigorously vortexed. Next, the T cells were let sit at room temperature for 10 minutes. After that, equal volume of calf serum was added to the T cells and the T cells were incubated at 37oC for 5 minutes to quench the reaction. Next, the T cells were washed twice using PBS and was resuspended in complete RMPI medium at a final concentration of 1X10^6 per ml. 1X10^6 or 1X10^5 T cells were then plated on a 24-well plate or 96-well round bottom plate. BMDC as stimulator cells were pulsed with chicken OVA protein and were stimulated by 1ug/ml LPS on Day 8 culture. These dendritic cells were then centrifuged to wash off cytokine medium, and was resuspended in the complete RPMI medium to make a final concentration of 1X10^5 per ml. The dendritic cells were cocultured with T cells at a ratio of 1:10 for 3 to 4 days. T cell proliferation was measured by CFSE dilution using flow cytometre.

2.6.3 Detection of OVA-specific cell lines proliferation by measuring IL-2 production using ELISA

Mock transduced or lentiviral vector transduced BMDC was pulsed with MHC class I-restricted peptide or MHC class II – restricted peptide at 1ug/ml, respectively. Next, these BMDC were cocultured with MHC class I restricted OVA-specific cell line RF33.70 or MHC class II restricted OVA-specific cell line BO97.10 at a ratio of 1:10 for 2 to 3 days. The supernatant of the coculture was collected and the proliferation was measured by IL-2 production by these cell lines.

2.6.4 Mixed lymphocyte reaction (MLR)

Balb/c splenic and lymph node T cells were sorted using CD90 (Thy1.2) MicroBeads (Miltenyi Biotec), and were labeled with CFSE as described previously. Allogeneic C57/B6 BMDC was treated by mitomycin C (15ug/ml) as previously described. (1) 1x10^6 Balb/c T cells and 1x10^5 C57/B6 BMDC were then cocultured on a 24-well plate for 48 hours to 72 hours. T cell proliferation was measured by CFSE dilution using flow cytometre.

2.6.5 in vitro T cell suppression assay

Lentiviral vector 66243, 66244, LKOsiEGFP and mock transduced Balb/c BMDC was pulsed with OVA protein (400ug/ml) and was matured by LPS(1ug/ml). Next, 1X10^6 BMDC was washed twice using PBS before they were injected into the footpad of naïve Balb/c mice. 7 days after immunization, 1X10^6 T cells from draining lymph nodes were purified and were resitmulated by 1X10^5 OVA protein pulsed and LPS matured BMDC

for 72 hours. The restimulated T cells were then added to the coculture of CFSE labeled DO11.10 T cells and wt Balb/c DC pulsed with MHC class II restricted OVA peptide and matured by LPS for 48 hours. The suppression of the proliferation of OVA-transgenic DO11.10 T cells was measured by CFSE dilution using flow cytometre.

2.66 Detection of CD4+CD25+Foxp3+ regulatory T cell

T cells collected from either primed animal or rechallenged animals were stained for CD4+CD25+Foxp3+ regulatory T cells using regulatory T cell staining kit (eBioscience) according to the manufacturer's manual. Briefly, T cells were harvested, and surface staining of CD4 and CD25 was performed according to the surface staining protocol using FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 before the intracellular staining of Foxp3. Next, T cells were washed in cold flow cytometry staining buffer. The pallet was resuspended in 1ml of freshly made fixation/permeabilization buffer, and was incubated at 4oC between 0.5 and 18 hours in the dark. After that, cells were washed 3 times using permeabilization buffer. APC-conjugated anti-mouse Foxp3 was added after the blockade of Fc receptors. Next, cells were washed twice using permeabilization buffer and was resuspended using flow cytometry staining buffer for acquisition.

2.7 Flow cytometry

Anti-CD11c FITC, anti-CD11c PE, anti-CD11c PECy5, anti-CD11c APC, Biotinated anti-CD11c, anti-CD80 PE, anti-CD86 PE, anti-CD40 PE, anti-CD40 PECy5, anti-CD 40 APC, anti-MHC class II PE, anti-CD3 FITC, anti-CD8 PE, anti-CD4 PE, anti-CD4 APC, anti-IL-12 PE were purchased from Biolegend. Samples were collected into the

flow cytometry suitable tubes, and were washed using 1XPBS supplemented with 2% CS and 0.2% sodium azide (Sigma) (FACs buffer). Supernatant was decanted and pallet was resuspended using liquid left over. Next, 1ul of Fc Blocker per test was added to the sample followed by 5-minute incubation at 4oC to minimize non-specific staining. After that, appropriate amount of antibody was added to the sample tube. The sample was mixed well, and was incubated at 4oC for 30 minutes in the dark. Next, sample was washed using 3ml of flow buffer, and was centrifuged at 1200rpm for 5 minutes. The supernatant was decanted and the pallet was resuspended using the buffer left in the tube. Sample now is ready for acquisition using flow cytometry. For samples which are not about to be acquired immediately, 200ul of 2% paraformaldehyde (Sigma) was added to the sample to fix them. Sample will be good for another 2 weeks before being aquired. For intracellular staining, samples were fixed using 2% paraformaldehyde after surface staining has been performed. Next, samples were permeablized by using saponin (Sigma) buffer to wash twice. After permeabilization, the antibody was added to the sample followed by 30-minute incubation at 4 oC in the dark. The sample was then washed with 1X PBS before acquisition.

Data acquisition was performed on either BD FACs Calibur or BD Canto. A minimal of 10,000 cells from the gated population were acquired for each sample. Collected data was analyzed using FACS Express 2.0 (De Novo Software, ON). Histogram and density plots were produced by the FCS Express 2.0 (De Novo Software, ON).

2.8 Apoptosis Assay

0.1X10^6 cells were washed and resuspended using 1X binding buffer (Guava Technologies). 1ul of Annexin V PE (BioVision, Mountain View, CA) and 2ul of 7-amino actinimycin (7-ADD) (Guava Technologies) was added to each cell sample. Data was acquired through Guava Nexin (Guava Technologies), and was analyzed using FACS Express 2.0 (De Novo Software, ON).

2.9 Cytokine production detected by ELISA

Recombinant murine cytokines, anti-cytokine capture mAbs, and biotinylated anti-cytokine detection mAbs were bought from Biolegend. The cytokine profile in the culture supernatant was quantified using matched antibody as described previously (Hoeck, J et al., 2001). Briefly, ELISA plate was coated with appropriate amount of purified antibody overnight. Next, the well was blocked using blocking buffer followed by 2 hour incubation at 37oC. After that, appropriate amount of recombinant cytokine and sample were added to the well with serial dilution. The plate was then incubated at 4oC overnight. After that, appropriate amount of detection antibody was added to the well followed by overnight incubation at 4 ^{o}C. Next, enzyme (alkaline phosphatise) was added to the well, and the plate was incubated at 37 ^{o}C for 3 hours. After that, substrate (PNPP) was added to the well, and the plate was incubated at room temperature till the color was fully developed. The optical density reading was taken through spectral Max 190 using softmax pro software. Cytokine proteins were quantified in reference to serial dilutions of recombinant standards falling within the linear part of the standard curve for each

specific cytokine measured, respectively. Each data point represents readings from a minimum of three independent assays performed in triplicate.

2.10 Detection of anti-KLH or anti-OVA IgG1/IgG2a

Serum from KLH or OVA pulsed DC immunized mice were collected for measurement of IgG1 or IgG2a. Briefly, 10μg/ml KLH or OVA was used for coating plate. Serum from immunized mice was diluted at different ratios staring from 1:100. Biotinylated anti-mouse IgG1 or IgG2a (Pharmingen) were used as detection antibody. Binding was detected by streptavidin-alkaline phosphatase and PNPP substrate (Sigma).

2.11 Detection of IL-12 production after CD40 cross-linking

after being stimulated by LPS for 18 hours, the suspenstion part of DC culture were collected, spun and resuspended in fresh medium before CD40 cross-linking. One of the duplicated well was added medium containing anti-CD40 antibody while the other duplicate was added medium only as control. 16 hours later, the supernatant in the cell culture was collected for ELISA.

Chapter 3 Results

3.1 Lentiviral vector mediated siRNA supports quantitative analysis of a molecule expressed on DC in defining DC function

3.1.1 Bone marrow-derived dendritic culture

Murine bone marrow cells were cultured in complete medium supplemented with 20ng/ml of murine recombinant GM-CSF that in favour of dendritic cell differentiation. After 9 days of culture, we observed that over 90% of the cells in the culture expressed CD11c along with MHC class II molecules (Fig. 1A). These $CD11c^{+}$ cells expressed no (CD40) or low costimulatory molecules (CD86), demonstrating an immature DC phenotype. Upon LPS stimulation, the mean fluorescence intensity of the expression of CD80, CD86, or CD40 was increased 6.65%, 74.11%, and 25.28%, respectively, representing a mature phenotype. The upregulation of costimulatory molecules upon stimulation is consistent with previous reports (Fig. 1B).

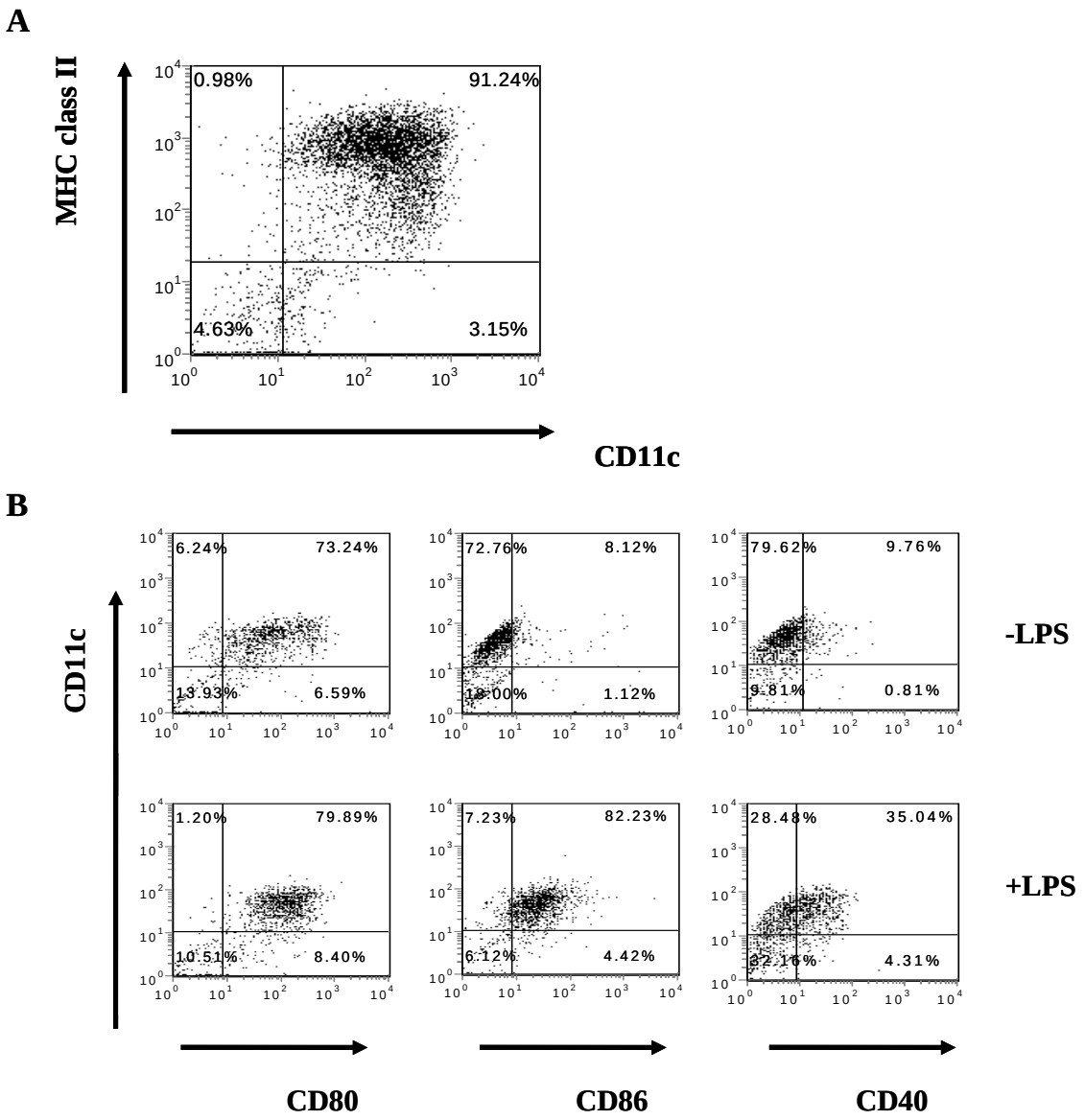


Figure 1: Phenotype of bone marrow-derived dendritic cell. Bone marrow-derived dendritic cells were stimulated with or without LPS on D8, and were harvested on D9. a) Surface staining of CD11c and MHC class II molecule. b) surface expression of co-stimulatory molecules with or without LPS stimulation.

3.1.2 VSV-G pseudotyped lentiviral vector allows stable and efficient gene delivery into primary murine BMDC

To evaluate transduction efficiency, the DC precursor in the bone marrow cells were transduced on day 2 with cppt2e, a VSV-G pseudotype replication incompetent lentiviral vector that overexpresses enhanced green fluorescence protein (EGFP) reporter gene, at a MOI of 10 IU/cell as previously described. The transduction efficiency was determined by EGFP expression, which was measured by flow cytometry 7 days post-transduction when the BMDC is fully differentiated. Here we demonstrated a transduction efficiency of over 80% of $CD11c^+$ murine BMDC (Fig. 2A). The transduced group had similar percentage of $CD11c^+$ population as compared with mock transduced group. Moreover, the lentiviral vector conferred a stable gene delivery into murine BMDC for 7 days.

We next examined whether this system can be used to deliver other transgene such as siRNA than EGFP into murine BMDC. Lentiviral vector FG12hisiCD40#4 that overexpresses shRNA sequence specifically against CD40 or FG12h1siluc that overexpressese shRNA sequence specifically targeting luciferase was used to transduce BMDC to evaluate the transduction capacity and efficiency as described above. We observed that over 80% of the $CD11c^+$ cells were transduced by FG12h1siluc or FG12siCD40#4 (Fig. 2B).

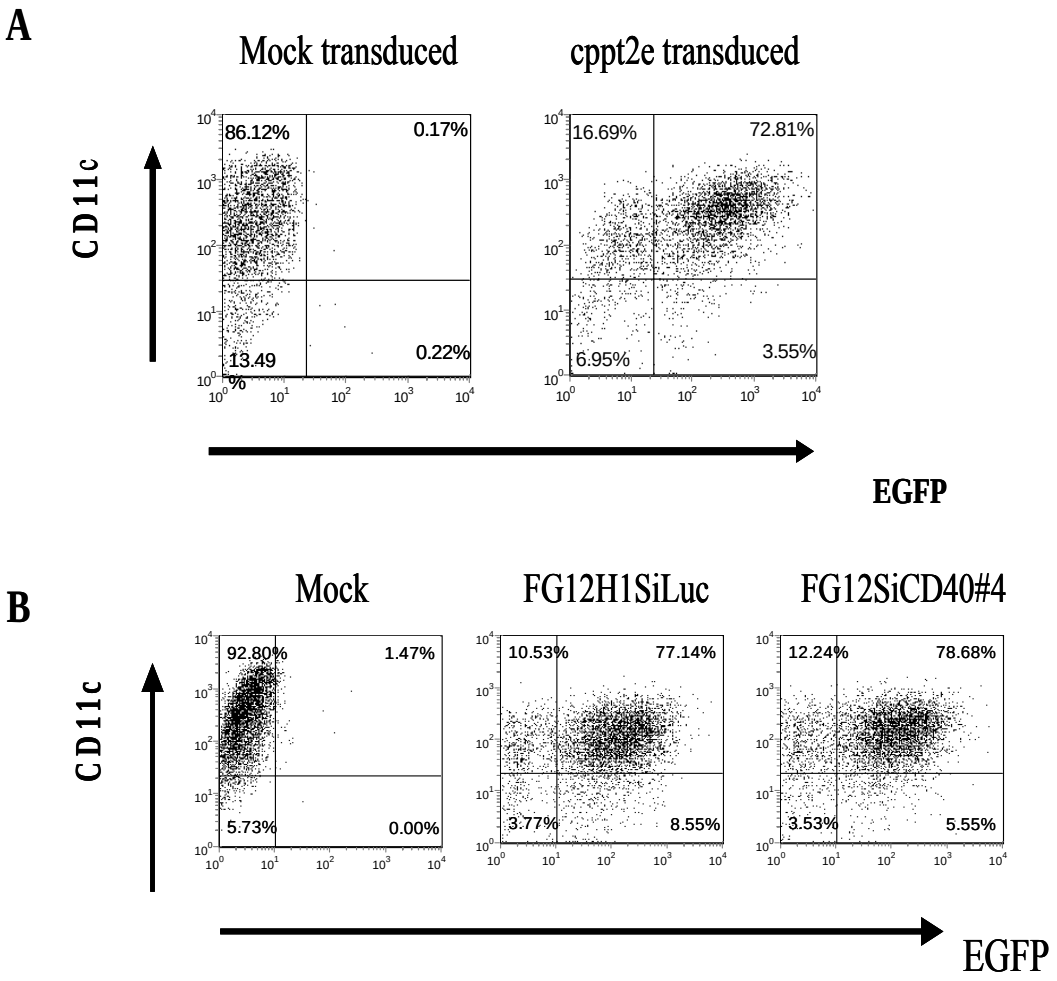


Figure 2: VSV-G pseudotyped lentiviral vectors allowed stable and efficient gene delivery in primary murine BMDC. BMDC were transduced with pseudotype lentiviral vectors on D2 culture. Mock transduced cells were used as negative control. Transduction efficiency was measured by EGFP expression. a) BMDC transduced with EGFP expressing vector, cppt2e. b) BMDC transduced with EGFP vectors encoding shRNA.

3.1.3 Lentiviral transduction does not induce spontaneous maturation or functional impairment

Although the employment of lentiviral vector to overexpress certain protein in DC has been well documented, the introduction of shRNA into DC generates concerns as the shRNA delivered by the lentiviral vectors might be recognized by toll like receptors such as TLR3, TLR7, TLR8, and TLR9 on transduced DC through pattern recognition mechanism, which in turn will cause spontaneous maturation or functional alteration of transduced DC. However, it has also been suggested TLR mediated RNA immunostimulation is dependent on the type of cell infected as well as the RNA length, sequence, and form existing in the infected cell. Therefore, we examined the specificity of lentiviral vector mediated gene silencing, and the potential of immunostimulation and functional alternation that could be induced by lentiviral transduction.

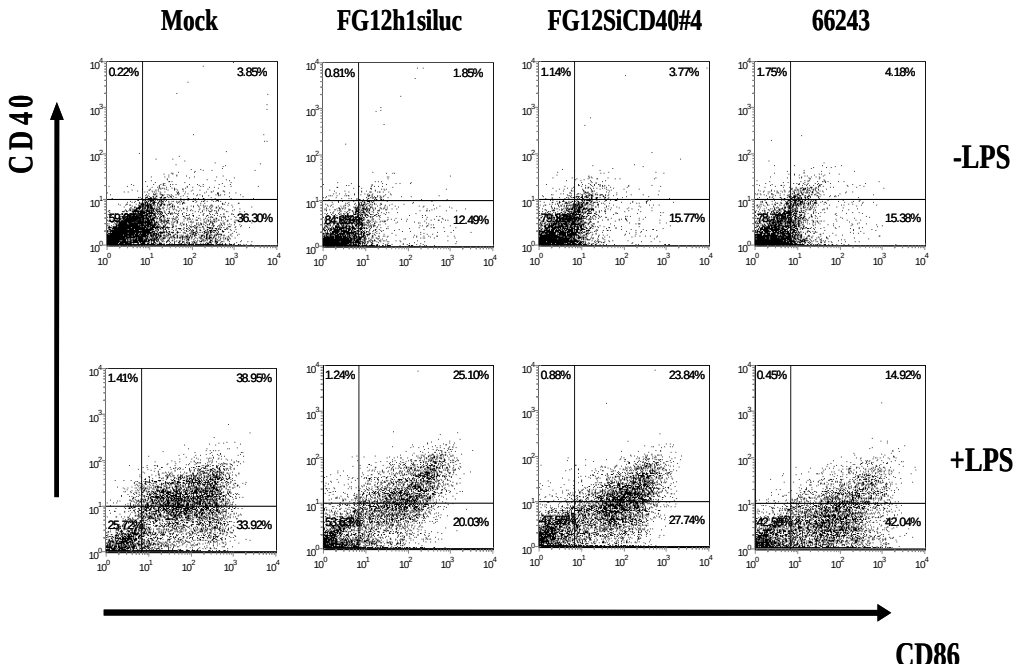
To test the specificity of gene silencing induced by lentiviral vectors, BMDC was transduced with FG12h1siluc (irrelevant target specificity control), FG12siCD40, or 66243, respectively. Surface expression of CD40 and another costimulatory molecule (CD86) on these transduced DC were detected by flow cytometry (Fig. 3A). We did not observe CD40 expression on neither transduced DC group nor mock transduced group. Upon LPS stimulation, FG12hisiluc transduced cell had comparable MFI of CD40 as compared with mock transduced DC, while FG12siCD40 and 66243 transduced DC had 30% or 72% lower MFI of CD40 respectively as compared with mock transduced or FG12h1siluc transduced DC. Meanwhile, the surface expression of CD86 on all transduced DC was comparable to mock transduced DC, suggesting that the gene

silencing was specific to CD40, and the lentiviral transduction itself did not mature DC phenotypically.

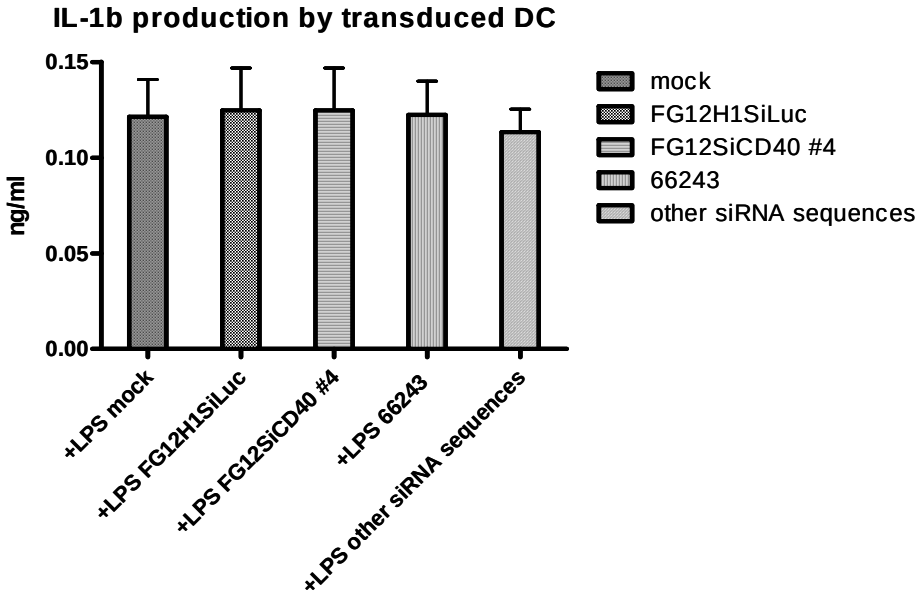
Besides upregulating surface expression of costimulatory molecules upon stimulation to provide second signal for naïve T cell activation, the major functions of dendritic cells also include presenting antigen to prime antigen-specific T cells and producing proinflammatory cytokines such as IL-12, IL-1β. We therefore transduced BMDC with a panel of lentiviral vectors that express shRNA sequence targeting different genes such as luciferase, CD80, CD40 and CD86 to examine whether there is any alteration in the cytokine production in transduced DC and their ability to prime antigen-specific T cell. By ELISA of the supernatant of transduced DC culture, we did not observe any change in IL-1β production (Fig 3B). Both mock transduced DC and shRNA transduced DC secreted comparable amount of IL-1β. Meanwhile, intracellular staining of IL-12 showed no difference in both mock transduced group and shRNA transduced group (Fig. 3C).

We also examined the apoptosis of shRNA transduced DC to determine whether lentiviral transduction will cause undesired effect on DC differentiation. D5 DC were stained with annexin V-PE and 7-ADD. The percentage of cell undergo apoptosis was determined by the percentage of annexin V single positive population on flow cytometry (Fig. 4). We did not observe any significant difference in the percentage of apoptosed cell between transduced DC group and mock transduced group.

A


Mock
FG12h1siluc
FG12SiCD40#4
66243
CD40
-LPS
+LPS
CD86
0.22%
3.85%
36.30%
0.81%
1.85%
12.49%
1.14%
3.77%
15.77%
1.75%
4.18%
15.38%
1.41%
38.95%
33.92%
1.24%
25.10%
20.03%
0.88%
23.84%
27.74%
0.45%
14.92%
42.04%

B


IL-1b production by transduced DC
ng/ml
0.15
0.10
0.05
0.00
+LPS mock
+LPS FG12H1SiLuc
+LPS FG12SiCD40 #4
+LPS 66243
+LPS other siRNA sequences
mock
FG12H1SiLuc
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other siRNA sequences

C

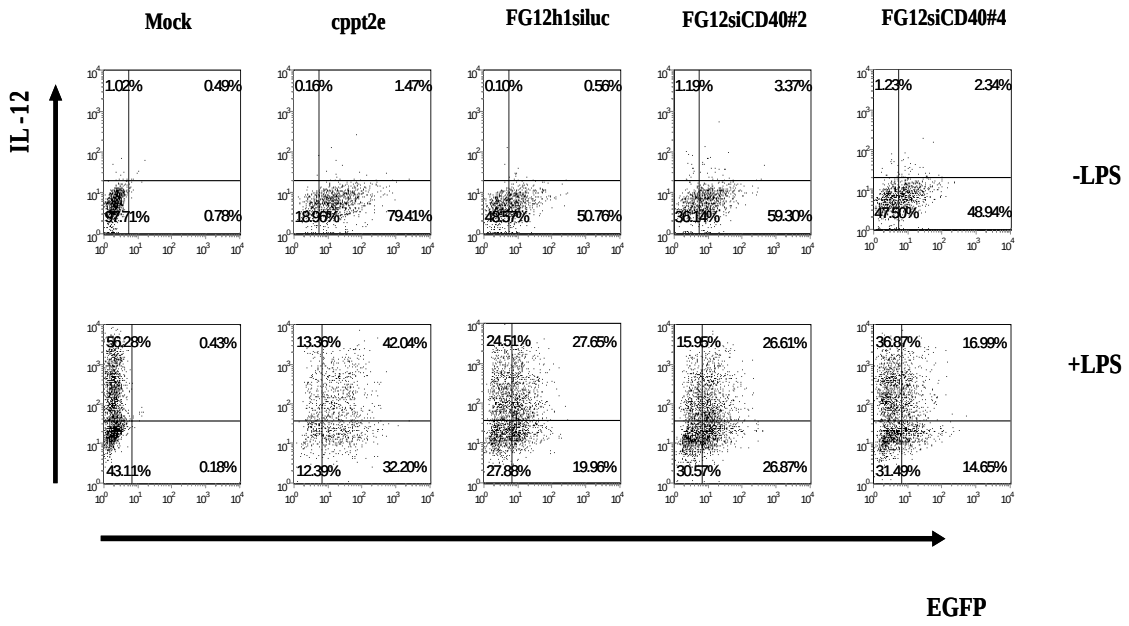


Figure 3: Lentiviral transduction did not induce spontaneous maturation or functional impairment. a) BMDC was transduced with EGFP vectors encoding shRNA specifically against CD40. FG12h1siluc was used as vector control and irrelevant siRNA control. Surface expression of CD40 and another co-stimulatory molecule, CD86, before or after LPS stimulation was detected by flow cytometry. b) IL-1β production in the supernatant of BMDC culture was detected by ELISA. c) Intracellular staining of IL-12 production in BMDC before or after LPS stimulation.

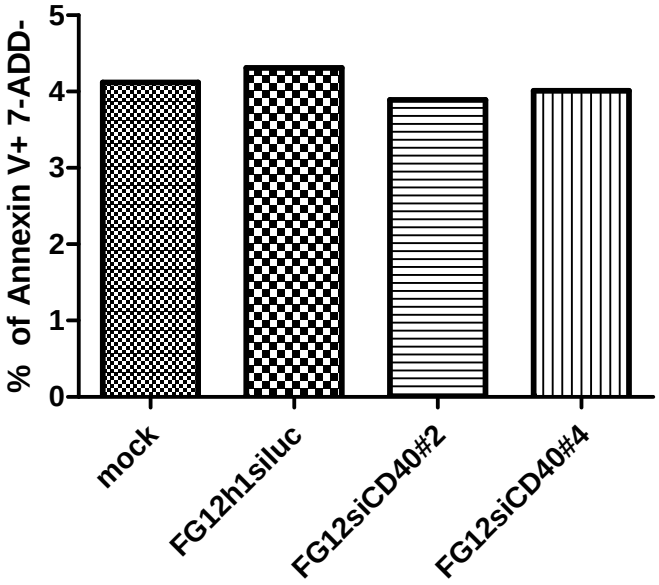


Figure 4: Lentiviral transduction did not induce spontaneous apoptosis. D2 BMDC were transduced with lentiviral vectors encoding shRNA sequences. Apoptosis assay were performed on D7 DC culture. The percentage of apoptosis was determined by the percentage of the Annexin $V^{+}7\text{-}ADD^{-}$ population.

Next, we examined the ability of lentiviral transduced DC to present allo-antigen. It has been previously reported that CD40 costimulatory signalling was essential in the activation of naïve allogeneic T cells in vivo but not in vitro (Hasse et al 2004). Therefore, the induction of allogeneic immune response in vitro should be irrelevant to the downregulation of CD40 on DC. We used 66243 vector to further examine the functional ability of transduced DC in the induction of allogeneic immune response in vitro. 66243 and FG12siCD40#4 lentiviral vectors co-transduced C57/BL6 DC were co-cultured with CFSE labelled allogeneic Balb/c T cells for 2 days. Mock transduced DC and $CD40^{-/-}$ DC were used as. The proliferation of allogeneic Balb/c T cells was determined by CFSE dilution measured by flow cytometry (Fig. 5). Consistent with previous report, $CD40^{-/-}$ DC was able to induce allogeneic immune response. 66243 and FG12siCD40#4 co-transduced DC were able to induce allogeneic immune response as well as mock transduced, $CD40^{-/-}$, suggesting that lenviral transduced DC remained their antigen presenting ability intact.

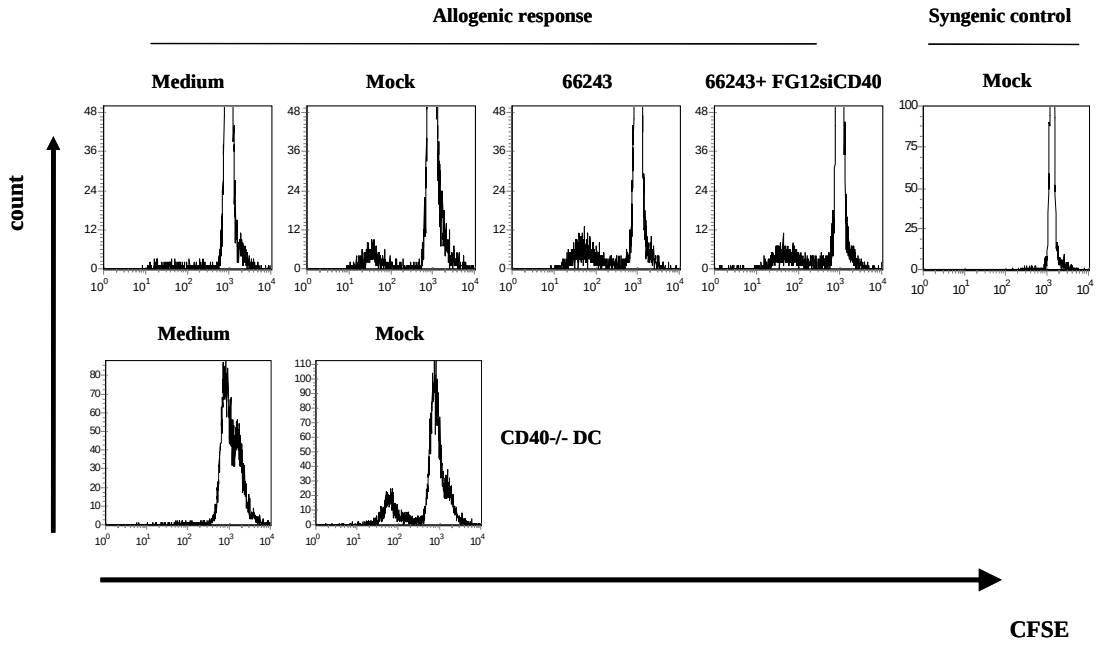


Figure 5: Lentiviral transduction did not impair the ability of DC to present allogeneic antigen. C57 BMDC transduced with 66243 or a combination of 66243 and FG12siCD40#4 were co-cultured with CFSE labelled allogeneic Balb/c T cells for 3 days. T cell proliferation were measured by CFSE dilution. wt C57 T cells were used as syngenic control. C57 $CD40^{-/-}$ DC was used as positive control.

3.2 Quantitative Expression of CD40 on Mature DC Revealed a Critical Threshold that Defines DC Functional

3.2.1 Generation of BMDC Expressing Different Levels of CD40

There is a notion that the potency of silencing effect on target mRNA is sequence dependent. Therefore, using different sequences of siRNA targeting the same mRNA alone or in combination in our lentiviral vector system would allow us to manipulate the expression levels of the target molecules in a stable and reproducible way, which hence will allow us to study the effect of different expression levels of co-stimulatory molecules on DC functions. To that end, we used CD40, the costimulatory molecule expressed on DC, as our model molecule to test whether we could regulate CD40 expression level in this way. We used 66243 or 66244 (lentiviral vectors that contain shRNA sequences against CD40 mRNA specifically) in the established transduction protocol to generate DC that express medium level of CD40 ($CD40^{med}$) and no/low level of CD40 ($CD40^{low}$). The MOI of $CD40^{med}$ DCand $CD40^{low}$ DC is 15.78 and 6.41, respectively, while the MOI of CD40hi DC is 28.17 (Fig. 6).

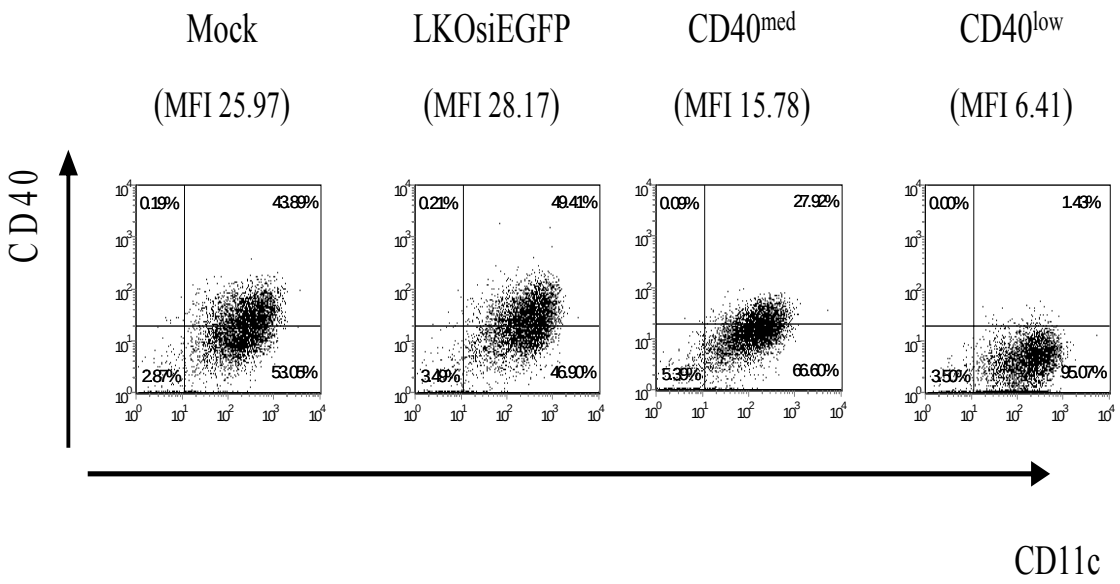


Figure 6: Generation of DC expressing different levels of CD40 D2 BMDC were transduced with different lentiviral vectors encoding different shRNA sequences that specifically target different regions of CD40 mRNA. LKOsiEGFP was used as vector and shRNA sequence control. The CD40 expression was detected by flow cytometry 7 days post transduction,

3.2.2 Downregulation of CD40 expression was specific

To further confirm that the downregulation of CD40 is specific, we performed surface staining of other costimulatory molecules known to be expressed on DC, such as CD80/86, B7-DC, B7-H3, and B7-RP1. We found that only CD40 but not other costimulatory molecules or MHC molecules were downregulated compared with mock transduced DC or LKOsiEGFP transduced DC (Fig. 7).

To examine whether these DC with down-regulated CD40 still remain their ability of presenting antigen to T cells, we next examined the antigen presentation ability of $CD40^{med}$ or $CD40^{low}$ DC using OVA-specific MHC class I and MHC class II cell lines, RF33.70 and BO97.10. $CD40^{med}$ or $CD40^{low}$ DC were co-cultured with RF33.70 or BO97.10 in the presence or absence of MHC class I-restricted peptide or MHC class II-restricted OVA peptide for 3 days. Antigen-specific T cell response was measured by IL-2 production using ELISA. We found no difference in IL-2 production by $CD40^{med}$ or $CD40^{low}$ DC primed hybridoma cell as compared with those primed by mock transduced or LKOsiEEGFP transduced DC (Fig. 8), indicating that the antigen presenting ability of the CD40 shRNA transduced DC is intact.

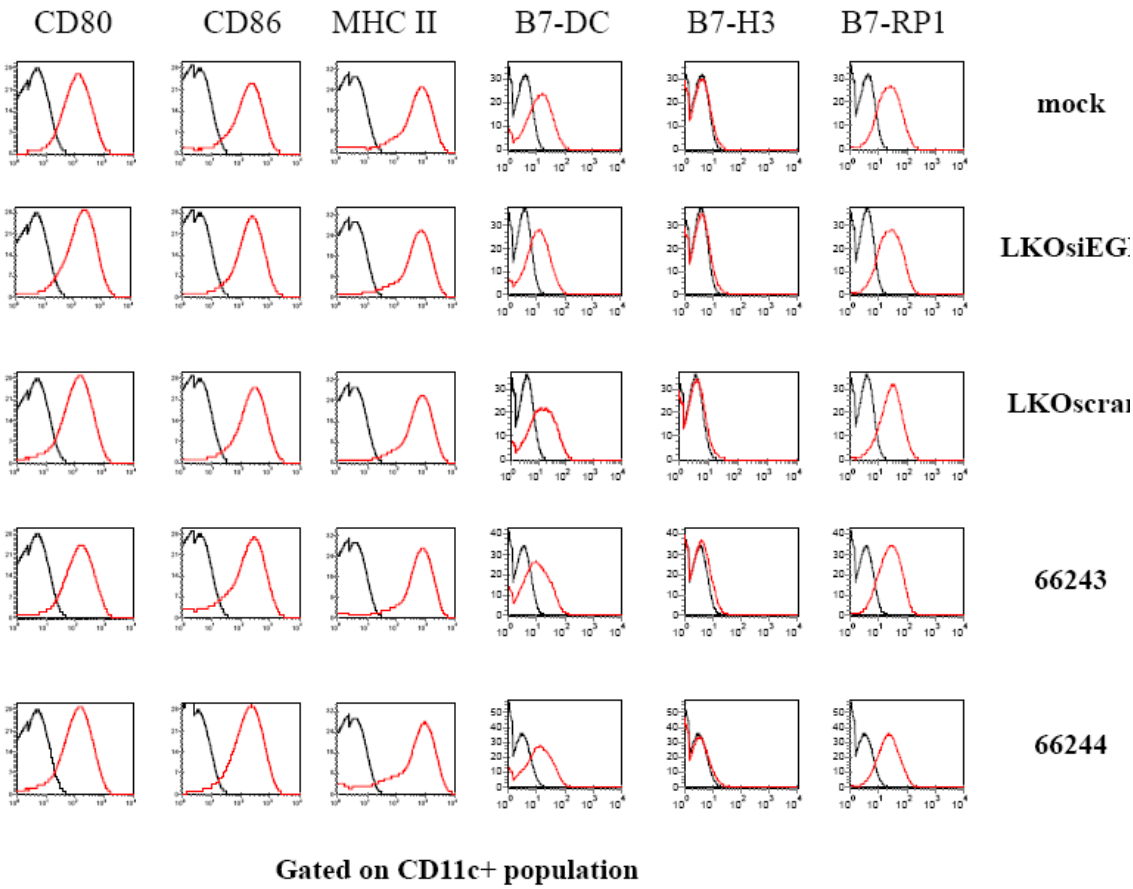


Figure7: Down-regulation of CD40 was specific. Surface staining of co-stimulatory molecules expressed on 66243 and 66244 transduced DC. Mock transduced DC were used as control, LKOsiEGFP and LKOscramble were used as vector and shRNA sequence control.

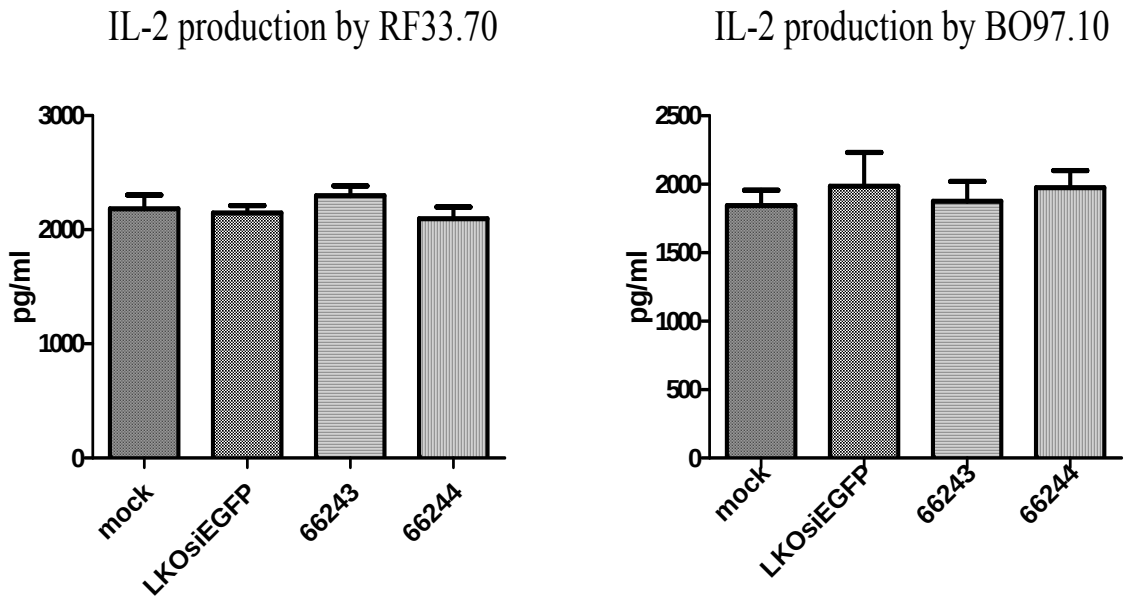


Figure 8: Antigen presenting ability of DC with down-regulated CD40 remained intact. Transduced DC were co-cultured with OVA-specific MHC I or II restricted cell line, RF33.70 and BO97.10, for 3 days in the presence of OVA257-264 or OVA323-339. IL-2 in the supernatant was measured by ELISA. (n=3)

3.2.3 Down-regulation of CD40 impaired OVA-specific CD4 T cell priming in vitro

We have demonstrated previously that down-regulation of CD40 using our protocol did not impair allogeneic T cell priming in vitro. Next, we examined whether down-regulation of CD40 would affect antigen-specific T cell in vitro. $CD40^{med}$ or $CD40^{low}$ Balb/c DC were co-cultured with CFSE labelled DO11.10 T cells in the presence of MHC class II-restricted OVA peptide II or MHC class I-restricted OVA peptide I (as control peptide) in order to test whether their ability to prime antigen-specific T cells remain intact. By measuring CFSE dilution on flow cytometry, we found that both $CD40^{med}$ and $CD40^{low}$ DC impaired OVA-specific T cell priming (Fig. 9). And the extent of impairment was correlated with the expression of CD40 on DC. The proliferation of $CD40^{med}$ DC primed T cell decreased 85% when compared with $CD40^{hi}$ DC primed T cells. The proliferation of $CD40^{low}$ DC primed T cells decreased 92% when compared with $CD40^{hi}$ DC primed T cells.

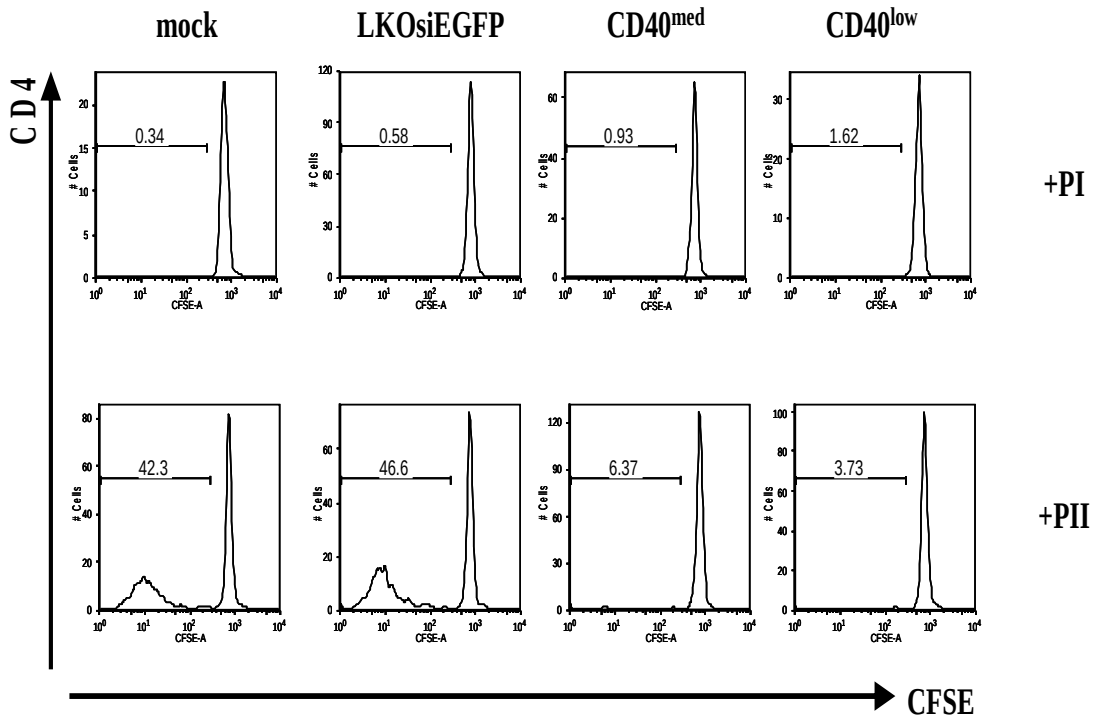


Figure 9: Down-regulation of CD40 impaired OVA-specific CD4 T cell priming in vitro. CD40med and CD40low DC were co-cultured with CFSE labelled CD4 T cells from OVA-transgenic DO11.10 mice in the presence of OVA323-339 for 3 days. OVA257-264 was used as antigen specificity control. LKOsiEGFP was used as vector and shRNA specificity control. DO11.10 T cell proliferation was measured by CFSE dilution on flow cytometry.

3.2.4 Impaired OVA-transgenic CD4 T Cell Priming Was Caused by Apoptosis

To understand the mechanism underlying the impaired T cell priming by $CD40^{med}$ or $CD40^{low}$ DC, we performed apoptosis test on the DO11.10 T cells primed by $CD40^{med}$ and $CD40^{low}$ DC as described in previous chapter. Briefly $CD40^{med}$ or $CD40^{low}$ DC were co-cultured with DO11.10 T cells in the presence of MHC class II-restricted OVA peptide for 3 days. Mock transduced or LKOsiEGFP transduced DC primed T cells served as control. Primed T cells were stained with Annexin V and 7-ADD. The percentage of apoptosis cell was determined by the percentage of Annexin V^+ 7-ADD^- population as detected by flow cytometry. We found that CD40low DC primed T cells had 2-fold increase in apoptosis compared with other groups, suggesting that apoptosis was one of the mechanism that caused impaired T cell priming (Fig. 10).

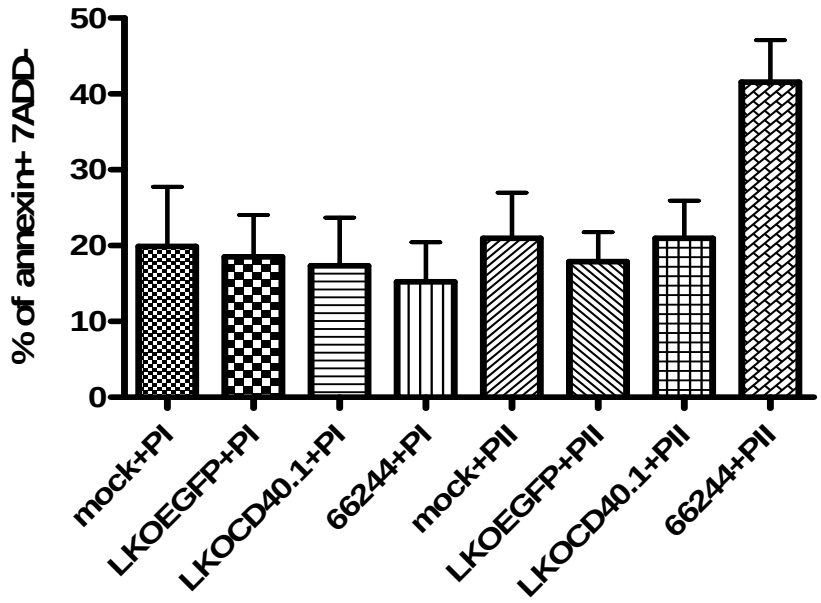


Figure 10: in vitro apoptosis assay of DO11.10 T cells primed by DC with down-regulated CD40. Balb/cDC transduced with mock, LKOsiEGFP, 66243, and 66244 were pulsed with MHC I-restricted OVA257-264 and MHC II-restricted OVA323-339, and were co-cultured with DO11.10 T cells for 3 days. T cells in the co-culture were stained with Annexin V and 7-ADD. The apoptosis population was defined as Annexin V^+ 7-ADD^-. (n=3)

3.2.5 Down-regulation of CD40 on DC impaired T cell priming in vivo

We have shown previously that down-regulation of CD40 on DC impaired OVA transgenic CD4 T cell priming in vitro. To further understand the effect of downregulation of CD40 on DC, we tested the ability of $CD40^{med}$ or $CD40^{low}$ DC to prime antigen-specific T cell response in a polyclonal setting. 66243 or 66244 transduced C57BL/6 DC were pulsed with OVA protein and were matured by LPS. These cells were then adoptively transferred into naïve C57BL/6 mice. Mock transduced DC were used as positive control, while LKOsiEGFP transduced DC were used as vector control and irrelevant RNA control. 7 days later, the T cells from immunized mice were separated and were analyzed for OVA-specific immune response in a CFSE-based proliferation assay where wild type LPS matured DC pulsed with OVA protein antigen was used as stimulator. Consistent with in vitro data, we found both $CD40^{med}$ and $CD40^{low}$ DC primed T cells showed impaired proliferation upon restimulation, even other co-stimulatory molecules on these DC remained intact. The proliferation of $CD40^{med}$ DC decreased 82.4% when compared with $CD40^{hi}$ DC, and the proliferation of CD40low DC decreased 94% when compared with $CD40^{hi}$ DC (Fig. 11).

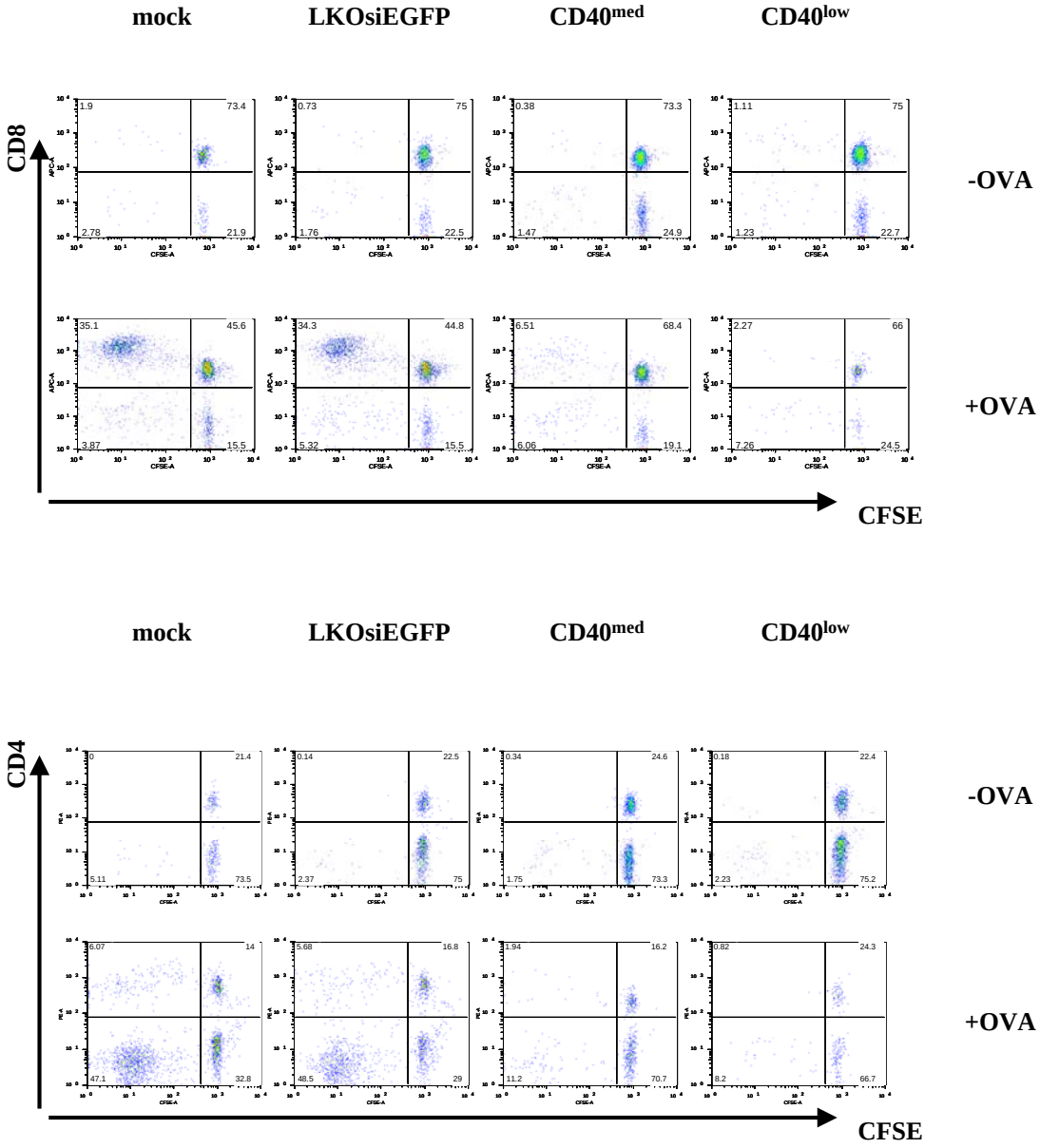
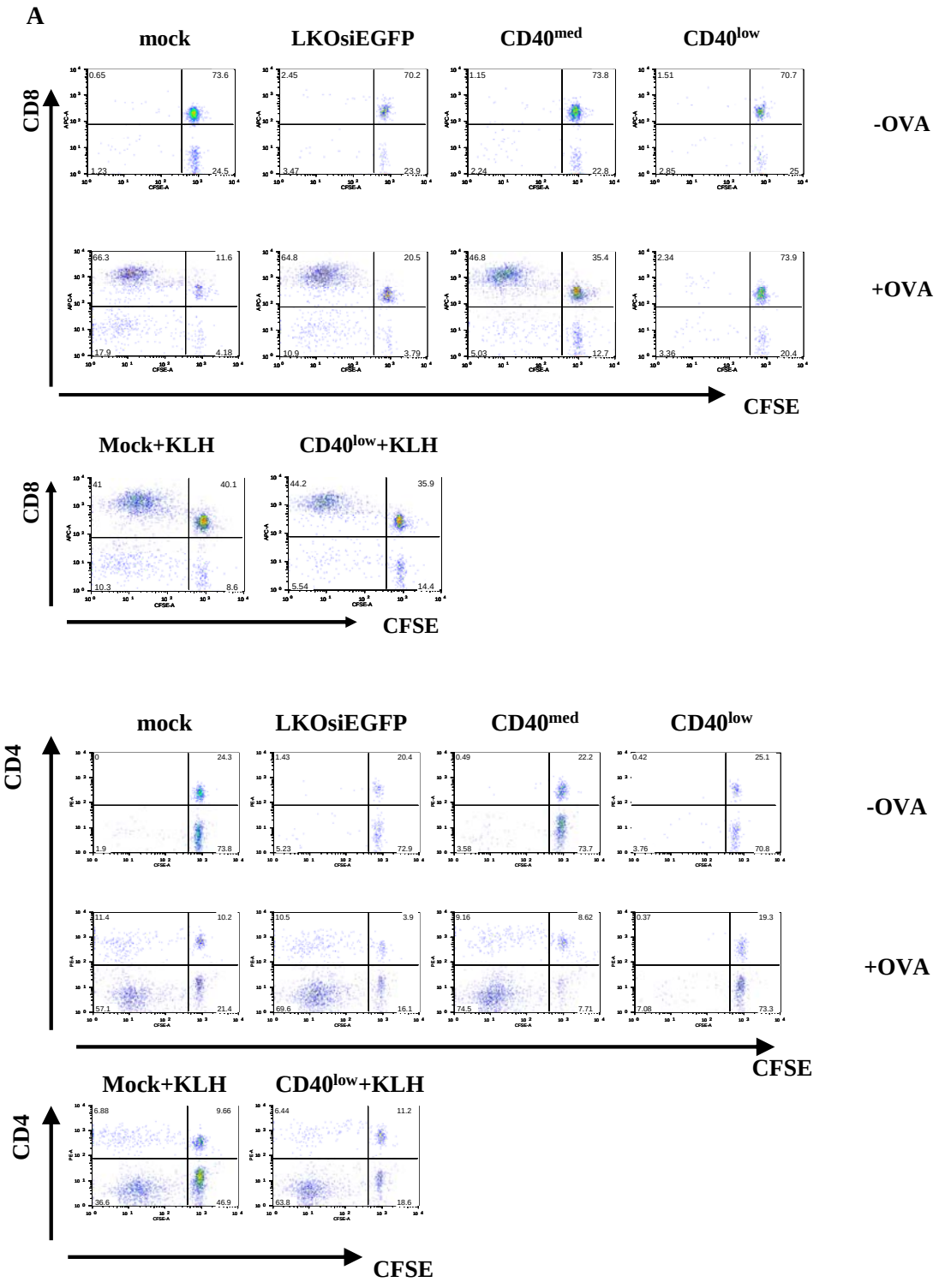


Figure 11: Down-regulation of CD40 impaired OVA-specific T cell priming in vivo. OVA antigen protein pulsed LPS stimulated CD40med or CD40low DC were injected into the foodpad of naïve animals. 7 days after immuniztion, T cells from draining LN were labelled with CFSE and co-cultured with OVA-pulsed wide type DC for 4 days. Mock transduced DC were used as positive control while LKOsiEGFP transduced DC were used as specificity control. T cells proliferation was measured by CFSE dilution on flow cytometry.

3.2.6 Only CD40^{low} DC Induced Antigen-specific Tolerance

It has been previously reported that CD40$^{-/-}$ DC is able to induce immunological tolerance to the antigen it is previously exposed to. To further understand whether the impairment of T cell priming by CD40 downregulated DC was induced by ignore or was induced by immunological tolerance, we rechallenged immunized mice with wild type, OVA pulsed mature DC 28 days after immunization. KLH pulsed DC were used as antigen specificity control. T cells from rechallenged mice were collected 5 days after rechallenge, and were analyzed for OVA-specific immune response in a CFSE based proliferation assay. As expected, the mock transduced DC immunized mice proliferated upon being rechallenged by the same antigen (Fig. 12A). There is no T cells proliferation without the presence of OVA protein. Moreover, T cells from KLH primed DC did not show proliferation upon OVA restimulation. Only CD40^{low} DC primed T cells failed to proliferation upon rechallenge. The CD40^{med} DC primed T cells showed the same proliferation as CD40^{hi} DC primed T cells did (Fig. 12A). Moreover, we observed proliferation of CD40^{low} DC primed T cells rechallenged with KLH upon KLH restimulation, indicating that the immunological tolerance induced by CD40^{low} DC is antigen-specific (Fig. 12B). Interestingly, we observed more CD8 T cells proliferation than that of CD4 T cells in this system.

Together, these data suggest that the CD40^{low} DC is able to induce antigen-specific tolerance. CD40^{med} DC was able to impair, however, failed to induce immunological tolerance.


A
mock
LKOsiEGFP
CD40med
CD40low
CD8
-OVA
+OVA
CFSE
Mock+KLH
CD40low+KLH
CD8
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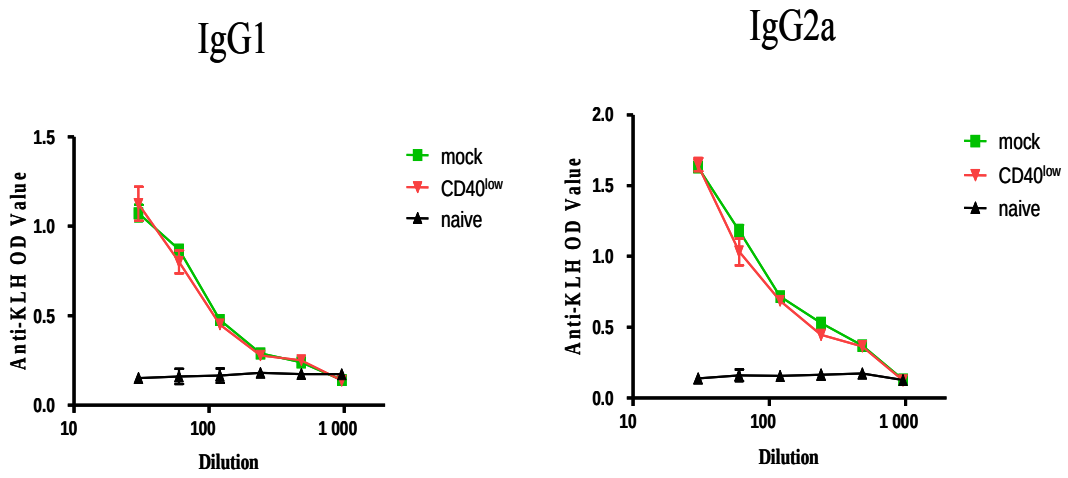

Figure 12: Only CD40^{low} DC induced OVA-specific immunological tolerance. A) Mice immunized by OVA-pulsed LPS-matured CD40med or CD40low DC were rechalleged with wide type DC pulsed with OVA protein antigen 28 days after priming. T cells from rechallenged mice were collected 7 days after rechallenge and were labelled with CFSE before they were co-cultured with OVA-pulsed wt DC. Mock transduced DC or 66244 transduced DC immunized mice were rechallegened with KLH protein antigen-pulsed wt DC as antigen specificity control. B) IgG1 and IgG2a in the serum were measured by ELISA. (n=3)

3.2.7 Regulatory T cells are involved in the induction of immunological tolerance by $CD40^{low}$ DC

Previous reports have shown that T cells with regulatory function are responsible for peripheral tolerance. To understand the mechanism by which $CD40^{low}$ DC was able to induce immunological tolerance, we examined whether the $CD40^{low}$ DC primed T cells can suppress T cell proliferation in vitro. T cells from immunized animals were collected and were restimulated with wt DC pulsed with OVA protein. These T cells were then added to a co-culture of wt DC and DO11.10 T cells at a ratio of 1:1 to test their suppressing ability. We observed only 10% OVA transgenic T cells proliferation in the presence of $CD40^{low}$ DC primed T cells while the control group had 60% of OVA transgenic T cells proliferated. There are 40% of the OVA-transgenic T cells proliferated in the presence of CD40med DC primed T cells which is slightly weaker than that of the group with the presence of LKOsiEGFP primed T cells,.

$CD4^{+}CD25^{+}Foxp3^{+}$ Regulatory T cells have been reported in many works for their regulatory function. To test if the $CD4^{+}CD25^{+}Foxp3^{+}$ Regulatory T cells were involved in the tolerance induced by $CD40^{low}$ DC, we preformed CD4+CD25+Foxp3+ regulatory T cells staining on the T cells from rechallenged mice. We observed that there were 17.84% of CD4+CD25+Foxp3+ regulatory T cells among $CD40^{low}$ DC primed T cells, which is one fold increase when compared with $CD40^{hi}$ or $CD40^{med}$ DC primed T cells (Fig. 13B), indicating that $CD4^{+}CD25^{+}Foxp3^{+}$ Regulatory T cells was involved in the mechanism underlying the tolerance induced.

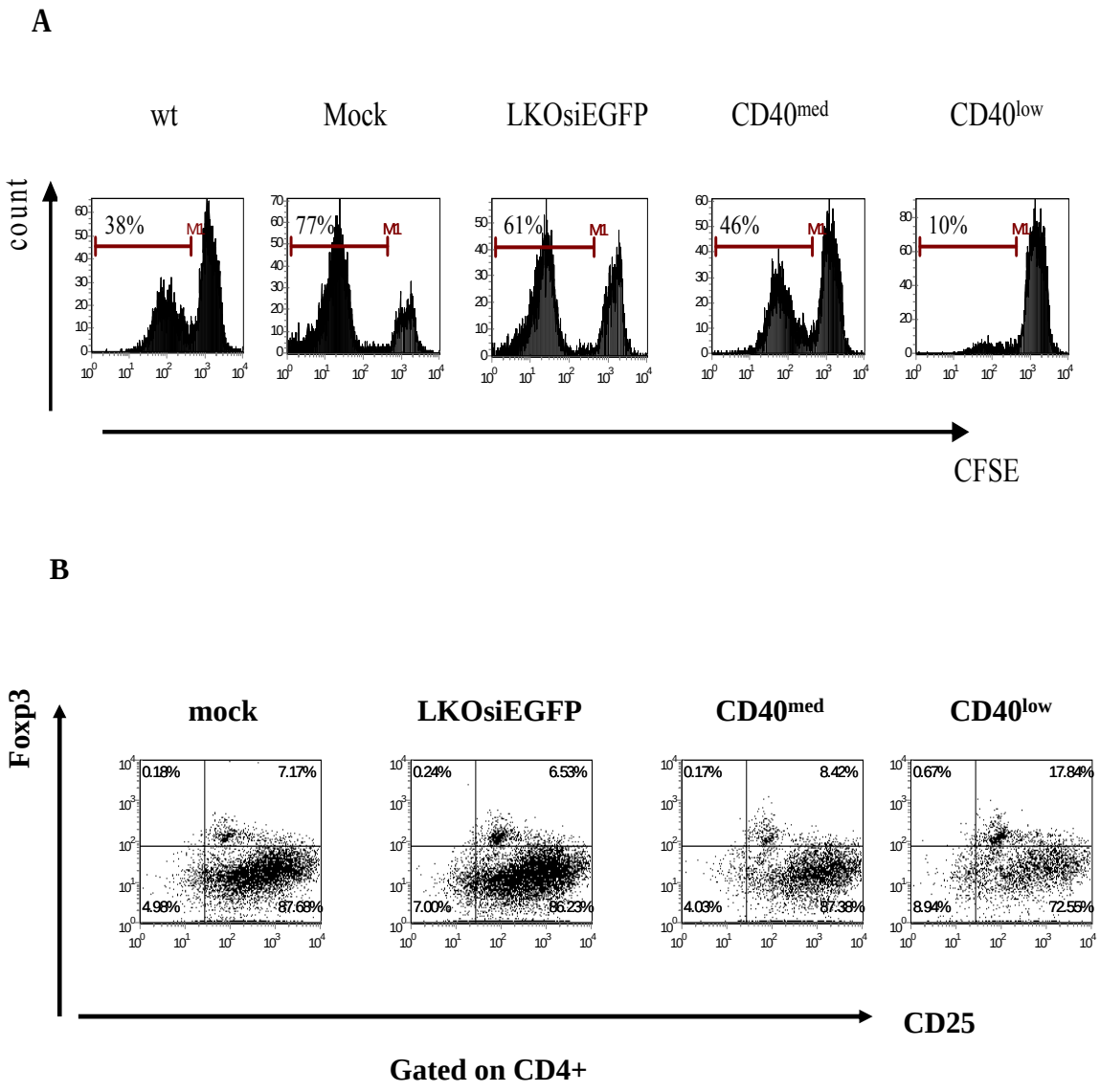

Figure 13: Regulatory T cells were induced in the immunological tolerance induced by CD40^{low} DC. A) in vitro suppression assay. T cells from dLN of CD40med or CD40low DC immunized animals were collected and were added into the co-culture of wt DC and DO11.10 T cells in the presence of OVA323-339. B) CD4+CD25+Foxp3+ regulatory T cells staining of T cells primed by DC with different CD40 levels.

3.2.8 CD40^{med} DC skewed T cell differentiation towards Th2 phenotype

Both CD40^{med} and CD40^{low} DC impaired antigen-specific T cell proliferation in vivo, but only CD40low DC induced immunological tolerance upon rechallenge. Therefore, it is of interest to understand whether or not CD40^{med} DC has its own different functional property than CD40^{low} DC or CD40^{hi} DC. Cytokine test of the supernatant of the cocultured T cells revealed that CD40^{med} DC primed T cells presented a different cytokine production pattern compared with both CD40^{hi} and CD40^{low} DC. CD40^{med} DC primed T cell produced one-third fold lower IFN-γ, but four folds IL-4 and IL-13 when compared with those primed by CD40^{high} DC (Fig. 14), suggesting a Th2 cytokine production phenotype. Moreover, we did not observe significant difference in IL-10 production.

The ELISA data from rechallenged mice showed similar results (Fig. 15A). T cells from rechalleged mice were restimulated with OVA protein antigen for 4 days. The IFN-γ, IL-4, IL-10 and IL-13 production in the supernatant were measured by ELISA. CD40med DC produced one-third fold of IFN-γ, but 2.5 folds of IL-4, and 4 folds of IL-13 when compared with CD40^{hi} DC primed T cells. Also, we found more OVA-specific IgG1 and less OVA-specific IgG2a in CD40^{med} DC immunized mice than that of the animals immunized by CD40^{high} DC while both IgG1 and IgG2a level remained at basal level in CD40^{low} DC immunized mice (Fig. 15B).

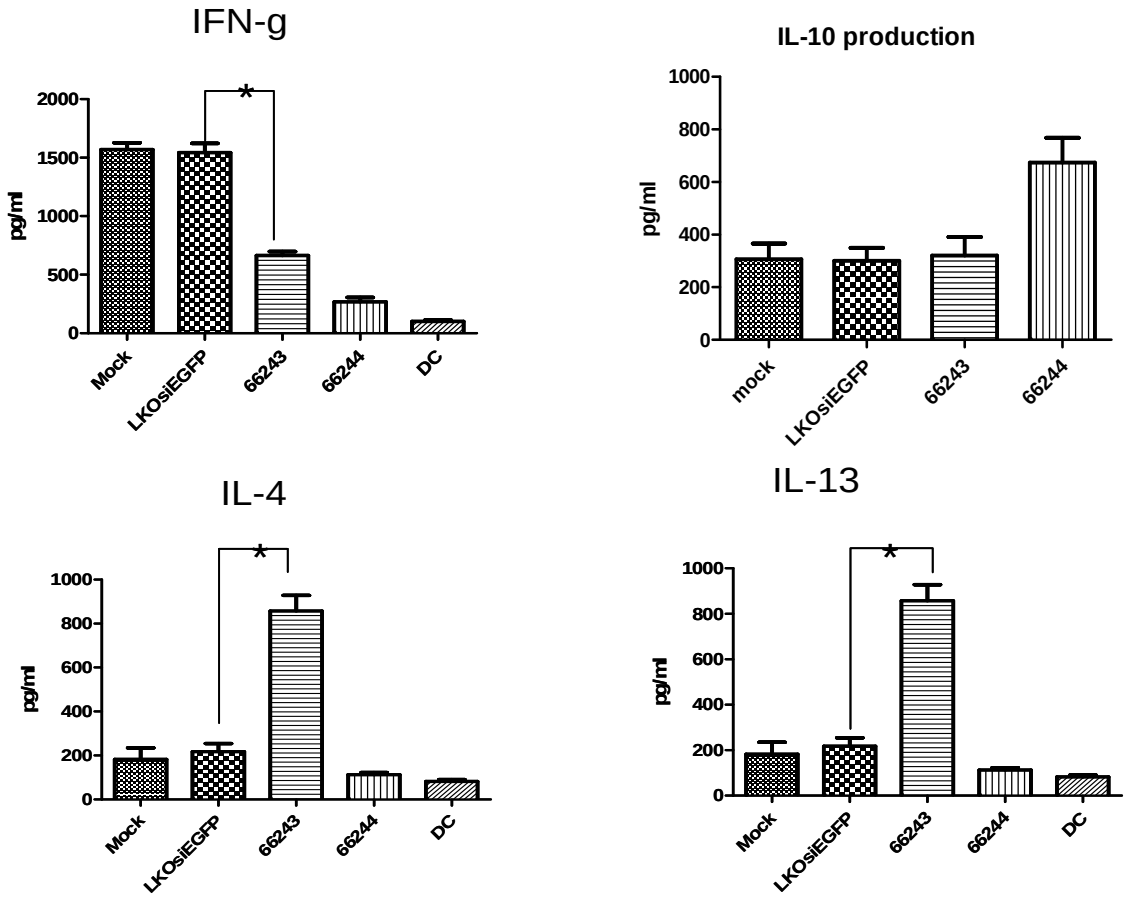

Figure 14: Cytokine profile of T cells primed by DC with different expressing levels of CD40. C57BL/6 DC were transduced with mock, LKOsiEGFP, 66243 and 66244, and were then pulsed with OVA protein. 1x10^6 cells were injected into the foodpand of naïve C57 mice after LPS maturation of these DC. 7 days after immunization, T cells from dLN were collected and were restimulated by wt DC with OVA antigen. Cytokine production in the supernatant (IFN-γ, IL-4, IL-10, IL-13) was measured by ELISA. (n=5) star=significance, where $p<0.05$

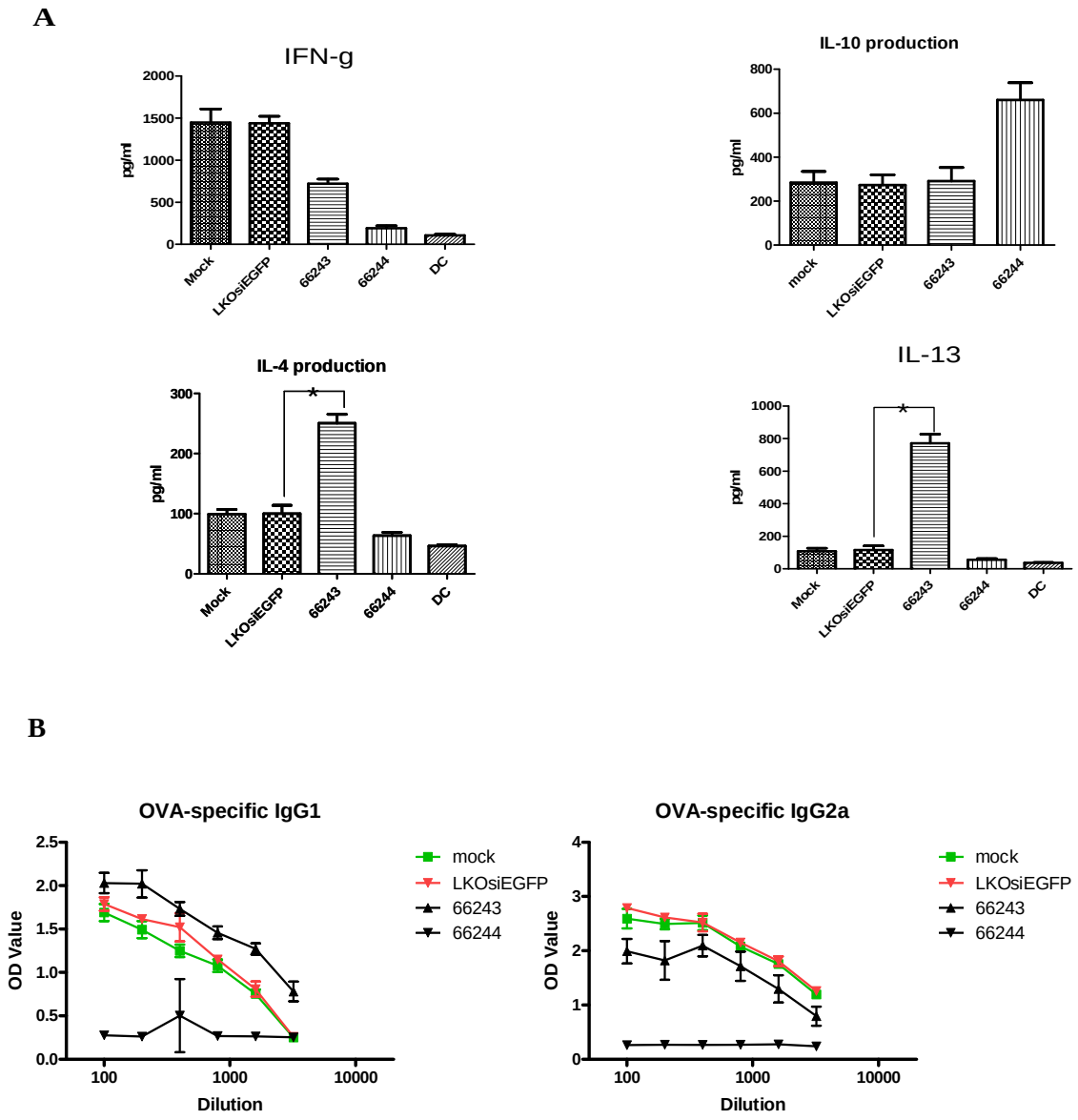

Figure 15: Cytokine profile of T cells from rechallenged mice. Immunized mice were challenged with wt DC pulsed with OVA protein 28 days after immunization. Wt DC pulsed with KLH and matured by LPS were used to challenge naïve mice and CD40low DC immunized mice as antigen specificity control. 7 days after rechallenge, T cells from rechallenged mice were restimulated by wt DC pulsed with OVA antigen, and KLH respectively. Cytokine production in the supernatant (IFN-γ, IL-4, IL-10, IL-13) was detected by ELISA (n=5). Star=significance where $P<0.05$. B) OVA-specific IgG1 and IgG2a in serum

3.2.9 CD40^{med} DC produced less IL-12 upon CD40 cross linking

It has been reported previously that IL-12 produced by DC can shape helper T cell differentiation towards Th1 [70], and the missing of IL-12 signals led to Th2 polarization [71]. We reasoned that CD40^{med} DC produced less IL-12 signal during DC and T cell interaction than CD40^{high} DC did, which in turn polarized the T cells they primed towards Th2. We therefore examined the IL-12 produced by DC with different CD40 expression levels using ELISA. We did not observe any difference among different groups of DC with different CD40 expression upon LPS stimulation. There was no IL-12 production before LPS stimulation in all 3 groups of DC that has different expression levels of CD40. These 3 groups of DC showed augmented IL-12 production after LPS stimulation, but at similar level, which is consistent with previous data (Fig. 3C). However, there is significant difference of IL-12 production among these 3 groups of DC after LPS stimulation. The IL-12 production decreased two third fold when compared with CD40^{hi} DC, and we did not observe significant increase or decrease of IL-12 production in CD40^{low} DC (Fig. 16).

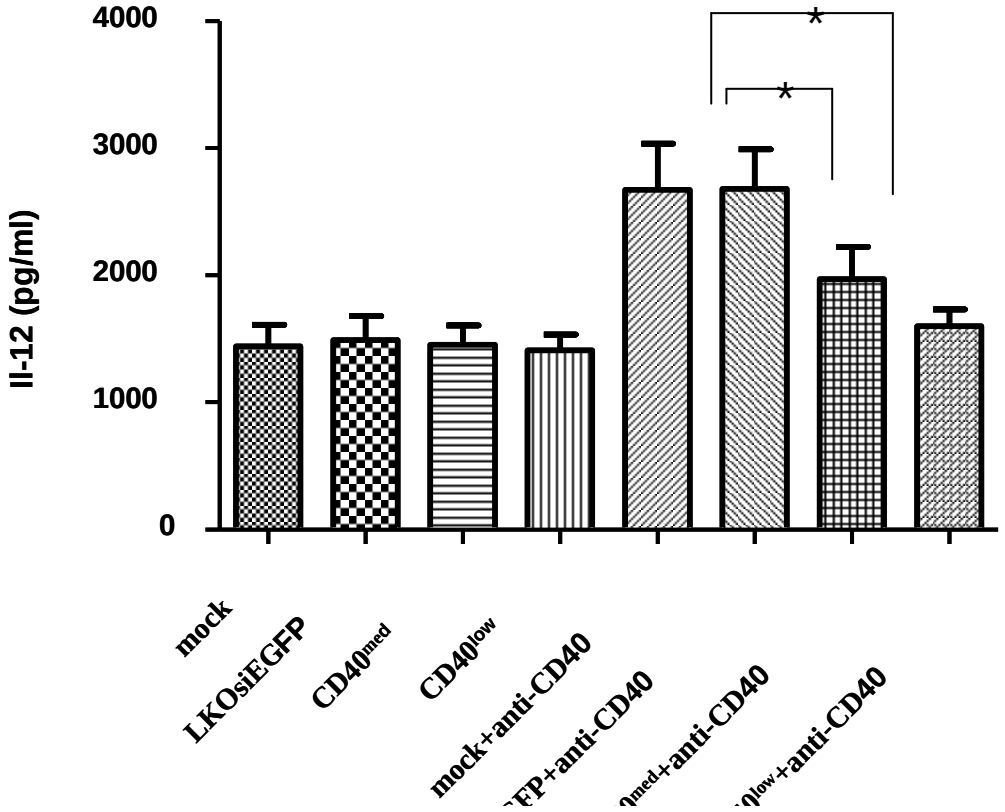

Figure 16: CD40med DC produced less IL-12 upon CD40 crosslinking. BMDC transduced with mock, LKOsiEGFP, 66243 and 66244. These DC were stimulated by LPS followed by anti-CD40 antibody crosslink. IL-12 production in the supernatant of DC culture was detected by ELISA (n=3). Star=significance where $P<0.05$.

Chapter 4 Discussion

Dendritic cells are professional antigen presenting cells with the ability to uptake, process and present antigens to resting lymphocytes in draining lymph nodes, which in turn induce immunity or tolerance. To date, the biology of dendritic cells has been well studied. Different DC subsets have been determined; more co-stimulatory molecules on DC have been identified; more DC effector functions have been studied, more activation pathways have been discovered. All these different aspects of studies in DC biology suggest the complex regulation mechanisms of dendritic function. We are interested in whether/how the quantitative expression of a co-stimulatory molecule expressed on DC will define its function. Our objectives are to develop a simple system that allows us to manipulate the expression levels of certain molecules on DC, and to study the functional outcome of the quantitative expression of one single or multiple co-stimulatory molecules on DC.

Although the employment of lentiviral vectors on DC transduction has been described previously, the undesirable effects of the lentiviral transduction on DC such as spontaneous maturation of DC or on the other hand, impairment of DC differentiation remain unclear. By using lentiviral transduction on DC, we genetically introduced a siRNA sequence into DC, which raises the concern that the introduced siRNA expressed inside the transduced cells might be recognized by the toll like receptors through pattern recognition mechanism of the innate immunity, which might lead to DC maturation or functional alternation. On the other hand, it has also been suggested that TLR-mediated immunostimulation by RNA is dependent on the type of affected cell as well as the

length and existing forms of RNA or the way that RNA is introduced to the cell. As a matter of fact, siRNA has been used by the cell as one of the mechanisms to regulate the synthesis of proteins. Our second objective of this study is to determine whether lentiviral transduction will induce spontaneous maturation or functional impairment.

The maturation of DC can be characterized by increased expression of co-stimulatory molecules and increased production of cytokines. By surface staining of the co-stimulatory molecules on DC, we detected neither up-regulation of CD40, CD80 and CD86 before LPS stimulation, nor altered expression pattern of co-stimulatory molecules after LPS maturation in transduced cells, indicating that lentiviral transduction does not alter DC maturation process, phenotypically. This observation agrees with most of the previous reports, but is contradictory to a previous report where the diminished expression of surface markers and impaired Th1 polarization were observed and DC function could be not restored by the up-regulation of CD80 and CD86. [223] The reason for this discrepancy might be because DCs were transduced at different stages of differentiation. In their system, DCs were transduced after the differentiation process started. The transduction could be a stress to the cells undergoing differentiation leading to less protein synthesis and alternation of gene expression, which might explain why the whole population was affected by the lentiviral transduction. On the other hand, we transduced DC precursors and the whole differentiation process of DCs remained intact. Surface staining of co-stimulatory molecules, intracellular staining of cytokine and ELISA of the cytokine secreted byDCs before and after LPS stimulation showed no

difference compared with mock transduced DC, suggesting the lentiviral transduction did not alter DCs differentiation or maturation.

In order to examine whether the antigen presenting ability of DC transduced with lentiviral vectors carrying shRNA remains intact, we performed mix lymphocyte reaction using either mock transduced DC or lentiviral vector transduced DC in an allogeneic setting. We found that lentiviral transduced DCs were able to present allogeneic antigen with no impairment observed. We also performed apoptosis assay on lentiviral transduced DC, and found no increased apoptosis rate in transduced cell compared with mock transduced cell. Taken together, these data suggests that lentiviral transduction on BMDC does not induce spontaneous maturation or functional alternation, both phenotypically and functionally.

As a professional antigen presenting cell, DC not only present antigen peptide : MHC class II complex to naïve T cells, but upregulate co-stimulatory molecules such as CD40, CD80, and CD86, expressed on their surface, providing co-stimulatory signals to fully activate naïve T cells. Recently, new cositmulatory molecules identified have shown different roles in regulating DC or T cell function. All these findings further demonstrate the importance of co-stimulatory molecules for DC function. We are interested in how difference expression levels of co-stimulatory molecules will affect DC functions, whether there is a threshold for the expression levels of co-stimulatory molecules that regulates DC towards immunity, tolerance or polarization of Th1/Th2. In this study, we used CD40 as a model co-stimulatory molecule to test our hypothesis.

It has been suggested that different siRNA sequences that target different regions of mRNA have different potencies of silencing. We reasoned that using lentiviral vectors that encode different shRNA sequences targeting different regions of CD40 mRNA will allow us to manipulate the expression levels of CD40 on DC. Therefore, we generated $CD40^{low}$ and $CD40^{med}$ DC using lentiviral vector to study how DC functions were regulated by the expression levels of CD40.

By co-culturing $CD40^{med}$ or $CD40^{low}$ DC with OVA-specific MHC class I restricted tumor cell line RF33.70 or OVA-specific MHC class II restricted tumor cell line, BO97.10, we found that the antigen presenting ability of the genetically manipulated DCs remained intact. Moreover, we examined the expression of other co-stimulatory molecules identified so far being expressed on DC, and found no significant changes in their expression after LPS stimulation, indicating the gene silence was specific to CD40 solely. Taken together, these data demonstrated that DCs with gene silencing of CD40 has intact antigen presenting ability and any functional alternation in these genetically modified DCs is due to the absence or impairment of CD40 signals.

According to the 2-signal model for T cell activation, naïve T cells activation requires co-stimulatory signals from co-stimulatory molecules expressed on APC in addition to the complex of MHC class molecules and the antigen processed by APC. Current model for the generation and maintenance of peripheral tolerance suggests that it is immature DCs that pick up self antigen and present it to naive T cells to maintain tolerance to self antigen. Several groups have tried to mimic this process in vitro by exposing allogeneic

antigen to immature DC or alternatively activated DC (AADC) that have low/no expression of co-sitmulatory molecules before allograft transplantation, and prolonged the survival of transplanted allograft. However, the immature DCs are sensitive to the variation or manipulation of culture conditions. Manipulation of DC culture condition has been shown to either mature DC or lead DC to no respond to stimuli. Therefore, it is necessary to generate DC with long-term stable phenotype for therapeutic purpose. Using lentiviral vector carrying shRNA against CD40, we were able to achieve long term and stable silence of CD40 expression.

We showed in this study that DC with low or media level of CD40 impaired T cell priming both in vitro and in vivo, and the extent of T cell priming was correlated to the expression level of CD40. Current data suggest that blockade of CD40 signal pathways induces apoptosis of T cells primed by DC. In vitro, we found more apoptosis induction in T cells primed by $CD40^{low}$ DC than that was primed by $CD40^{med}$ DC or the control group. Interestingly, we did not observe increased apoptosis of T cells primed by $CD40^{low}$ DC in vivo, suggesting that the absence of CD40 might employ different pathways to regulate T cells. However, $CD4^{+}CD25^{+}Foxp3^{+}$ T cell staining of the T cells from draining lymph node restimulated by DC pulsed with the same antigen showed increased regulatory T cell induction. Studies using $IL\text{-}10^{-/-}$ mice have shown that $CD40^{low}$ DC induced regulatory T cells exert their function through secreting anti inflammation cytokine IL-10. In this study, the IL-10 produced by $CD40^{low}$ DC primed T cells is lower than those primed by $CD40^{med}$ or $CD40^{hi}$ DC. Considering the fact that $CD40^{low}$ DC primed T cells had the lowest or nearly no proliferation, the IL-10 produced

by single T cell primed by $CD40^{low}$ DC might be comparable or even higher than those primed by $CD40^{med}$ or $CD40^{hi}$ DC. Moreover, the T cells primed by $CD40^{low}$ DC suppressed the T cell proliferation stimulated by wild type DC pulsed with the same antigen, demonstrating regulatory property of the T cells primed by $CD40^{low}$ DC.

Several groups have suggested that the ligation between CD40 and its Ligand has a direct or indirect role of up-regulating other co-stimulatory signals, where the primed T cells accept signals from CD40 through CD40L, and thus augment the level of the interaction between CD80/86 and their Ligand, or enhance the expression of the co-stimulatory molecules and their ligands on primed T cells. Therefore, another explanation for the impaired T cell priming is that the lack of CD40 expression on DC interrupted or impaired the signals through CD40: CD40L ligation, and prevented the induction or enhancement of co-stimulatory signals on these cells. To investigate whether the ligation of CD40 augment the interaction between CD80/86 and their Ligand, we examined the surface expression of those two co-stimulatory molecules on DC after they presented antigen to the co-cultured T cells. However, we did not detect any up-regulation of CD80 or CD86 on the mock transuced or control vector transduced DC co-cultured with T cells, suggesting that CD40 signals might solely enhance the interaction between CD80/86 and their Ligand but have no effect on their up-regulation after DC presented antigen to T cells. It could be also possible that the T cells primed by $CD40^{low}$ DC failed to up-regulate key co-stimulatory signals in response to its ligand, but induced regulatory T cells instead.

It is worthy of noting that only did the $CD40^{low}$ DC primed T cells, but not the $CD40^{med}$ DC primed T cell present immunological tolerance property upon rechallenge with the same antigen. This suggests that there might be a threshold of the expression of CD40 that dictates the consequences of antigen presentation to T cell by DC. Studies using CD40-/- DC or DC generated from RelB KO mice demonstrated the ability of these DCs to suppress T cell proliferation and pre-primed T cell response. Therefore, it suggests the requirement of low or no expression level of CD40 in order to induce immunological tolerance. $CD40^{low}$ DC induced $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells with active inhibition ability that suppressed T cell activation upon rechallenge, $CD40^{med}$ DC, on the other hand, failed to induce any regulatory T cells. Therefore, the impaired T cell priming is due to the insufficient CD40 signals, and the impaired T cell function was restored once they were primed by DC with full co-stimulatory signals.

The fact $CD40^{med}$ DC failed to induce immunological tolerance upon rechallenge makes it interesting to understand what these cells have done to T cell activation, whether it affects differentiation or polarization of T cells. To understand the possible effect of these $CD40^{med}$ DC on the T cells primed, we examined their cytokine profile. The ELISA data revealed that T cells primed by $CD40^{med}$ DC produced less IFN-γ but more IL-4 than those primed by $CD40^{low}$ or $CD40^{high}$ DC. Same cytokine production pattern was found in the rechallenged T cells that were primed by $CD40^{med}$ DC. Moreover, higher level of OVA-specific IgG1 and lower level of OVA-specific IgG2a was found in the serum of $CD40^{med}$ DC immunized mice upon rechallenge. Several groups have reported that the avidity of the ligation between CD40 and its Ligand on DC polarized the T cells it primed.

However, there is no consensus whether there is a threshold that regulates such change. This study demonstrated the existence of such threshold, that is, intermediate level of CD40 expression on DC favours Th2 polarization, high level of CD40 expression favours Th1 polarization, and low level of CD40 will induce tolerance.

CD40 ligation through its Ligand on T cells has been shown to induce IL-12 dependent IFN-γ production and Th1 polarization. Changes on CD40 signaling might be able to alter the cytokine profile of DC, which in turn change the functional consequence on T cells primed. Disruption of CD40 signaling is known to decrease CD40-mediated IL-12 production that is important for Th1 polarization regulated by DC. Indeed, we observed less increase of IL-12 after CD40 crosslinking on DC transduced with 66243 and 66244, and the extent of the lack of IL-12 increase was correlated to the CD40 expression levels. However, we did not observe any difference of IL-12 production before CD40 crosslinking. This suggests that IL-12 production is regulated by CD40 signaling instead of LPS stimulation, and there is a threshold for IL-12 to polarized Th1 phenotype.

In our study, we used LPS to mature DC to upregulate CD40 that does not express on the DC grown under our protocol in order to manipulate the expression levels of this cositmulatory molecule. We did not observe any change of IL-12 production by DC transduced with 66243 or 66244 after LPS stimulation. However, we did find alternation of IL-12 production after CD40 crosslinking using CD40 agonist antibody. Our data suggests that LPS maturation of DC does not impair DC function or induce spontaneous maturation on DC. Thus the DC transduced with 66243 or 66244 produced comparable

amount of IL-12 as well as other cytokines that non-manipulated DC will do. CD40 crosslinking, on the other hand, is involved in the regulation of IL-12 production. Therefore, the decrease of IL-12 production on 66243 or 66244 transduced DC was due to the impairment of CD40 signal mediated by the shRNAs..

The impairment of CD40 ligation has profound effect on DC function. The studies triggering CD40 expression or blocking CD40 signal pathway strongly suggested that CD40 could be the key determinant for the functional consequence of T cell activation, either immunity or tolerance. However, it was also reported that CD40 signaling was not required for the Th1 response to *Proprionibacterium acnes*[224] or *Histoplasma capsulatum.*[225] The reason of this discrepancy may be due to different antigens employed in different studies, which activated different signaling pathways downstream of CD40. In this study, we observed antigen-specific immunological tolerance with total absence of CD40 signaling, and Th2 polarization with medium level of CD40 signaling. Meanwhile, the expression of CD80 and CD86 kept intact. Moreover, CD80 or CD86 expression on DC co-cultured with T cells was not up-regulated or down-regulated during co-culture, suggesting that the bidirection regulation between DC and activated T cells was not involved in our system. Taken together, these data suggests that CD40 alone is enough to have impact on DC function. Although tolerance has been reported to be observed while B7-CD28 pathway is blocked, CD40 expression was not evaluated in those reports. In addition, the partially activation of CD40 signaling might activated different downstream signaling pathway from NF-κB that favours the production of Th2 cytokines.

In summary, we established a platform using lentiviral transduction on murine BMDC to regulate the surface expression of certain molecule(s) on BMDC. Using shRNA sequences with different potencies against the target molecule CD40, we were able to generate $CD40^{med}$ and $CD40^{low}$ DC that allowed us to quantitatively analyze the function of this molecule. In this study, we found that CD40 alone is the key determinant for the functional consequence of T cells in response to DC presentation. DC with low CD40 expression level led to immunological tolerance by inducing $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells while DC with intermediate level of CD40 polarized T helper cell towards Th2 type. Polarized T cells produced higher level of Th2 cytokines such as IL-4 and IgG1. CD40 crosslink experiment revealed that impairment of CD40 signals resulted in the decrease of IL-12 production, which is responsible for the Th1 polarization mediated by DCs.

Chapter 5 Implication and Future direction

Our observations in this study have significance for DC based immunotherapy. Lentiviral transduction has been demonstrated to be a reliable approach that can achieve long-term stable gene silencing or over-expression of transgene into DC without impairing their functions. By using shRNA that targets certain molecule expressed on DC, we can study the biological function of that particular molecule, and such understanding can be used in disease treatment. For instance, we observed in this study that CD40low DC mediated peripheral tolerance by inducing regulatory T cells with active inhibition ability. This finding has implication in treatment of autoimmune diseases, allograft rejection and graft-versus-host disease (GVHD), where ongoing antigen presentation is associated with chronic inflammation. Knowledge of the traffic of induced regulatory T cells to peripheral inflamed organs will be of more help regarding the immunotherapy for autoimmune diseases.

We also observed in this study that CD40med DC polarized T cells toward Th2 phenotype, producing more Th2 cytokines and less Th1 cytokines. This finding can be beneficial for the treatment of Th1 diseases. It has been reported that Th2 cytokines can alleviate disease symptoms and obtained better control on progression. in vitro generating DC with intermediate level of CD40 might have the systematic effects on Th1 diseases by introducing IL-4, IL-5 and IL-13 systematically. Future studies can be testing the functional consequence of Th2-inducing CD40med DC in disease models that favor Th1.

The system used in this study can also be employed on the study of novel molecules that are recently identified on DC, such as the heterogeneous nuclear ribonucleoprotein K (hnRNP K), and JLP. shRNA sequence against hnRNP K has been encoded into a lentiviral vector. Primilary studies on BMDC transduced with this lentiviral vectors showed down-regulated co-stimulatory molecules expression resembling immature DC. Functional analysis of this transduced DC will be involved in future studies.

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