The Role(s) of JLP Scaffolding Protein in Regulating LPS- vs. Poly(I:C)-activated Mature Dendritic Cell Functions

by

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Abstract

Dendritic cells (DC) recognize pathogen via toll-like receptors (TLRs) and become activated, leading to cytokine production, co-stimulatory molecule up-regulation, antigen processing and presentation as well as survival in the absence of growth factor. It has been demonstrated that TLR3- and TLR4-mediated DC activation lead to distinct functional properties of mature BMDC, in which MAPKs pathway is involved. JNK-associated leucine zipper protein (JLP) was reported to associate with JNK/p38 phosphorylation, however the functional role of JLP in immune cells is not well understood. We found that either LPS or poly(I:C) up-regulates JLP expression in BMDC. Therefore we hypothesized that JLP plays a differential role in regulating TLR-3 and TLR4-mediated BMDC maturation and functions. In our study, We demonstrated that JLP facilitated LPS- and poly(I:C)-induced CD86 and CD40 up-regulation, LPS-induced CD80 up-regulation and poly(I:C)-induced MHC-II up-regulation. JLP also played a role in down-regulation of IL-12 in LPS-stimulated BMDC, and up-regulation of IL-6 production in poly(I:C)-stimulated BMDC. Our data also showed JLP negatively regulated MHC-II antigen presentation in LPS- and poly(I:C)-stimulated BMDC and JLP was involved in promoting LPS-activated BMDC survival without GM-CSF, but not in poly(I:C)-activated BMDCs. Therefore our current data suggested a multi-functional role of the scaffold protein JLP in the regulation of TLR3- and TLR4-mediated DC maturation.

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List of abbreviations

APC	antigen presenting cell
Ag	antigen
AKAP	A-kinase anchor proteins
AP2	adaptor protein complex 2
ASC	apoptosis-associated speck-like protein containing a CARD
BM	bone marrow
BMDC	bone marrow-derived dendritic cell
CARD	caspase-recruitment domain
cDC	conventional dendritic cell
CMP	common myeloid progenitor
CLP	common lymphoid progenitor
CLR	C-type lectin receptor
CTL	cytotoxic T lymphocyte
ER	endoplasmic reticulum
ELISA	enzyme-linked immunosorbent assay
FcR	Fc receptor
FBS	fetal bovine serum
IFN	interferon
IKKβ	IκB kinase-β
ITAM	immunoreceptor tyrosine-containing activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
JIP4	JNK-interacting protein-4
JLP	JNK-associated leucine zipper protein
KLC1	kinesin light chain 1
KSR	kinase suppressor of RAS
LC	Langerhans cell
LN	lymph node
LPS	lipopolysaccharide
LPDC	lamina propria dendritic cell
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
NFAT	nuclear factor of activated T cell
NK cell	natural killer cell
NMEO	NK-κB essential modulator
NLR	NOD-like receptor
pDC	plasmacytoid dendritic cell
PKA	protein kinase
PRR	pattern-recognition receptors
PAMP	Pathogen-associated molecular pattern
RLR	Rentinoic acid-inducible gene-I-like receptors
RIP	receptor-interacting protein
RHIM	receptor-interacting protein (RIP) homotypic interaction motif

ssRNA	single-stranded RNA
SARM	sterile-alpha and Armadillo motif-containing protein
SPAG9	sperm associated antigen 9
Sirp-α	signal regulatory protein-α
SR	scavenger receptor
TCR	T-cell receptor
TLR	Toll-like receptor
TIR	Toll-interleukin 1 receptor
TRIF	TIR domain-containing adaptor inducing IFN-β
TRAM	TRIF-related adaptor molecule
TRAF6	TNFR-associated factor 6
TAK1	TGF-β-activated kinase 1
TRADD	TNFR-associated death domain protein
β_2m	β2-microglobulin
2-ME	2-mercaptoethanol
7-AAD	7-amino actinimycin

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Chapter 1 Introduction

In 1973 R. Steinman and Z. Cohn reported their discovery of a novel cell type from lymphoid organs of mice for the first time [1]. According to its unique morphological features, this cell type was named “Dendritic cells”. Since then, dendritic cells (DC) have been studied in great detail by many research groups around the world, and their role in immunity has been intensely investigated.

Hematopoietic progenitors in bone marrow (BM) give rise to DC precursors and they reside as immature DC at blood, lymphoid tissues and non-lymphoid tissues. Immature DC become mature when they recognize invading pathogens via various pattern-recognition receptors (PRRs), and this maturation process associates with pro-inflammatory cytokine production, co-stimulatory molecule up-regulation and migration toward secondary lymph organs. These events result in activation of lymphocytes and initiation of adaptive immunity.

DC are defined as a family of professional antigen-presenting cells (APCs) that bridges innate and adaptive immunity and regulates T-cell mediated immune response. DC are capable of processing antigens, presenting them on major histocompatibility complex (MHC) to initiate naïve CD4 and CD8 T cell response, as well as regulating T cell-mediated immune response [2-5]. Moreover DC are capable of inducing central and peripheral tolerance, activating NK cells as well as B cells directly. Therefore, DC are important initiators and regulators in both innate immunity and adaptive immunity.

1.1 Classification of murine DC

DC are distributed in peripheral tissues as well as non-lymphoid and lymphoid organs. Though cells classified as DC have many common features such as origin of bone marrow, antigen presentation capacity and regulation of adaptive immune responses, DC represent a population of highly heterogeneous leukocytes distinct in development, morphology and function. Classification of DC can be based on their phenotype, localization and development pathway.

1.1.1 Conventional DC (cDC) vs. plasmacytoid DC (pDC)

DC can be divided into two main classes described as conventional DC (cDC) and plasmacytoid DC (pDC). Both of these two DC subsets are bone marrow derived.

Conventional DC refers to DC displaying phenotypic and functional features originally identified by R. Steinman and Z. Cohn [1]. cDC are cytoplasmic veiled showing dendritic morphology. cDC express high levels of CD11c and MHC-II as well as a broad range of pattern-recognition receptors (PRR) , and have a unique ability of priming naïve T cells. cDC have been further subdivided into many subsets due to various criteria, like lymphoid tissue-resident cDC which encountered antigen inside lymphoid organs as well as migratory cDC which resided in non-lymphoid tissues and migrate to the draining lymph nodes (LNs) after microbial encounter. Based on CD8α expression cDC are segregated into two subsets: $CD8^+$ cDC capable of antigen cross-presentation to CTLs (cytotoxic T lymphocytes) and $CD8^-$ cDC displaying high capability for antigen-presentation via MHC-II [6]. The functional roles of these two subsets in immunity are further discussed in the following section.

Plasmacytoid DC are the main cell type responsible for type-I interferons (type-I IFNs) upon viral infection production for antiviral immunity. In 1958 human pDC were identified in human lymphoid tissue and named T-associated plasma cells and plasmacytoid T cells [7, 8]. They were also called plasmacytoid monocytes because their expression of T-cell marker and myeloid-cell markers [8]. Murine pDC at steady state display the morphology of lymphocytes with low expression of CD11c, MHC-II and co-stimulatory molecules but high level of B220/CD45RA, which are defined as B-cell marker [9, 10], and they exhibit low capacity of T cell stimulation. pDC recognize foreign nucleic acids such as viral single-stranded RNA (ssRNA) and CpG-containing DNA via TLR7 and TLR9 and become activated, as a result the expression of MHC-II and co-stimulatory molecules is up-regulated upon activation of pDC. In addition to TNF-α and IL-12 production as well as enhanced antigen presentation ability, secretion of type-I IFNs is the most distinct response to stimuli [11]. In addition to TLR7 and TLR9, mouse pDC also express TLR1, 2, 4 and 6, whereas human pDC express TLR7 and TLR9 exclusively [12]. Furthermore activated pDC contribute to the activation of natural killer cells (NK cells) and plasma cells [13, 14].

1.1.2 Classification of DC by localization

DC can be found in all lymphoid and non-lymphoid tissues and they show tissue-specific functions besides their regulation of innate and adaptive immune responses, such as DC assist to enhance the homing of effector T cells to target sites by imprinting T cells with organ specificity during the priming process in lymphoid organs. For example, DC from intestinal lymphoid organs promote gut-homing receptors CCR9 and α4β7 on T cells by producing retinoic acid.

In non-lymphoid tissues, Langerhans cells (LCs) and dermal DC reside in the skin, LCs is distinct from dermal DC based on their location and expression of langerin. The majority of the dermal DC lack langerin expression [15]. However recent evidence showed that a population of dermal-resident DC independent of LCs also expresses langerin [16-21]. Mucosal tissue-associated DC exist in the mucosa of intestinal tract, respiratory tract, and interstitial DC can be found in liver, kidney, lung and other connective tissues. These subsets of DC differentiate from precursors in the blood and function as sentinel system in the peripheral tissues.

Lymphoid tissue-resident DC include thymic DC, splenic DC and lymph nodes DC. Murine thymic DC can be divided into two subsets as $CD11c^{int}CD45RA^{+}$ pDC and $CD11c^{+}CD45RA^{-}$ cDC [22]. And cDC population can be further segregated on the basis of surface expression of CD8 and signal regulatory protein-α (Sirp-α) [22]. The $CD8^{+}Sirp^{-}$ cDC represent about 70% of thymic cDC and they differentiate from intrathymic DC precursors, whereas the minor $CD8^{-/lo}Sirp\text{-}\alpha^{+}$ cDC are generated from the periphery migratory cDC [23]. In mouse spleen three subsets of $CD11c^{hi}MHC\text{-}II^{+}$ cDC have been identified based on their surface expression of CD8α and CD4: $CD4^-CD8^-$, $CD4^+CD8^-$ and $CD4^-CD8^+$ [24]. $CD4^-CD8^+$ cDC are localized in T-cell areas, whereas $CD4^-CD8^-$ and $CD4^+CD8^-$ cDC are mainly localized in the marginal zone and can migrate to T cell areas upon LPS or antigen stimulation [25]. The $CD8^-$ DC stimulate $CD4^+$ and $CD8^+$ T cell efficiently in vitro and induce Th2 response in vivo [26]. The $CD8^+$ DC are responsible for peripheral tolerance when they are in steady state [27], whereas when they are activated, they become the major producers of IL-12 [28], and activate $CD8^+$ T cells [29, 30] and trigger Th1 response in vivo [31, 32]. In addition to cDC, pDC also exist in mouse spleen and they are the major producers of large amount of type-1 interferon after viral infection [9, 33-36]. These pDC are identified as $CD11c^{int}CD45RA^+B220^+$ based on their surface molecule expression. Studies have shown that splenic pDC may be originated from peripheral blood [37, 38]. The constitution of DC populations in LNs is more complicated. The three murine splenic cDC subsets are found in mouse LNs: $CD8^-$ cDC are localized in the subcapsular sinus and immediate perifollicular zone of LNs and $CD8^+$ cDC are present in the paracortical region of LNs [39, 40]. Moreover two additional DC subsets have been described as $CD8^{lo}CD205^{hi}$ and $CD8^{lo}CD205^{int}$ cDC representing the mature tissue-derived cDC that migrate from the epidermis and dermis to the lymph nodes [28, 41]. These migratory DC are responsible for presenting antigens captured in peripheral tissues to the draining LNs.

1.1.3 Classification of DC base on origin

Myeloid and lymphoid have been identified as two different DC development pathways, the origin of DC was used to be utilized to classify DC subsets as myeloid DC and lymphoid DC. DC8α, DEC205 and CD11b were used as markers to distinguish these two subsets: $CD11c^+CD8\alpha^+DEC205^+CD11b^-$ for lymphoid generated DC and $CD11c^+CD8\alpha^-DEC205^-CD11b^+$ for DC originated from myeloid progenitors [42]. But the distinction between lymphoid and myeloid DC was challenged and disproved by series studies demonstrating the fact that DC subsets can be originated form either myeloid or lymphoid progenitors [42].

Myeloid origin of DC was demonstrated directly by transplantation of murine bone marrow common myeloid progenitors (CMPs) into irradiated mice, and CMPs reconstructed the cDC and pDC in the spleen and thymus of recipients [43-45]. And further studies indicated that $Flt3^+$ CMPs are capable of generating all cDC and pDC population [46]. Firm evidence of lymphoid origin was established by studies on murine bone marrow common lymphoid progenitors (CLPs) demonstrating that DC can be originated from CLPs both in vivo and in vitro [45, 47, 48], and they can give rise to all DC subsets in spleen and thymus [45, 49].

1.2 Functions of DC in immunity and tolerance in mice

DC are professional antigen presenting cells that bridge innate and adaptive immunity. In innate immunity, DC recognize invading microorganism through PRRs and capture the antigens, and upon activation DC perform protective response by producing cytokines such as type I IFN and interleukins, moreover DC are also responsible for activation of

NK cells. Activated antigen-bearing DC migrate to lymph organs and prime primary and secondary T-cell response. Furthermore, DC are critical regulators in maintenance of immune tolerance.

1.2.1 Antigen capture

Immature DC reside ubiquitously throughout the body as sentinels and display highly efficient antigen capture capacity [50]. They keep sampling the peripheral tissue and capture pathogens, infected and injured cells as well as dead cells for antigen presentation.

Receptor-mediated endocytosis, phagocytosis and macropinocytosis are three distinct mechanisms adopted by DC for antigen uptake [51, 52]. Mouse immature DC express various types of endocytic receptors for macromolecule uptake through coated pits, which are specialized plasma membrane. FcγRI, FcγRII abd FcγRIII are receptors for Fc portion of immunoglobulins expressed on mouse DC that capture immune complex or opsonized particles [53, 54]. Scavenger receptors (SRs) are glycoproteins expressed on DC surface, which are critical for internalization of apoptotic bodies and bacteria such as *Escherichia coli* and *Staphylococcus aureus* [55, 56]. Another type of receptor involved in endocytosis is C-type lectin receptor, such as mannose receptor and DEC-205 [51, 52].

Phagocytosis is actin dependent effective engulfment induced by the engagement of specific receptors. Immature DC were demonstrated to phagocytose intracellular parasites such as *Lishmania major* [57, 58], various GRAM$^+$ and GRAM$^-$ bacteria [59], apoptotic and necrotic bodies derived from fibroblasts [60], infected macrophages [61] and allogenic B cells [62] etc.

Macropinocytosis is a critical constitutive cytoskeleton-dependent fluid-phase endocytosis mechanism in immature DC [63]. DC can engulf large amounts of soluble antigens rapidly and non-specifically via macropinocytosis.

It is noteworthy that pathogens have evolved strategies to enter DC by utilizing their endocytic receptors and use migratory capacity of DC for dissemination of pathogens. For example, measles virus infect DC via Fc receptors (FcRs) [64], *Leishmania major* interacts with CR3 located on surface of DC [65]. Internalization of HIV by DC has been extensively studied, it was demonstrated that CCR5 on fresh LCs and CXCR4 on mature DC [66] as well as DC-SIGN are involved in HIV anchor on DC surface and trans-infection of T cells [67].

1.2.2 Antigen processing and presentation

DC are well equipped to process and present antigens (Ags), Ags are degraded into peptides and loaded onto MHC molecules in the cytoplasm of DC. The intracellular pathways Ags processing and loading onto MHC class I and class II have been studied in detail and well understood.

1.2.2.1 Antigen presentation via MHC-I by DC

Newly ubiquitinated cytosolic proteins are directed to proteasome and degraded into peptides through an ATP-dependent proteolytic system. The resulting peptides are transferred to endoplasmic reticulum (ER) ATP-dependent specialized transmembrane peptide transporter TAP1/2, and are loaded onto nascent MHC-I molecules via a loading complex composed by of tapasin, calnexin and calreticulin [68]. In the ER, MHC-I molecules comprised of β2-microglobulin and Class I α chain, become fully assembled MHC-I/peptide complex after the insertion of a peptide. Peptide-bearing MHC-I molecules are then transferred to plasma membrane by the Golgi apparatus.

In addition to endogenous Ags, exogenous Ags also can be presented by MHC-I molecule, which is termed cross-presentation. Two routes of cross-presentation have been reported [69]. One is TAP-independent pathway and the peptides are loaded in endocytic compartments [70]. Another pathway is TAP-dependent in which peptides are loaded in ER. Moreover, in this pathway transportation of Ags from phagosome to cytosol is necessary [71, 72].

1.2.1.2 Antigen presentation via MHC-II by DC

Immature DC are capable of internalizing exogenous soluble and particulate Ags efficiently directing the captured Ags to MHC-II compartments [39, 62, 63, 73, 74]. MHC-II dimers associate to invariant (Ii) chains [75]. The resulting nonamers are transported form ER to endosomes and lysosomes through Golgi apparatus directed by signals present in Ii chains. In endosomes and lysosomes, the Ii chains are degraded by proteolytic enzyme of the cathepson family [76], and then MHC-II dimers can bind to Ag-derived peptides. In mouse this process is under control of H2-M and H2-O [77], which facilitate the removal of MHC-II-associated Ii chain and promote binding of antigenic peptides to MHC-II molecules [75, 78]. After loading of peptides, MHC-II/peptide complexes relocate to the surface of plasma membrane.

The level of surface MHC-II/peptide complexes is highly regulated in the life cycle of DC. Immature DC display a very low level of surface MHC-II/peptide complex. Because of low efficiency of several proteases involved in antigen degradation in immature DC, captured Ags are degraded inefficiently, leading to the low availability of antigenic peptides to load on MHC-II molecules [79, 80]. Moreover, accumulation of MHC-II-Ii complexes in endosomes and lysosomes also contributes to the low surface MHC-II/peptide complexes level [81, 82], due to low protease activity of a main protease responsible for Ii degradation named cathepsin S inhibited by cystain C [83-85]. Furthermore, MHC-II molecules on the surface of immature DC are rapidly internalized with a short half-life. Those internalized MHC-II molecules can be loaded with new peptides in endosomes and translocated to cell surface again [82, 86, 87]. Otherwise they can be directed to lysosome and degraded finally [88, 89].

Maturation signals modify the formation, transportation as well as stability of MHC-II/peptide complexes. Upon DC maturation, synthesis of MHC-II molecule is enhanced transiently [88, 90], and the level of cystain C is downreguated, which highly increase the protease activity of the cathepsins [80]. As a result, MHC-II/peptide complexes are formed at a high efficiency due to the increased availability of MHC-II dimers [79]. Decreased endocytosis activity of mature DC inhibits the internalization of MHC-II/peptide complexes and contributes to stabilization of MHC-II/peptide complexes located on the cell surface [88, 89].

1.2.1.3 CD1-restricted antigen presentation in DC

Besides MHC class I and class II molecules, the CD1 family of MHC-I-like glycoproteins has been identified as Ag-presenting molecules responsible for T cell response to microbial pathogen- and self-derived lipids as well as glycolipids [91, 92]. CD1 molecule consists of a heavy chain containing α1, α2 and α3 extracellular domains which are associated with β2-microglobulin (β_2m) noncovalently. Three groups of CD1 isoforms have been classified based on sequence analysis: CD1a-c as group 1, CD1d as group 2 and CD1e as group 3. All CD1a-e were detected in human, whereas only CD1d was found expressed in mouse. CD1a-d are displayed on the cell surface, whereas CD1e mainly exists in the Golgi apparatus of immature DC and are detected in the endosomes and lysosomes of mature DC as functional soluble form. New synthesized CD1 molecules become glycosylated and locate in the lumen of ER binding the calnexin and calreticulin [93-95]. In the ER endogenous self-lipids are loaded onto CD1 molecules, which are supported by the research evidences that CD1b associate with phosphatidylcholine [96] and CD1d associate with phosphatidylinositol [96-98]. Those self-lipids may be presented as Ags or may function as chaperones to facilitate the assembly and stabilization of CD1 molecules in the ER [96, 98]. Following assembly in the ER, CD1 molecules relocate to the cell surface via Golgi due to the association with β_2m [93, 99, 100], however other transportation pathways have also been identified [97, 101-103].

CD1 molecules at cell surface are internalized constitutively, facilitated by adaptor protein complex 2 (AP2) and calthrin-coated pits [104-107]. After internalization CD1 molecules are diverted into endocytic recycling compartments or late endosomes and lysosomes through distinct trafficking pathways depending on the isoform of CD1 [106, 108]. Following all these events exchanging of bound self-lipid antigen with other new lipid antigens occur. And then recycled CD1 molecules return back to cell surface, although the mechanism and routes have not been clarified. Unlike antigen presentation of MHC-II, the antigen presentation via CD1 is not affected dramatically by DC maturation and recycling of CD1 appears not repressed upon DC maturation [51, 109, 110].

1.2.2 Priming of T cells by DC

As professional APCs, DC play as principal inducer in T cell priming and this is one of prominent functions of DC because of their unique capacity of initiating primary immune responses of naïve T cells. Activation of naïve $CD4^+$ T cells by DC has been demonstrated by injection of Ag-bearing DC into mice [111], furthermore direct Ag presentation by pulsed DC was also revealed by immunohistology of PALS showing interactions between DC and helper T cells [112]. Priming of naïve CD8$^+$ T cells by DC has also been studied in vitro and in vivo. Potent proliferation of allogeneic CD8$^+$ T cells can be induced by DC without the assistance of CD4$^+$ helper T cells [113-115]. CTLs can be triggered in mice by injection of DC pulsed by various Ags such as tumor, allografts and viral proteins presented via MHC-I. Under some situation assistance of CD4$^+$ helper T cells are required for CD8$^+$ T cell activation. These CD4$^+$ helper T cells upregulate CD40-L expression level on DC to facilitate CD8$^+$ T cell activation by DC [116-118].

To achieve optimal activation of Ag-specific T cell responses, three distinct signals are required. The first signal is triggered by interaction of MHC-I and MHC-II molecules with T-cell receptor (TCR) on CD8$^+$ and CD4$^+$ T cell respectively. Ag recognition via TCR restricts the Ag specification of the T cell response. The second signal called co-stimulation is mediated by crosslinking of co-stimulatory molecules on DC and their ligands on T cells. One of the well-studied co-stimulation is triggering of CD28 by CD86 and CD80 expressed by DC, which initiates T-cell activation and proliferation [119]. Accumulating evidence show that the actual effect of co-stimulation on T cells is likely to be a fine balance of positive and negative co-stimulatory signals mediated by many receptors [120]. Signal 3 refers to the polarizing signal mediated by membrane-bound soluble factors, which determines differentiation of T cells into effector cells. For example IL-12 is a mediator that promotes development of helper 1 T cells or CTLs [121], IL-27 and TGF-β produced by DC drive the differentiation of regulatory T cells [122, 123], and Th17 differentiation can be promoted by TGF-β,IL-6 and IL-23 [124].

1.2.3 Activation of NK cells by DC

Early study of Fernadez et al. showed that DC can directly activate NK in vivo [125], and following work showed that both mouse and human DC could activate NK in vitro [126]. Moreover recent studies provided evidence of interaction between NK and pDC [127].

Soluble factors produced by DC have been demonstrated to be involved in DC-induced NK activation. IL-12 produced by DC in respond to LPS and poly(I:C) was demonstrated to be crucial for induction of INF-γ produced by NK cells [128, 129]. In synergy with IL-12, IL-18 produced by human CD34$^+$-derived DC enhances both INF-γ production and cytotoxicity of NK cells [130]. Moreover type-I IFN has been shown to play a role in NK activation [131, 132]. pDC are the major population that produce large amount of type-I IFN upon recognition of microbial nucleic acid through TLR7 and TLR9 [133]. Therefore, after virus infection, type-I IFN produced by DC trigger the IFN-γ secretion and cytotoxicity of NK cells to further augment the antiviral immunity. In addition to IL-12, IL-18 and type-I IFN, other cytokines are also involved in DC-induced NK activation. IL-15Rα on murine DC was demonstrated to be required for NK activation [134], and IL-15 produced by monocyte-derived DC may be involved in NK proliferation. And IL-2 produced by mature BMDC may also affect NK activation [135].

Besides soluble factors, cell-cell contact between DC and NK cells has also been shown to be required for NK activation. This has been demonstrated by in vitro transwell separation experiment showing physical segregate of these two types of cells could inhibit DC-dependent induction of NK cytotoxicity [125]. The formation of synapse can also further enhance IL-12 production by DC [136], mediated by NKp30, KIR and NKG2A [137]. In addition to DC activation, synaptic formation also leads to NK-mediated killing of DC. In previous work, it has been observed that NK cells can recognize and lysate monocyte-derived DC, which was cell-cell contact dependent [128, 138-140].

1.2.4 Induction of tolerance by DC

Though DC play a critical role in activating T-cell immune responses, DC are responsible for regulating immunity by inducing tolerance, which is important for immune homeostasis and maintenance of self-tolerance.

In thymus, DC are important for negative selection of T cells. The expression of MHC-II molecule on thymic DC has been demonstrated to be sufficient for negative selection of I-E reactive CD4$^+$ T cells and a portion of CD8$^+$ T cells [141]. Elimination of CD11c$^+$ DC in thymus impairs the clonal deletion of T cells [142]. Moreover thymic DC can also mediate negative selection of T cells via induction of regulatory T cells. It has been demonstrated that thymic CD8^{lo}Sirpa$^+$ DC efficiently induced differentiation of regulatory T cells and negative selection [143]. Recent studies showed that Hassall's corpuscles instruct development Foxp3$^+$ regulatory T cells [144].

In addition to its role in central tolerance, DC also promote peripheral tolerance by induction of regulatory T cell development and T-cell anergy or deletion. The maturation state of DC is critical for tolerogenic capacity of DC. By promoting regulatory T cell development and deleting antigen-specific T cells, immature DC induce tolerance in vivo [145-148]. Bonifaz L. et al demonstrated that DC in a steady state led to peripheral antigen-specific CD8$^+$ T cell tolerance [149]. Regulatory CD4$^+$ T cells are defined by the constitutive expression of CD25, and they are potent inhibitors of T cell activation. CD4$^+$CD25$^+$ T cells are capable of inhibiting activity of both CD4$^+$ and CD8$^+$ T cells in an antigen-nonspecific manner. Previous study indicated that CD4$^+$CD25$^+$ inhibited autoimmune diabetes [150] and immune response against alloantigens [151, 152]. Insufficient co-stimulation and non-inflammatory cytokine such as IL-10 and TGF-β induce T cell anergy. Studies have demonstrated that DC treated with IL-10 produced less IL-1β, IL-6 and TNF-α, and no IL-12 [153-155]. Moreover such IL-10-treated DC can induce T cell anergy and Ag-specific tolerance, which is characterized by reduced production of IL-2 and IFN-γ, down-regulated CD25 as well as inhibited antigen-specific proliferation [155, 156]. However, matured DC are not sensitive to IL-10 effect any longer [155, 157, 158]. It has been shown that DC are capable of inducing T-cell apoptosis. FasL expressed by murine splenic CD8α$^+$ DC can induce apoptosis in allogenic CD4$^+$ T cells [159]. TRAIL expressed by IFN-γ- or TNF-α-activated DC can induce apoptosis of activated effector T-cell by recognizing its ligand on target cells [160].

1.3 Activation of DC

Two functional states of DC were defined as immature DC and mature DC. Immature DC has an efficient endocytosis ability facilitating antigen uptake, low surface MHC as well as co-stimulatory molecule expression level, and poor cytokine secretion and migration capability. On the contrary, mature DC display a phenotypic and functional transformation. Matured DC do not capture antigens actively, but can migrate to secondary lymph organs with stable high level expression of MHC and co-stimulatory molecules on cell surface as well as up-regulated cytokine production, which favor T cell priming. Recognition of pathogens-derived stimuli via PRRs and sensing of inflammatory cytokines and cytosolic compound are two types of signals that induce response of DC. Upon stimulation of these signals, DC switch to mature state and become potent T cell stimulators.

1.3.1 Pathogen-associated molecular pattern (PAMP) induced DC activation

Various microbe-derived pathogenic compounds induce DC maturation, such as lipopolysaccharide (LPS), lipoproteins, double-stand and nucleic acids [161]. Most of them are recognized by germline-encoded PRRs [162, 163]. So far, four types of PRRs have been identified, such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and Rentinoic acid-inducible gene-I-like receptors (RLRs). TLR is a big well-characterized family of PRRs expressed on cell surface and endosome, and more than 10 subsets of TLRs have been defined in DC. Different types of pathogen-associated molecular pattern are specifically distinguished by one or a combination of TLRs. For example, LPS is recognized by TLR4, lipoproteins of bacteria and mycoplasma are sensed by TLR2 with combination of TLR1 or TLR6 [164, 165], and viral and bacterial nucleic acids are detected by a set of TLRs including TLR3, TLR7, TLR8 as well as TLR9.

1.3.2 Lymphocytes-induced DC activation

DC maturation can be induced by crosstalk with lymphocytes such as B cells and T cells. Previous studies showed CD4$^+$ T cells induce DC maturation in vivo and in vitro. For example ligation of CD40 on DC with CD40L on T cells triggers DC maturation [166, 167]. Furthermore functional maturation can also been induced by crosslinking of OX40L and Fas on DC with OX40 and FasL on T cells respectively [168, 169]. Research evidence also showed B-cell modulated DC maturation [170]. Splenic DC in B cell-deprived mice could not induce IL-4 producing helper T cells [171, 172]. Moreover a consistent reduction of DC in spleen of B cell-deprived mouse was observed [171, 173]. Previous studies indicated that B cell regulate maturation, migration and survival by providing various cytokines and chemokines such as B cell-produced IL-16, MIP-1α and MIP-1β [173-176], and lymphotoxin expressed on the surface of B cells was also proved to affect DC homing [177, 178].

1.3.3 FcR-mediated DC activation

Various subsets of FcR have been identified on human and mouse DC including FcγR, FcεR and FcβR. FcγRI, FcγRIII and FcγRIIB are epxressed in mouse BMDC, splenic DC and Langerhans cells [53, 179-181]. DC activation can be mediated by engagement of FcγR by immune complex and Abs, and this process is mediated by cytoplasmic immunoreceptor tyrosine-containing activation motif (ITAM) in FcR-associated γ-chain [179, 182, 183]. On the contrary, engagement of FcγRIIB induces inhibition of DC maturation, and this is mediated by immunoreceptor tyrosine-based inhibitory motif (ITIM) which inhibits ITAM-mediated activation signal by recruiting the inositol-phosphatase SHIP [184, 185]. Therefore immune complex-induced DC response via FcγR is determined by balance of activating and inhibitory signal in DC.

1.3.4 Danger signal-induced DC activation

Inflammatory mediators such as TNF-α, IL-1β and PGE2 [4, 186] secreted by macrophages and neutrophils and cell death can be sensed by DC as activation signal. For example TNF-α production can be induced by virus infection, studies showed in TNF-α-deficient mice, DC isolated from local lymph node were relatively immature compared to DC in WT mice, moreover TNF-α is required for DC maturation upon virus stimulation in vitro [187]. Furthermore, studies showed that intracellular compounds released by necrotic and apoptotic cells, such as nucleotides from dead cell and necrotic tumor cells, induced DC maturation [188].

1.4 TLR-mediated DC maturation and signaling pathways

1.4.1 TLR expression of DC

TLR is one of best-characterized families of PRRs. TLRs are type I transmembrane proteins which consist of N-terminal leucine-rich repeats, transmembrane domains and intracellular Toll-interleukin 1 receptor (TIR) domains. So far 10 TLRs have been identified in humans and 12 TLRs in mice [189]. Studies have revealed distinct functions of TLRs in recognition of different PAMP [162, 163] and self-components [188] as well as in induction of immune response [161]. Varieties of PAMP can be recognized by TLRs including lipids, lipidproteins, proteins and nucleic acids derived from bacteria, various, fungi and parasites [161].

According to cellular localization and PAMP ligands, TLRs can be divided into two classes. One class is cell-surface-expressed TLRs which recognize mainly microbe-derived lipids, lipoproteins and proteins, including TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11[161]. The other class of TLRs, comprising TLR3, TLR7, TLR8 and TLR9, are localized in intracellular vesicles such as ER, endosomes, lysosomes as well as endolysosomes, recognizing pathogen-derived nucleic acids [161].

TLR2 recognize wide range of components from bacteria, virus, fungi and parasites [161], such as lipoproteins from bacteria and mycoplasma, lipoteichoic acid and peptidoglycan rom Gram-positive bacteria, hemagglutinin protein from measles virus, zymosan from fungi and t-GPI-mucin from *Trypanosoma cruzi* [190]. TLR2 recognize its ligands generally by forming heterodimers with TLR1 or TLR6. Distinct ligands can be sensed by the TLR2/TLR1 and TLR2/TLR6 complexes, such as triacyl lipoproteins from Gram-negative bacteria and mycoplasma and diacyl lipoppoteins from Gram-positive bacteria and mycoplasma respectively [191, 192]. Moreover, TLR2 can associate with other co-receptors, such as CD36, to facilitate pathogen recognition [193]. In DC, activation of TLR2 induce production of inflammatory cytokines, but not type I interferon.

Together with MD2, TLR4 responds to LPS that is derived from Gram-negative bacteria and can cause septic shock [194]. The resulting TLR4-MD2-LPS complex initiates signal transduction. In addition to LPS, TLR4 also recognize fusion protein of respiratory syncytial virus, pneumolysin of *Streptococcus pneumoniae* and paclitaxel [161]. Moreover TLR4 recognizes endogenous molecules of damaged cells induced by H5N1 avian influenza virus infection and modulates the resultant pathogenesis [195].

TLR5 is largely expressed by CD11c$^+$CD11b$^+$ lamina propria DC (LPDC) in the small intestine [196]. TLR5 reacts to flagellin protein derived from flagellated bacteria [161] and activated LPDC promote antigen-specific Th17 response and induce B cell differentiation into IgA-producing plasma cells [196]. TLR11 expressed in mouse but not human, has been shown to recognize *Toxoplasma gondii*-derived profiling-like molecule as well as uropathogenic bacteria [197].

TLR3 was identified to detect viral dsRNA and poly(I:C), which induce production of type I interferon and inflammatory cytokines and promote antiviral immune responses [198]. However TLR3 is critical for IL-12p40 but not type I IFNs in sera [199]. Therefore it is suggested TLR3 plays an essential role in antiviral infection. TLR7 recognizes guanine analogs like loxoribine as well as ssRNA from RNA virus such as influenza A virus and HIV [161, 200]. Some small interfering RNAs and synthetic poly(U) RNA can also be recognized by TLR7 [201]. Moreover in cDC, TLR7 senses RNA from bacteria like group B Streptococcus [202]. TLR9 detects bacterial and viral unmethylated DNA with CpG motifs. Though synthetic CpG oligodeoxynucleotides can activate DC via TLR9, the DNA sugar backbone is essential for TLR9 recognition [203]. Moreover hemozoin produced by the malaria parasite and a crude exptract of malaria parasite can activate TLR9 in endosome [204]. Both TLR7 and TLR9 are highly expressed in pDC, and nucleic acids recognition via TLR7 and TLR9 can induce pDC to produce large amount of type I interferon and initiate antiviral responses.

1.4.2 TLR signaling pathways

Recognition of PAMPs via TLRs initiates transcriptional up-regulation of distinct inflammatory genes. Intracellular TIR domains in TLRs are responsible for activating the signaling cascades, by recruiting TIR domain-containing adaptor proteins to TLRs [161]. So far, five adaptor proteins involved in TLR signal transduction have been identified, including MyD88, TIRAP/Mal, TIR domain-containing adaptor inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM) and Sterile-alpha and Armadillo motif-containing protein (SARM). MyD88, utilized universally by TLRs except TLR3, is the first identified TIR domain-containing adaptor, which ultimately activates mitogen-activated protein kinases (MAPKs) and NF-κB and initiates production of inflammatory cytokines [161]. TRIF associates with TLR3 and TLR4 signaling transduction and induces activation of IRF3 and NF-κB resulting in secretion of type I interferon and inflammatory cytokines. TIRAP participates the recruitment of MyD88 to TLR2 and TLR4, moreover TRAM assists recruitment of TRIF to TLR4. Therefore, TLR signaling pathways are generally divided into two categories: MyD88-dependent pathways and TIRF-dependent pathways.

Upon recognition of PAMPs of TLRs, IL-1 receptor-associated kinases IRAK4 is recruited by MyD88, and subsequently other IRAK members IRAK1 and IRAK2 are activated by IRAK4 [205]. And then IRAKs dissociate from MyD88 and interact with TNFR-associated factor 6 (TRAF6), an E3 ubiquitin protein ligase that catalyzes the synthesis of polyubiquitin chain linked to Lys63 (K63) on TRAF6 and IRKA1, together with E2 ubiquitin-conjugating enzymes Ubc13 and Uev1A [206]. The K63 polyubiquitin chains bind to TAB2 and TAB3 and activate TGF-β-activated kinase 1 (TAK1), allowing phosphorylation of IκB kinase-β (IKKβ) by TAK1 [207]. And then IκBa is phosphorylated by IKK complex comprising IKK-α, IKK-β and NK-κB essential modulator (NMEO), and undergoes degradation, which frees NF-κB to translocate into nucleus and initiate transcription of proinflammatory cytokine genes. Moreover TAK1 also phosphorylates the MAPKs including ERK1/2, p38 and JNK, inducing activation of another transcription factor complex AP-1. NF-κB and AP-1 activation via MyD88-dependent pathways are responsible for inflammatory cytokine production such as IL-6 and IL-12p40 [208, 209], moreover TLR7 and TLR9 in pDC induce type I interferon production in a MyD88-dependent manner.

TLR3 recruits TRIF after recognition of dsRNA, leading to recruitment of TRAF3 and TRAF6 via TRAF-binding motifs, which activates TAK1 by ubiquitination-dependent manner and finally activates NFκB. TRIF also interacts with RIP1 and RIP3 via a C-terminal receptor-interacting protein (RIP) homotypic interaction motif (RHIM). RIP1 undergoes K63-linked polyubiquitination with the association of TNFR-associated death domain protein (TRADD), required for NF-κB activation. Moreover TRIF-dependent pathways mediate IRF3 activation and IFN-β expression. In response to stimulation of TLR3, TLR7 and TLR9, TRIF recruits TBK1 and IKKi that catalyze IRF3 phosphorylation and promote its translocation into nucleus, in which participation of TRAF3 is required [210-212]. TLR4 also interacts with TRIF indirectly in a TRAM-dependent mechanism. Therefore, TRIF signaling is mediated by multiple proteins, which in turn activate NF-κB and IRFs [198].

1.4.3 TLR-mediated regulation of DC function

Transformation from immature DC from activated DC upon PAMP recognition via TLRs indicates that TLR activation play a crucial role in DC function modulation. In addition to up-regulated inflammatory cytokine production and surface molecule expression, TLR recognition also affects antigen uptake, migration as well as antigen presentation.

Previous studies indicated that a few hours after LPS stimulation, mouse BMDC or splenic DC displayed transient enhanced endocytosis and phagocytosis efficiency [213]. It is demonstrated that both Erk1/2 and p38 play essential role in this process [213], however the molecular mechanism has not been investigated. Afterwards endocytosis capability of DC decreases gradually, however DC show enhanced migration ability. After maturation DC migrate to spleen T-cell zone and lymph nodes, where activated antigen-bearing DC start to prime T cell response. Both endocytosis and migration are tightly regulated by cytoskeleton organization. Though the molecular mechanism of cytoskeleton rearrangement is not well understood, two molecules MARCO receptor in mouse splenic DC, and actin-bundling protein fascin have been demonstrated to associate with this process [213-215]. On the other hand up-regulated chemokine receptors expression after maturation also guides DC migration, such as CCR7 that responds to CCL19 and CCL21 [216, 217].

TLR activation also regulates antigen processing. Compared to macrophages and neutrophils, cDC display a lower antigen processing efficiency [218], because lysosomal proteases are not recruited efficiently to the phagosomes, and activity of lysosomes and phagosomes is inhibited by cysteine protease inhibitors produced by cDC [219, 220]. On the contrary, with TLR activation, antigen peptide generation is enhanced due to increase of proteasome and lysosomal function efficiency [221-223]. Previous studies have shown that LPS-conjugated antigens were presented preferentially [224]. Moreover cross-presentation and $CD8^+$ T cell priming are can be augmented when antigen is captured together with TLR agonists [225, 226].

1.4.4 TLR-mediated regulation of DC survival

DC survival and apoptosis are regulated by TLR activation. TLR9 engagement up-regulates expression of Bcl-2 and Bcl-xL and inhibits activity of caspase 3 via PI3K pathways, which sustain DC survival [227]. Moreover LPS can enhance DC survival with absence of growth factor in an Erk-dependent mechanism [90].

After T-cell priming, DC undergo apoptosis thereby maintaining homeostasis of T cell responses. One mechanism is CD14-dependent, which activates src kinases and phospholipase c $\gamma 2$ and elevated intracellular Ca^{2+} concentration, followed by translocation of nuclear factor of activated T cells (NFAT) and ultimately apoptotic death of DC. Another mechanism reported is death receptor-dependent. Engagement of FAS and TRAIL on surface of DC with their ligands on T cells triggers DC apoptosis.

1.5 Scaffold proteins in immune system

1.5.1 Function of scaffold proteins

Scaffold proteins are a group of highly heterogeneous proteins that bind multiple signaling components and facilitate their communication or interaction with each other. Scaffold proteins can function as platforms that assemble signaling molecules, or localize signaling molecules at specific sites, or modify the signaling pathways by coordinating positive and negative feedback signals, or activate signaling molecules. Therefore scaffold proteins augment complexity to signaling cascade and regulate signaling transduction.

Assembly of signaling molecules is a basic function, which enhance signaling transduction efficiency and specificity [228] in signaling cascade by strengthening spatial interaction of protein kinase and its substrate. In addition to enhancing kinase specificity, scaffold proteins that have multiple binding sites can limit signal amplification by binding distinct kinases at all sites and restricting kinase to phosphorylate no more than one target molecules [228]. Moreover mathematical model studies of multiple-molecule-binding scaffold proteins in MAPKs have suggested that they can either amplify or inhibit signaling cascade [229-232], and this capability is related to the expression levels of the scaffold proteins and signaling molecules [229], affinity and interaction stability between the scaffold protein and each binding component as well as location of signal cascade transducers in the cell [230]. Studies on engineered yeast MAPK scaffold protein Ste5 showed that scaffold protein enhanced the signaling transduction when binding to a positive regulator, whereas binding to a negative regulator suppressed signaling response [233]. Research on A-kinase anchor proteins (AKAPs) investigated that AKAPs facilitated local regulation of cyclic AMP-dependent protein kinase (PKA) and phosphorylation of its substrates by localizing PKA to various sites in a cell [234].

1.5.2 Scaffold protein in immune cells

So far, various types of scaffold proteins have been identified in immune cells such as T cells, B cells and DC. Though further studies are necessary to describe their function and regulation mechanism precisely, it is demonstrated that different families of scaffold proteins display complex function by participating the regulation of distinct signaling pathways.

In MAPKs pathway, diversified scaffold proteins regulate signal cascades mediated by ERK, JNK and p38. Kinase suppressor of RAS (KSR) has been identified to regulate RAS-MAPK pathway positively, by binding to RAF and MEK1 in a pseudokinase domain-dependent manner and ERK in a serine/threonine-rich domain-dependent manner [235]. KSR can also bind 14-3-3 proteins at serine 297 and serine 392 to localize to the cytoplasm, and upon activation two sites are dephosphorylated resulting in release of KSR to cytoplasm [236, 237]. These features allow KSR to interact with activated RAS and localize the activated ERK to the plasma membrane. In mammalian cells two isoforms of KSR have been identified: KSR1 and KSR2 [238, 239]. In KSR1-deficient mice, a defective ERK activation was observed in T cells which leading to an attenuated cytokine production and impaired T cell proliferation [240]. However, development of thymocytes seemed to be unaffected in KSR1-deficient mice [240], though KSR1 was demonstrated highly expressed in thymus [225, 240]. In neutrophils and macrophages, KSR1 is indispensible for ERK activation in response to simulation of TNF, IL-1 or osmotic shock [241]. Moreover, KSR1-deficient mice are shown to be insusceptible to serum-induced arthritis [241]. MEKK1, which can bind JNK, MAPKK4, MAPKK7 and NF-κB, has been shown to be a crucial scaffold protein in activation of JNK, ERK and p38 [232, 242]. Recent research has been shown that MEKK1 is also important in CD40-dependent JNK activation [242, 243]. BCL-10 composed of an N-terminal caspase-recruitment domain (CARD), and a serine/threonine-rich domain, functions as a scaffold protein for JNK2 activation specifically downstream of TCR signaling [244].

Calcium signaling is also crucial for regulation of immune cell functional properties. Calcium influx plays an essential role in translocation of NFAT to nucleus, which is important for activation and proliferation of T cells [245]. AHNAK1 has been defined as a scaffold protein facilitating localization of calcium channels at the plasma membrane for efficient calcium signaling and NFAT activation in T cells [246]. In AHNAK1 deficient mice, T cells displayed a defective NFAT translocation and impaired proliferation and cytokine production, due to a defect in the calcium influx. However, the exact mechanism has to be further studied. Another family of scaffold proteins functioning in calcium signaling named Homer scaffold proteins, originally described in neuronal cells, have been shown plays a role in T cells recently [247]. In activated T cells, Homer2 and Homer3, down-regulated NFAT activation and IL-2 production by competing with calcineurin to bind to the N-terminus of NFAT therefore inhibiting dephosphorylation of NFAT [247]. In HOMER-deficient mice, upregulated NTAF activation and IL-2 production were observed. Moreover, compared to wild type mice, higher number of memory T cells accumulated in the peripheral tissues and this may be responsible for a severe autoimmunity in pneumonitis-disease model [247].

Innate immunity is also regulated by scaffold proteins, demonstrated by identification of scaffold proteins functioning in the signaling pathways of PRRs. Three members of

Pellino proteins have been identified in human and mice. Research in recent years has demonstrated that they can bind IRAK1, TAK1 and TRAF6 after TLR activation [248, 249]. Moreover evidence also suggested Pellino proteins can bind NIK [250] and BCL-10 [251], though the function is unclear. NLR is another family of PRRs, detecting toxins, bacterial DNA and other chemical stimuli in cytosol [252]. NALP is a subset of NLR, containing an N-terminal pyrin domain, a NACHT domain, a NACHT-associating domain and a leucine-rich repeat domain [253]. NALP1 has been shown to function as scaffold protein mediating inflammasome assembly [254]. Activation of NALP1 leads to binding of adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), resulting in transformation of pro-caspase1 to caspase1 and release of functional IL-1β and IL-18 [255]. Furthermore CALP1 can bind Bcl-2 and Bcl-xL, which suppress activation of caspase1 [256].

A family member of DGL proteins DGL1 is highly expressed in lymphocytes, which plays an important role in T-cell activation [257]. It is reported that DGL1 associates with ζ-chain of TCR and ZAP70, LCK, VAV1, Casitas B-linage lymphoma, WASP, and p38 [258-261]. Previous studies showed hyperproliferative T-cell response upon TCR activation in DLG1-deficient mice, indicating DGL1 might negatively regulate T cell proliferation [257]. Moreover NFAT, p38 phosphorylation and cytokine production were both inhibited in DLG1 knockdown TCR-transgenic $CD8^+$ T cells [259, 260], and overexpression of DGL1 can enhance NFAT activation [259]. Therefore these studies indicate that DGL1 positively regulate p38 and NFAT activation.

In DC, scaffold protein spinophilin has been identified. Upon DC maturation, spinophilin associates with the formation of immunological synapses following contact with T cells [262]. And antigen-specific $CD4^+$ T cells cannot be activated by spinophilin-deficient DC in vivo or in vitro [262]. Spinophilin is also detected in B cells and T cells, but its role in these cells is still unclear.

1.6 biological role of JLP in signal transduction

JLP is a novel member of JNK interacting protein family, composed of leucine zipper domains, a C-terminal domain as well as three SH2- and SH3-binding sites [263]. A screen for Myc/Max-interacting protein indicated that JLP interact with Max through the first leucine zipper domain, whereas with Myc through a domain next to the two leucine zipper domains and a domain adjacent to the N-terminus [263]. Moreover JLP also interacts with MEKK3, MKK4 and the MAPKs downstream components JNK1 and p38MAPKα [263]. It is reported that JLP can enhance the JNK activation in several cell types [263-266].

It has been demonstrated that JLP physically interact with α-subunit of G12 family, which is involved in JNK activation. JLP interacts with $G_{\alpha12}$ or $G_{\alpha13}$ through C-terminal region [264, 265] and it has been shown that JLP is indispensable for JNK activation by $G_{\alpha12/13}$ coupled LPA receptors [267]. Furthermore interaction of JLP with kinesin motor proteins through kinesin light chain 1 (KLC1) has been observed [266]. Therefore, JLP plays a crucial role in the regulation of JNK signaling pathway. Moreover, a recent study has demonstrated that JLP played a role in gep oncogene-regulated neoplastic signaling pathway, in which JLP was required for the stimulation of JNK by $G_{\alpha 12}$ [268].

JLP was identified to interact with Cdo-intracellular domain in a yeast two-hybrid screen for Cdo-interacting protein [269]. Studies in mouse myoblast cell line and primary mouse myoblast cell showed JLP played a crucial role in Cdo-mediated p39MAPK activation and myogenic differentiation. Furthermore interaction of JLP with MKK4 and MEKK3 that can activate p38MAPK suggest that JLP associates with p38MAPK activation. Recently a novel physical interaction between JLP and N-cadherin has been identified, and this study also showed that N-cadherin negatively regulated JLP-mediated p38 activation in neurons [270].

JNK-interacting protein-4 (JIP4) [271] and sperm associated antigen 9 (SPAG9) [272, 273] have been identified as two splice variants of JLP. They share common 3’ exons but contain different 5’ exons. JIP4 was highly expressed in brain, liver, kidney and testis [271]. Previous studies suggested that JIP4 is involved in MEKK3- and MKK4-mediated JNK activation, and in MKK3- and MKK6-mediated p38MAPK activation [271]. Previous studies have shown that SPAG9, an 84 kDa splice variant, interacts with JNK1, JNK2 and JNK3 [272, 273]. However, SPAG9 does not interact with other MAPKs. It has been shown that interaction of SPAG9 with JNK plays a role in spermatid development [274].

1.7 Dendritic cell and autoimmune diseases

Recognition of self-component by T and B lymphocyte can induce autoimmune responses. Self-reactive T cell and B cell can be eliminated through negative selection of central tolerance and other mechanisms of peripheral of peripheral tolerance. The failure of any of these mechanisms in elimination of self-reactive clones of lymphocytes can result in autoimmunity. Moreover, bacterial and viral antigens that cross-react with self-antigens may initiate or enhance autoimmune response [275, 276]. Thus autoimmune diseases are result of de-regulated self-reactive immune responses mediated by diverse types of immune cells including lymphocytes as well as antigen presenting cells.

Accumulative evidences from studies on autoimmune disease models indicated that self-antigen bearing DC displayed a mature phenotype and are capable of triggering autoimmune responses through inducing Th1 and Th17 responses [277]. Activation of self-reactive T cells by DC loaded with self-antigen also lead to imbalance of self-tolerance and autoimmunity. The pro-inflammatory environment at sites of autoimmunity can shift tolerogenic DC towards immunogenic DC, and augment autoimmune diseases.

Studies of DC in patients have shown that aberrant DC activation and functions facilitate diverse autoimmune diseases including Rheumatiod Arthritis (RA), Systemic Lupus Erythematosus (SLE), Inflammatory Bowel Disease (IBD), Multiple Sclerosis (MS) as well as Psoriasis.

The observation of increased DC number in peripheral blood, synovial fluids and tissues of RA patients was reported [277]. Further studies have shown that DC from synovial fluid of RA patients expressed high level of HLA-DR and co-stimulatory molecules and these DC associated with T cells in a manner similar to germinal centers [278]. DC are also involved in promoting synovial inflammation by secreting pro-inflammatory cytokines [279]. In SLE hyper-activation of DC leads to chronic inflammation [280] and these DC contribute to effector T cell activation due to their mature phenotype [281, 282] and facilitate the sustaining of autoimmune responses via stimulating production of IFN-γ and IL-17 by T cells [283]. In Crohn's disease and ulcerative colitis patients, abnormal accumulation of DC expressing BDCA-1 in intestine has been observed in several studies, and these DC induced excessive T cell activation [284-286]. Observation of activated DC in the central nervous system of MS patients was reported [287], and their presence in the demyelinating lesions suggests that DC are involved in activation of T cell response to myelin in central nervous system [288]. Moreover, DC isolated from peripheral blood of MS patients were reported to produce higher level of pro-inflammatory cytokines [289] and these DC induce $CD4^+$ T cell differentiation into IFN-γ producing T cells [289, 290]. 30-fold increased frequency of DC in psoriatic lesions compared to normal skin was observed [291]. These DC activate production of IL-1 and IL-6 by fibroblasts by secreting TNF-α, IL-12, IL-23 and inducible nitric oxide synthase [292], therefore contributing to induction of effector Th1 and Th17 cells and dermal inflammation as well as epidermal hyperplasia [293, 294]. These evidences demonstrate that DC play a role of key factor in promoting progression of autoimmune diseases by inducing and augmenting self-reactive T cell responses.

The critical role of DC in progression of autoimmune diseases indicates that DC can be target for treatment of autoimmune diseases. So far, two approaches have been introduced to modulate DC in autoimmune diseases. Monoclonal antibodies are currently under clinical investigation as a method to reduce the DC immunogenicity. For example, administration of Anakinra in combination with methotrexate showed good effect in RA patients [295, 296]. Anti-TNF-α-mAb also has been demonstrated to be helpful for treatment of RA, Crohn's disease and psoriasis [297-299]. Blocking the co-stimulatory molecules by using antibodies has been demonstrated to be effective in pre-clinical models. Though therapies with monoclonal antibodies to inhibit pro-inflammatory cytokines or co-stimulatory molecules are effective, this method require long-term administration with side effect. Another strategy for treatment of autoimmunity is to enhance the tolerogenic DC function. Various agents have been to modify the functional properties of DC. Previous studies have shown that vitamin D3 [300], dexamethasone [301], or cytokines such as IL-10 [156, 302-304] or TNF-α [305, 306] are capable to modify the phenotype and restore the function of tolerogenic DC. Moreover protocols to generate clinical grade human tolerogenic DC with IL-10 [307], vitamin D3 and dexamethasone [308] have been developed.

Previous efforts on the study of DC pathological role in autoimmune diseases have demonstrated that self-antigen bearing DC is critical for the autoimmune diseases progression and modulation of aberrant DC function became a therapeutic approach for autoimmune diseases. Therefore, studies on the factors that regulate DC maturation and functions will contribute to understanding the mechanism of de-regulation of DC function as well as development of therapies for autoimmune diseases.

Rationale

JNK-associated leucine zipper protein (JLP) is a novel scaffold protein. Previous studies have demonstrated that JLP associates with JNK/p38 phosphorylation and further regulates the biological function of various cell lines and tissues, such as HeLa cells, P19 embryonic carcinoma cells and human brain tissues. However functional role of JLP in immune cells has never been reported.

Previously, our lab demonstrated a novel role of JLP in regulating the transportation of CD40 molecules between plasma membrane and cytosol of DC. Therefore this study showed JLP play a role in regulating DC function. Furthermore, we found JLP expression was up-regulated in BMDC activated via TLR4 or TLR3, by LPS or poly(I:C) respectively. However it is demonstrated that LPS and poly(I:C) activated DC via different signal pathways and lead to distinct functional properties of mature BMDC, though MAPKs pathway is shared by TLR4 and TLR3. Therefore it suggested that JLP play differential roles in BMDC matured by LPS and poly(I:C) respectively.

Global hypothesis

We hypothesize that JLP plays a differential role in regulating LPS- and poly(I:C)-induced BMDC maturation and functions.

Objectives

We analyzed functional role of JLP in BMDC by silencing JLP expression with established lentiviral gene silencing system:

1. Examine surface molecule expression on JLP-silenced BMDCs activated by poly(I:C) and LPS.
2. Detect the cytokine production on JLP-silenced BMDCs activated by poly(I:C) and LPS.
3. Examine the antigen presentation ability of JLP-silenced BMDCs activated by poly(I:C) and LPS.
4. Detect the survival of JLP-silenced BMDCs activated by poly(I:C) and LPS

Chapter 2 Material and Methods

2.1 Mice and cell lines

Six- to eight-week-old female C57BL/6 mice were purchased from GMC and were maintained in the animal facilities at the University of Manitoba according to the recommendations of the Canadian Council of Animal Care.

293T cell line, derived from human embryonic kidney cells, was cultured in IMDM medium supplemented with 1% PSG and 10% fetal bovine serum (FBS). The OVA 257-264-specific and K^b-restricted T cell hybridoma, RF33.70, was cultured in RPMI 1640 medium containing 1% PSG, 10% FBS. The T cell hybridoma BO97.10, recognizing OVA 323-339 that binds to I-A(d) MHC-II, was also cultured in RPMI 1640 medium containing 1% PSG, 10% FBS. DC2.4 cell line was cultured in RPMI 1640 medium supplemented with 1% PSG, 10% FBS and 2-mercaptoethanol (2-ME).

2.2 Generation of bone-marrow-derived dendritic cells

Bone marrow cells were extracted from the femur and tibiae of C57BL/6 mice by flushing the bones with RPMI 1640 medium through a cell strainer, and centrifuge at 1200 rpm for 5 minutes. Resuspend cell pellet with ACK buffer lysis buffer and centrifuge at 1200 rpm for 5 minutes. And then wash the pelleted cells with 5ml RPMI 1640 medium and collect the cells by centrifuging at 1200 rpm for 5 minutes. Resuspend pelleted cell in complete RPMI 1640 medium supplemented with 20ng/ml GM-CSF, and culture the cells in 12-well plate 0.5×10^6 cell/well at 1ml, and add 1ml culture medium on day 3. At day 7 the percentage of CD11c$^+$ cells should be about 80%-90% as measured by FACS analysis. BMDC are ready for experimental use.

2.3 Production, concentration and titration

AgeI/EcoRI-digested pLKO.1-puro vector containing JLP-shRNA-sequence expressing hairpin sequence (GenBank accession no. AF327451) was used for pLKO.1 HIV-based lentiviral vector preparation in 293T cells. And pLKO.1 HIV-based lentiviral vector expressing EGFP-shRNA was used as the negative control. 20×10^6 293T cells in 20ml complete IMDM medium were plated in a T175 flask (BD Falcon) on the day before transfection. On the day of transfection, 25ml of complete IMDM medium containing 100μl chloroquine (Sigma) was added to the flask before adding plasmid mix and incubate at 37 °C and 5% CO_2, to prevent the degradation of DNA plasmids in cell lysosome. To make plasmid mix, 12.5μg 8.2Δvpr, 5μg p-VSVG and12.5μg gene therapy vector were mixed in a 50ml Falcon tube for one T175 flask use. Add cell culture grade water to a final volume of 997μl. Shake the mixture vigorously and incubate on ice for 10 minutes. And then add 133μl 2M $CaCl_2$ drop by drop and mix well. The solution was incubated on ice for 5 minutes. After incubation, 1110μl 2× HBS buffer was added to the solution drop by drop and was mix well, and then the solution was incubated on ice for 20 minutes. Then the transfection solution was added to the roof of T175 flask and was mixed well gently. The flask was then incubated at 37 °C and 5% CO_2. 6-8 hours later, the transfection solution was removed by aspiration and 40ml IMDM medium containing 1% PSG and 10% calf serum was added to the flask mildly. 3 days after transfection, supernatant was harvested and centrifuged at 1200 rpm for 5 minutes to remove the debris of 293T cells. The centrifuged supernatant was then filtered with Nelgene filters.

To concentrate the produced lentivirus, the filtered supernatant was transferred to ultracentrifuge tube and was centrifuged at 17,000 rpm for 1.5 hours at 4 °C. And then the supernatant was discarded and 200µl IMDM medium to the pellet. The pellet was left overnight at 4 °C and was then resuspended and collected. The concentrated lentivirus was stored at -80 °C.

The titer of virus was tested by titration on 293T cells. 293T cells were plated in 24-well plate 5×10^4 cells/well on the day before titration. On the day of titration, the lentivirus was diluted to 1× and 1/10× using complete IMDM medium. Polybrene was added to the diluted virus to a final concentration of 8µg/ml. The medium in 24-well plate was removed and 250µl diluted virus was added into each well. And then the 293T cells were incubated at 37 °C and 5% CO_2 for 2 hours. After incubation the diluted virus was replaced by fresh complete IMDM at a volume of 1ml/well. For pLKO.1 vectors, puromycin was added to the transduced 293T cells after 1 day of transduction. And 2 days after adding puromycin, transduced 293T cells were harvested, and number of viable cells and dead cells were counted by trypan blue staining. And the formula of titer is show as:

$$\text{Titre} = (1\text{x}10^5 \text{ cells}) \text{ x } (\text{\% of transduced cells}) \text{ x } (4) \text{ x } (\text{dilution factor})$$

2.4 Lentiviral transduction of BMDC

Bone marrow cells were tranduced at MOI of 10 on the day 2 of culture. Generally, the medium was replaced by 0.5ml mixture of 100× lentivirus, serum-free RPMI 1640 medium and 4μl polybrene for each well. And the cells were incubated at 37 °C and 5% CO_2 for 2 hours. After incubation, the 0.5ml mixture was removed form the well, and 1ml complete RPMI 1640 medium containing 20ng/ml GM-CSF was added to the cell culture. On day 4 of culture add another 1ml complete RPMI 1640 medium containing 20ng/ml GM-CSF. For LKO vector, on day 5 puromycin was added to the culture to a final concentration of 4μg/ml for selection of transduced cells. On day 7 the transduced cells were ready for experimental use.

2.5 Stimulation and treatment of BMDC

The 7-day immature BMDC were harvested and plated into 24-well or 96-well plates at a standardized number in a standardized volume of complete RPMI 1640 medium containing 20ng/ml GM-CSF. And then the cells were stimulated with LPS at a final concentration of 1μg/ml or poly(I:C) at a concentration of 10μg/ml for 24 hours. And then the medium was removed and fresh complete RPMI 1640 of same volume was added to the culture. In some experiments, the cells were further stimulated with mouse anti-CD40 antibody for another 24 hour after changing medium.

2.6 Western blot for detection of JLP expression in BMDC

BMDC were harvested by using 1mM EDTA and washed with cold PBS, and cell pellet was collected in 1.5ml eppendorf tube. Cells were lysed with 0.5 triton-X lysis buffer 20μl per 1×10^6 cells and incubated on ice for 30 minutes. The calibration of protein concentration was performed following the manual instruction of Quick Start^{TM} Bradford Protein Assay kit. The protein concentration was adjusted to 4mg/ml with lysis buffer. Then the samples were diluted to 2mg/ml with 2× loading buffer and boiled at 100 °C for 10 minutes. Equivalent amounts of samples were separated by 8% SDS-PAGE and transferred to polyvinylidene difuoride membranes. Membranes were blocked with 10% nonfat dry milk, washed and incubated in primary antibody overnight at 4 °C with gentle agitation. Primary antibodies were diluted 1:2000 in 5% nonfat dry milk. The primary antibody was a poly-clonal rabbit anti-mouse JLP antibody. The secondary antibody was anti-rabbit IgG conjugated with HRP. Loading controls were determined using anti-mouse GAPDH diluted 1:2000 in 5% nonfat dry milk. Visualized bands can be developed by ECL plus/advanced kit and the figures were acquired by flourchem 8800 system.

2.7 Flow cytometry analysis of cell surface phenotype

Samples were collected into the tubes for FACS, and were washed with 1× PBS supplemented with 2% CS and 0.2% sodium azide (FACS buffer). Supernatant was discarded and pellet was resuspended with 100μl FACS buffer. 20μl Fc blocker was added to each tube and incubated on ice for 10 minutes. And then appropriate antibodies cocktails were added into samples. The samples were mixed well and were incubated on ice for 15 minutes. The samples were washed with 1ml FACS buffer for each tube, and were centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended with 200μl FACS buffer. Finally the samples were ready for test and the results were analyzed with Flowjo.

2.8 Assay of BMDC apoptosis

LPS- and poly(I:C)-stimulated cells with or without CD40 crosslinking were harvested and washed with 1× binding buffer. 0.5μl Annexin-V-APC and 0.5μl 7-amino actinimycin (7-AAD) was added to each sample. The samples were detected by FACS and data was analyzed with Flowjo. Annxin-V$^-$ 7-AAD$^-$ cells were defined as viable cells, Annxin-V$^+$ 7-AAD$^-$ cells were defined as apoptotic cells and Annxin-V$^+$ 7-AAD$^+$ cells were defined as dead cells.

2.9 IL-12 and IL-6 measurement by ELISA

Supernatant of LPS- and poly(I:C)-stimulated cells with or without CD40 crosslinking were harvested and kept frozen (-20°). IL-12 and IL-6 were quantified in supernatants from BMDC culture by enzyme-linked immunosorbent assay (ELISA). Primary antibodies were purified anti-mouse IL-12 and IL-6 monoclonal antibodies purchased form ebioscience. Secondary antibodies were biotin-conjugated anti-mouse IL-12 and IL-6 monoclonal antibodies. And the samples were developed with alkaline phosphatase system. The results were measured with spectral Max190 system and were analyzed with SoftMax Pro software.

2.10 Antigen presentation assay

BMDC were pulsed with MHC-I-restrcted OVA 257-264 peptide, MHC-II-restricted

OVA 323-339 peptide at 1μl/ml and whole OVA protein at 600μg/ml, respectively with or without LPS and poly(I:C) stimulation. Then BMDC were co-cultured with K^b-restricted T cell hybridoma RF33.70 and MHC-II-restricted hybridoma BO97.10 T cell line at a ratio of 1:10 for 3 days. BMDC antigen-presentation ability through either MHC-I or MHC-II were determined by measuring IL-2 produced by these hybridoma cells [309, 310]. The supernatant of the co-culture was collected and the IL-2 was determined in supernatant by ELISA.

2.11 Statistical analysis

All data were reported as the mean±S.E.M. These data were analyzed using GraphPad® Prism 5.0. For multivariant comparison among groups, a one-way ANOVA was performed with the post analysis using Newman-Keuls test for comparing all pairs of columns. For two group comparison, t test was used. Differences were considered significant at $p<0.05$.

Chapter 3 Results

3.1 Titration of optimal poly(I:C) concentration for BMDC stimulation

To acquire mature BMDC, we used LPS and poly(I:C) as stimuli. We first determined the optimal concentration of LPS and poly(I:C) for DC maturation, defined by surface up-regulation of key co-stimulatory molecules, and lowest toxicity because it has been reported that activation of TLR3 was capable of inducing apoptosis in various cell types [311-313]. The optimal concentration of LPS was determined as 1μg/ml according to the protocol of our laboratory, whereas the optimal concentration of poly(I:C) was not identified.

To test optimal concentration for the up-regulation of surface molecule expression, we stimulated BMDC with poly(I:C) at various concentrations, and measured expression of MHC-II, CD40, CD86 and CD80 on the surface of BMDC. From the result (Fig. 1), we can tell poly(I:C) at both 10μg/ml and 15μg/ml displayed accessible up-regulation of MHC-II, CD40, CD86, moreover up-regulated CD80 was also detected on BMDC stimulated by poly(I:C) at 15μg/ml.

To further determine the optimal concentration for poly(I:C) stimulation, we tested the survival of BMDC treated by poly(I:C) at different concentrations. We stimulated the BMDC with poly(I:C) at different concentrations (1μg/ml~20μg/ml) for 24 hours, and then we acquired the absolute cell number of viable cells using trypan blue. The cell counting indicated that viability of BMDC decreased when poly(I:C) concentration reached 15μg/ml (Fig. 2).

Therefore, according to surface molecule up-regulation and cell viability, 10μg/ml ply(I:C) was identified as the optimal stimulant concentration. Furthermore this concentration was also adopted in the work of Jones L.A. et al [314] as the optimal concentration for DC maturation.

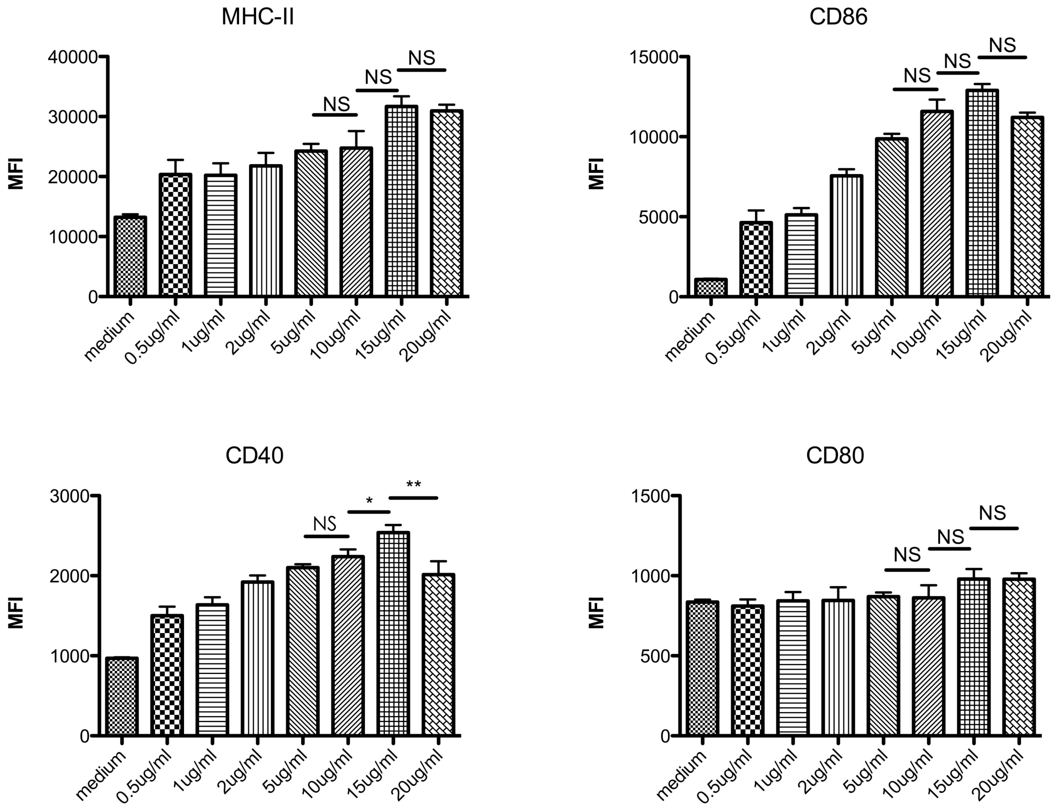


Figure 1: Up-regulation of surface molecule expression induced by distinct concentrations of poly(I:C). BMDC were stimulated with distinct concentrations of poly(I:C) (0.5μg/ml~20μg/ml) for 24 hours. Cells were harvested and stained with CD11c, MHC-II, CD86, CD40 and CD80, and detected with FACS. Molecule expression level was analyzed in term of MFI. (Results are the mean ± S.E.M. of n=3 treatments)

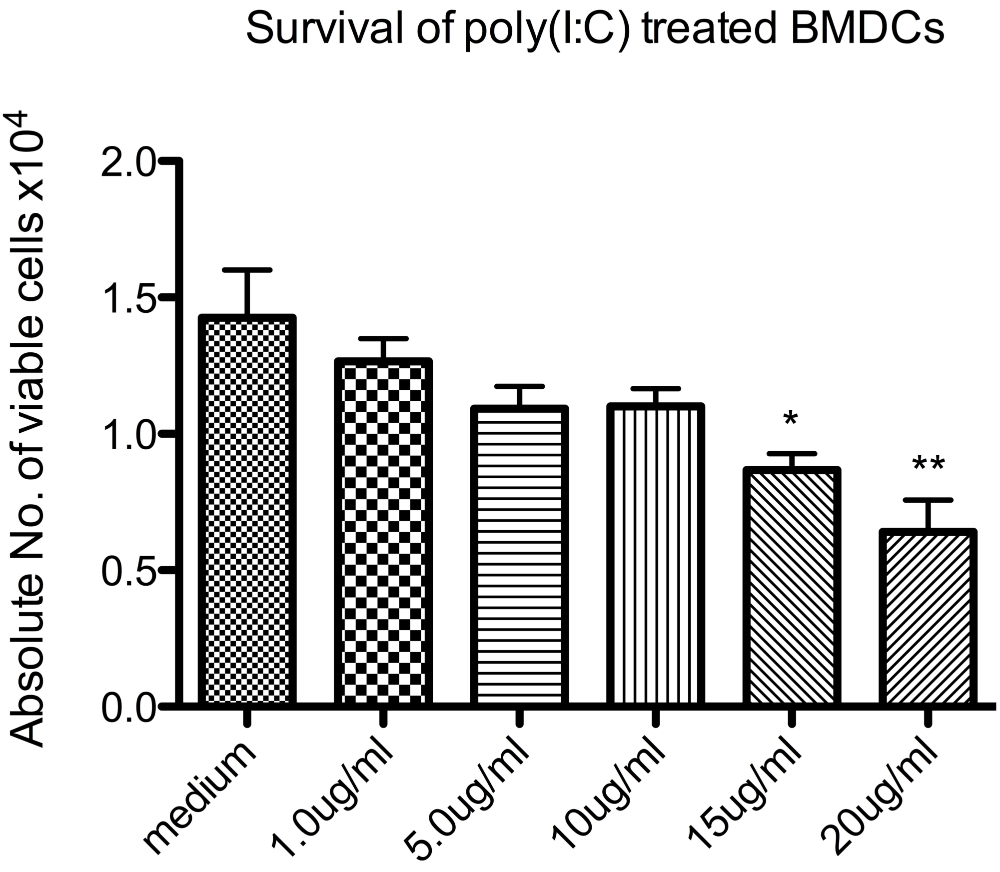


Figure 2: Viability of BMDC stimulated by poly(I:C) at various concentrations. BMDC were cultured (1.25x104/well) at volume of 0.5ml, and stimulated with poly(I:C) at different concentrations (1µg/ml~20µg/ml) for 24 hours, and then cells were collected and counted using trypan blue. (Results are the mean ± S.E.M. of n=3 treatments)

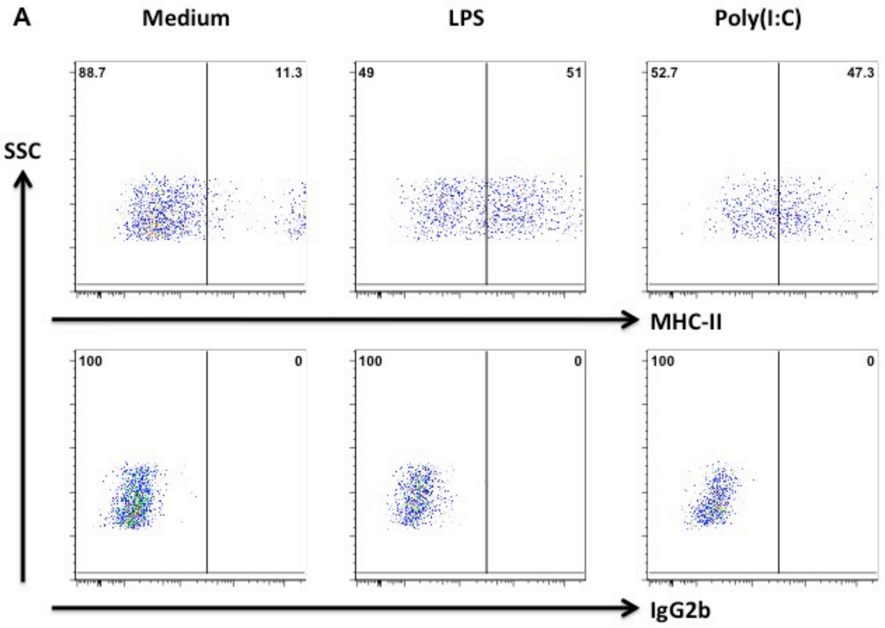
3.2 TLR4- and TLR3-mediated maturation induced differential surface molecule expression and IL-12 and IL-6 production in BMDC

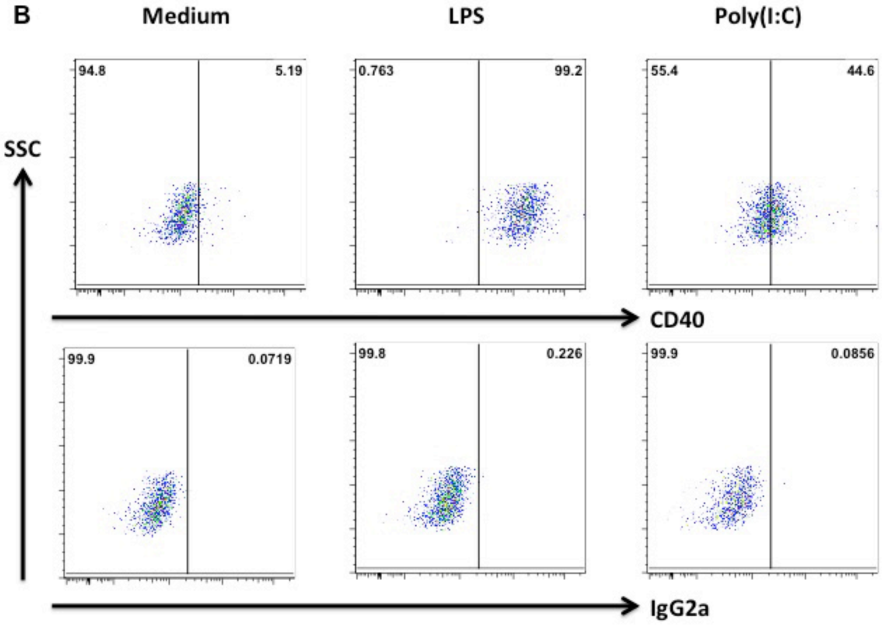
More than 10 subtypes of TLR have been identified in mice, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are displayed mainly on the cell surface detecting microbial molecules, and TLR3, TLR7, TLR8 and TLR9 are found localized endosome recognizing nucleic acids. Previous studies have demonstrated that activation of DC via distinct TLRs including TLR3 and TLR4, induced differential functional consequences of DC [314-316]. Therefore activation of DC induced via activation of TLR4 or TLR3 induce distinct DC phenotypes and functional properties.

To confirm the phenotype of BMDC activated by LPS and poly(I:C), we stimulated BMDC with LPS and poly(I:C), and analyzed surface molecule expression (Fig. 3&4). From our data, we observed that CD40, MHC-II and CD86 were significantly up-regulated by LPS and poly(I:C). However, MHC-II, CD40 and CD86 up-regulation induced by poly(I:C) was weaker than LPS stimulation. However CD80 expression was not elevated on surface of poly(I:C)-stimulated BMDC, but CD80 expression was up-regulated by LPS. Hence LPS was a more potent stimulus than poly(I:C) to induce up-regulation of MHC-II, CD40, CD80 and CD86.

We also examined the IL-12 and IL-6 production level of immature, LPS- and poly(I:C)-stimulated BMDC (Fig. 5). In supernatant of immature BMDC very low level of IL-12 was detected, a slight up-regulation of IL-12 was observed in poly(I:C)-stimulated BMDC but statistically not significant, whereas LPS was capable of enhancing IL-12 production in BMDC. IL-6 was not detectable in supernatant of immature BMDC, and IL-6 was up-regulated by either LPS or poly(I:C) stimulation, however, IL-6 produced by LPS-activated BMDC was significantly higher than poly(I:C)-stimulated BMDC. Therefore LPS stimulation induced higher level of IL-12 and IL-6 production comparing to poly(I:C) stimulation.

Conclusively, maturation of BMDC via TLR4 and TLR3 resulted in differential surface molecule expression and IL-12 and IL-6 production.


A
Medium
LPS
Poly(I:C)
88.7
11.3
49
51
52.7
47.3
SSC
MHC-II
100
0
100
0
100
0
IgG2b


B
Medium
LPS
Poly(I:C)
94.8
5.19
0.763
99.2
55.4
44.6
SSC
CD40
99.9
0.0719
99.8
0.226
99.9
0.0856
IgG2a

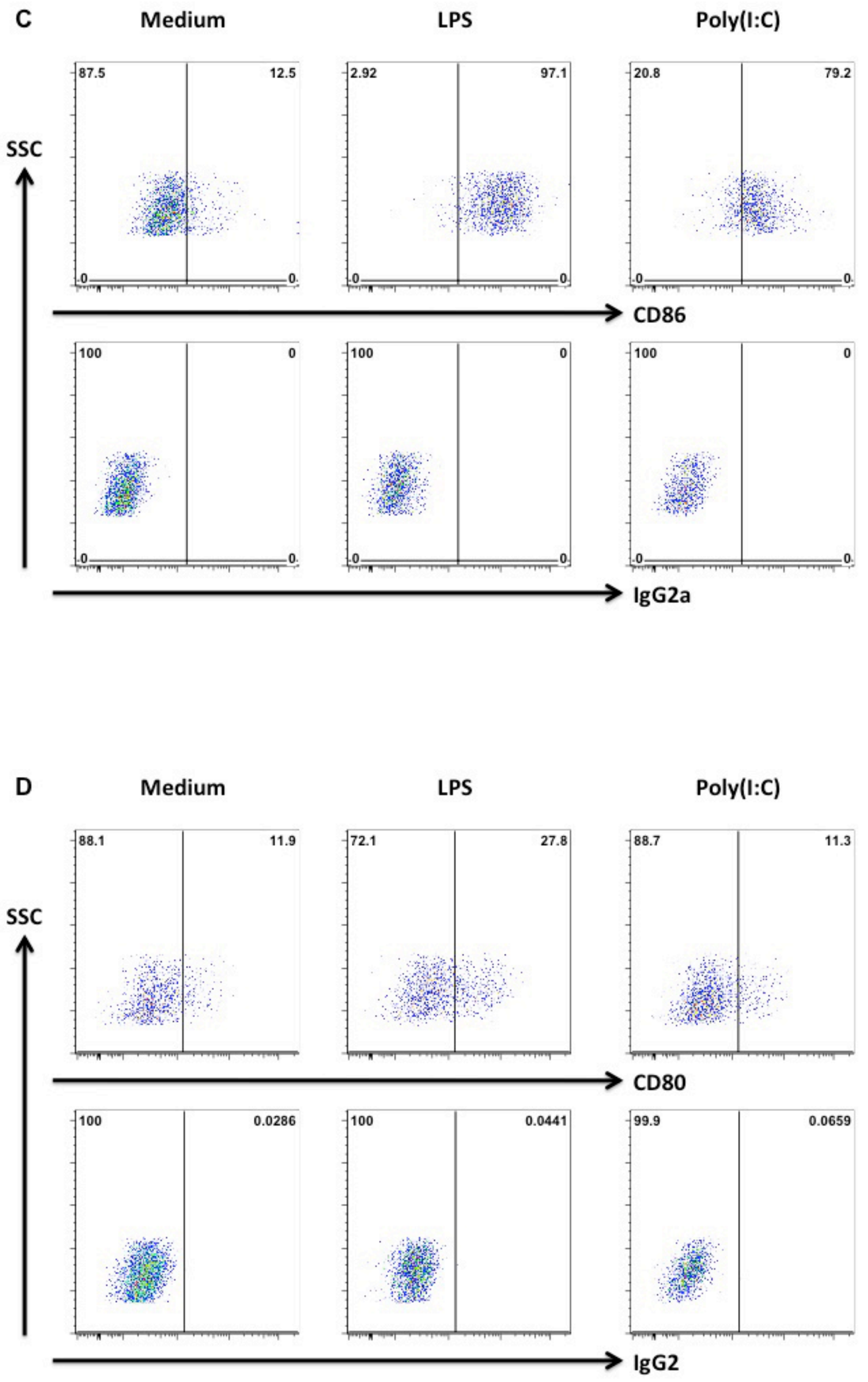


Figure 3: Representative data of up-regulation of (A)CD40, (B)CD86, (C)CDCD80 and (D)MHC-II on the surface of BMDC. BMDC were stimulated with either LPS (1μg/ml) or poly(I:C) (10μg/ml) for 24 hours. And surface CD40, CD80, CD86 and MHC-II were detected by performing FACS.

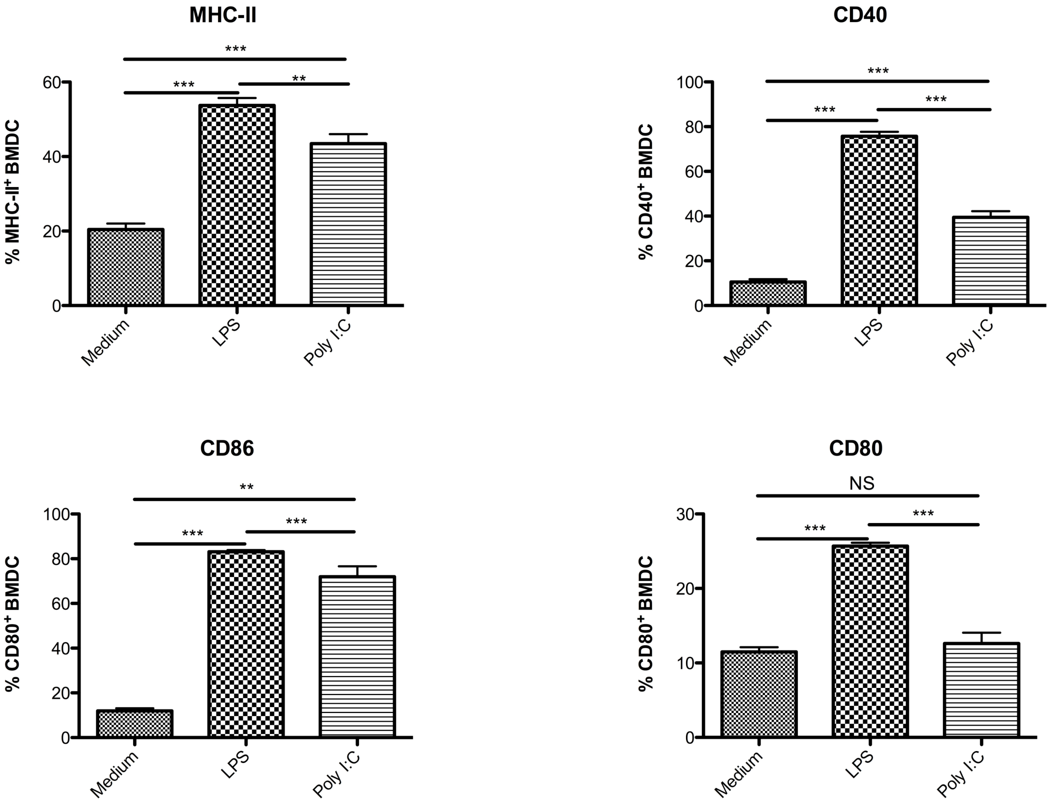


Figure 4: Differential levels of MHC-II, CD86, CD40 and CD80 expression on the surface of LPS- and poly(I:C)-activated BMDC. BMDC were stimulated with either LPS (1µg/ml) or poly(I:C) (10µg/ml) for 24 hours. Cells were harvested and stained with CD11c, MHC-II, CD86, CD40 and CD80, and detected with FACS. Molecule expression level was analyzed in term of MFI. (Results are the mean of n=12 treatments; * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

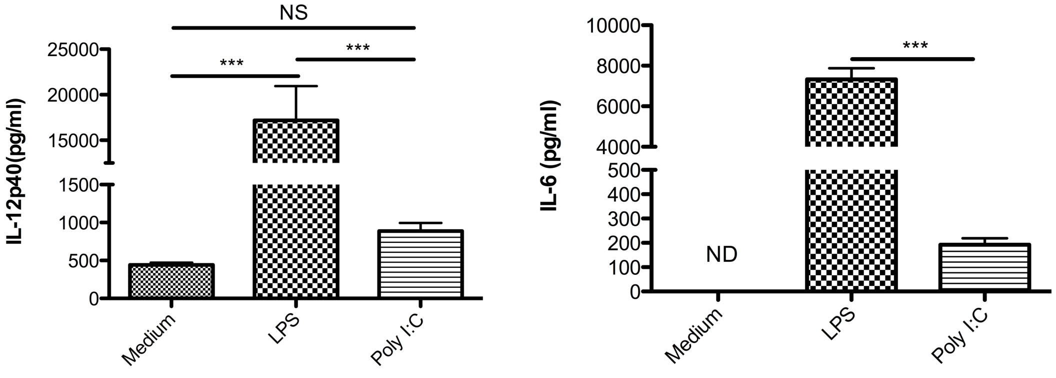


Figure 5: Differential IL-12 and IL-6 secretion level produced by LPS- and poly(I:C)-activated BMDC. Immature BMDC were cultured (0.05x106/well) in a total volume of 0.2ml in 96-well plates with GM-CSF (20ng/ml) and with/without LPS (1µg/ml) or poly(I:C) (10µg/ml) for 24 hours, and then supernatants were collected and kept frozen (-20°). IL-12 and IL-6 was quantified in supernatants from BMDC culture by enzyme-linked immunosorbent assay (ELISA). (Results are the mean of A: n=15, B: n=20; * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

3.3 Both LPS and poly(I:C) up-regulated JLP expression in BMDC

Function of JLP has been studied in various cell lines and different types of tissues, and previous studies have demonstrated that JLP was expressed ubiquitously in tissue of brain, lung, testis as well as spleen. However expression of JLP has never been reported in DC.

Therefore, we detected JLP protein expression in immature, LPS- and poly(I:C)-stimulated BMDC by western blot (Fig. 6). Very low level of JLP was detected in immature BMDC, and expression of JLP was up-regulated upon LPS and poly(I:C) stimulation. Since we used CD40 antibody for CD40 crosslinking on mature BMDC, we also detected whether JLP expression was enhanced by CD40 ligation. However, significant up-regulation of JLP expression via CD40 ligation was not observed.

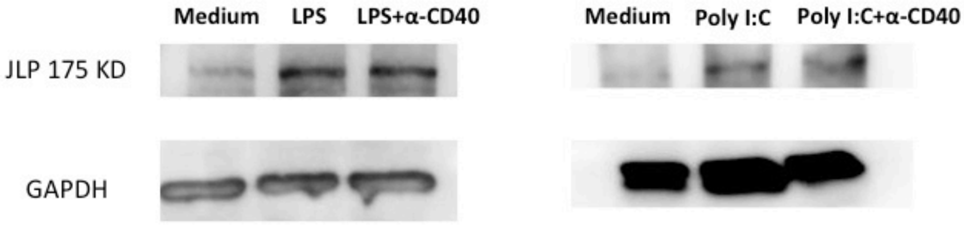


Figure 6: JLP expression in immature, LPS- and poly(I:C)-stimulated BMDC. BMDC was stimulated by LPS (1μg/ml) or poly(I:C) (10μg/ml) for 24 hours, then cells were cultured in fresh complete medium with/without anti-CD40 mAb (5μg/ml) for another 24 hours, after which cells were collected for western blot.

3.4 JLP-silencing by lentiviral transduction in DC2.4 cell line and BMDC

To study the function of JLP in BMDC, we silenced JLP expression in BMDC using established lentiviral gene silencing system. In order to check the efficiency of JLP knockdown and if transduction of irrelevant silencing sequence affects JLP expression, we detected JLP expression in JLP-silenced and EGFP-shRNA-transduced BMDC.

Previously we detected high level of JLP expression in DC2.4 cell line. We firstly tested JLP silencing in DC2.4. shown as in the figure 7, JLP expression in EGFP-shRNA-transduced cells was not affected and JLP expression was silenced in DC2.4 transduced with JLP silencing sequence. Since we work with BMDC, JLP silencing efficiency was also tested in LPS and poly (I:C) matured BMDC (Fig. 7). Western blot results of JLP-silencing sequence transduced BMDC indicated that expression of JLP can be silenced in both LPS- and poly(I:C)-stimulated BMDC, moreover in BMDC transduction of irrelevant gene silencing sequence did not affect JLP expression up-regulation in BMDC matured by LPS and poly(I:C). Therefore by using lentiviral gene silencing system, JLP expression in LPS- and poly(I:C)-stimulated BMDC was successfully silenced.

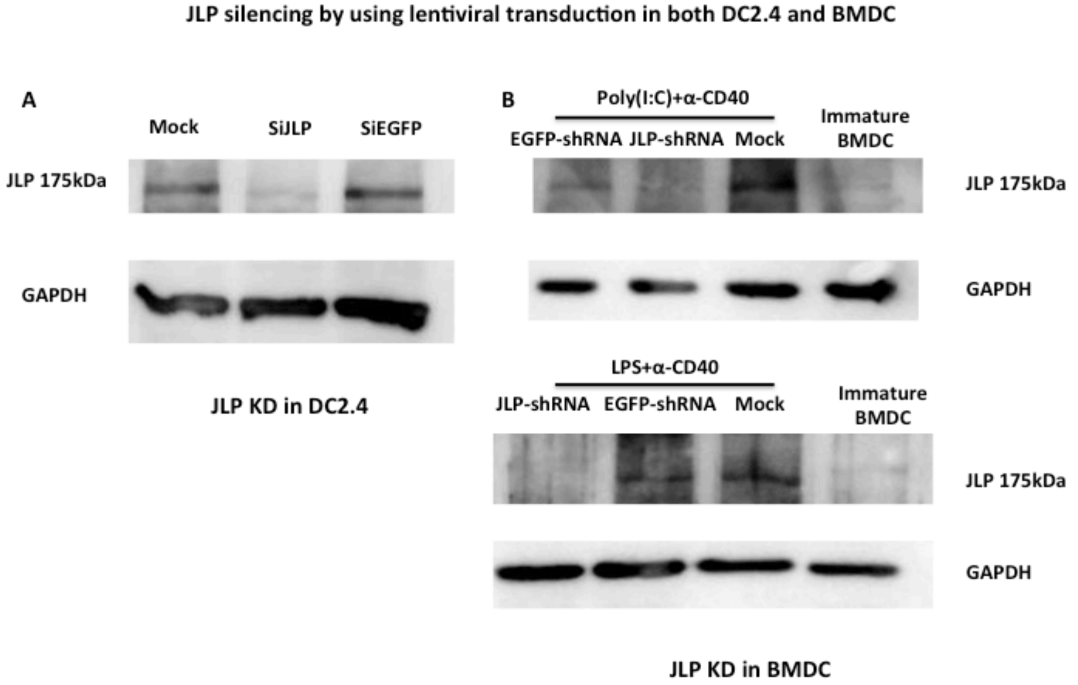


Figure 7: JLP expression was silenced by lentiviral gene silencing system in both DC2.4 and BMDC. Silenced JLP expression level was detected in (A) DC2.4 cell line as well as in (B) LPS- and poly(I:C)-stimulated BMDC. Moreover transduction of irrelevant gene silencing sequence did not affect JLP expression in either DC2.4 or mature BMDC.

3.5 JLP facilitates CD86 and CD40 up-regulation upon LPS and poly(I:C) stimulation, poly(I:C)-induced MHC-II up-regulation and LPS-induced CD80 up-regulation in BMDC

As antigen presenting cells, antigen presentation and co-stimulation are two fundamental functions of DC in T cell priming, which are mediated by MHC molecules and co-stimulatory molecules respectively. Therefore, we analyzed up-regulation of MHC-II, CD40, CD86 and CD80 in JLP-silenced BMDC upon LPS and poly(I:C) stimulation, respectively.

We measured MHC-II, CD40, CD86 and CD80 expression on the surface of immature, LPS- and poly(I:C)-stimulated mock, EGFP-shRNA-transduced as well as JLP-silenced BMDC and the percentage of $CD40^+$, $CD86^+$, $CD80^+$ and $MHC\text{-}II^+$ immature and stimulated BMDC were analyzed respectively(Fig. 8&9). As described previously, MHC-II, CD40 and CD86 were up-regulated in mock BMDC upon either LPS or poly(I:C) activation, whereas CD80 up-regulation was only observed in LPS-stimulated BMDC. The same result was also observed in EGFP-shRNA-transduced BMDC. However, comparing to mock and EGFP-shRNA-transduced BMDC, considerably lower percentage of $CD40^+$ and $CD86^+$ JLP-silenced cells were observed after LPS and poly(I:C) stimulation. Moreover lower percentage of $CD80^+$ was observed in LPS-stimulated JLP-silenced BMDC, however the percentage of $CD80^+$ of poly(I:C)-stimulated BMDC with or without JLP was comparable. For $MHC\text{-}II^+$ BMDC stimulated by either LPS or poly(I:C), no difference was observed among mock, EGFP-shRNA-transduced and

JLP-silenced BMDC with or without stimulation. Fig. 9 showed the up-regulation of CD40 and CD86 in JLP-silenced BMDC was inhibited upon either LPS or poly(I:C) activation. Furthermore CD80 up-regulation induced by LPS in JLP-silenced BMDC was also impaired. Though statistically the percentage of MHC-II^{+} JLP-silenced BMDC was comparable to mock and EGFP-shRNA-transduced BMDC upon poly(I:C) stimulation (Fig. 8A), up-regulation of MHC-II in JLP-silenced BMDC induced by poly(I:C) was not significant when compared to un-stimulated JLP-silenced BMDC (Fig 8B).

Hence, up-regulation of CD40 and CD86 of LPS- and poly(I:C)-stimulated JLP-silenced BMDC were significantly impaired compared to mock and EGFP-shRNA-transduced BMDC. Moreover, MHC-II up-regulation upon poly(I:C) stimulation and LPS-induced CD80 up-regulation on surface of JLP-silenced BMDC were also inhibited.

Conclusively, JLP is required for the up-regulation of CD40 and CD86 induced by either LPS or poly(I:C), poly(I:C)-induced MHC-II up-regulation as well as LPS-induced CD80 up-regulation in BMDC.

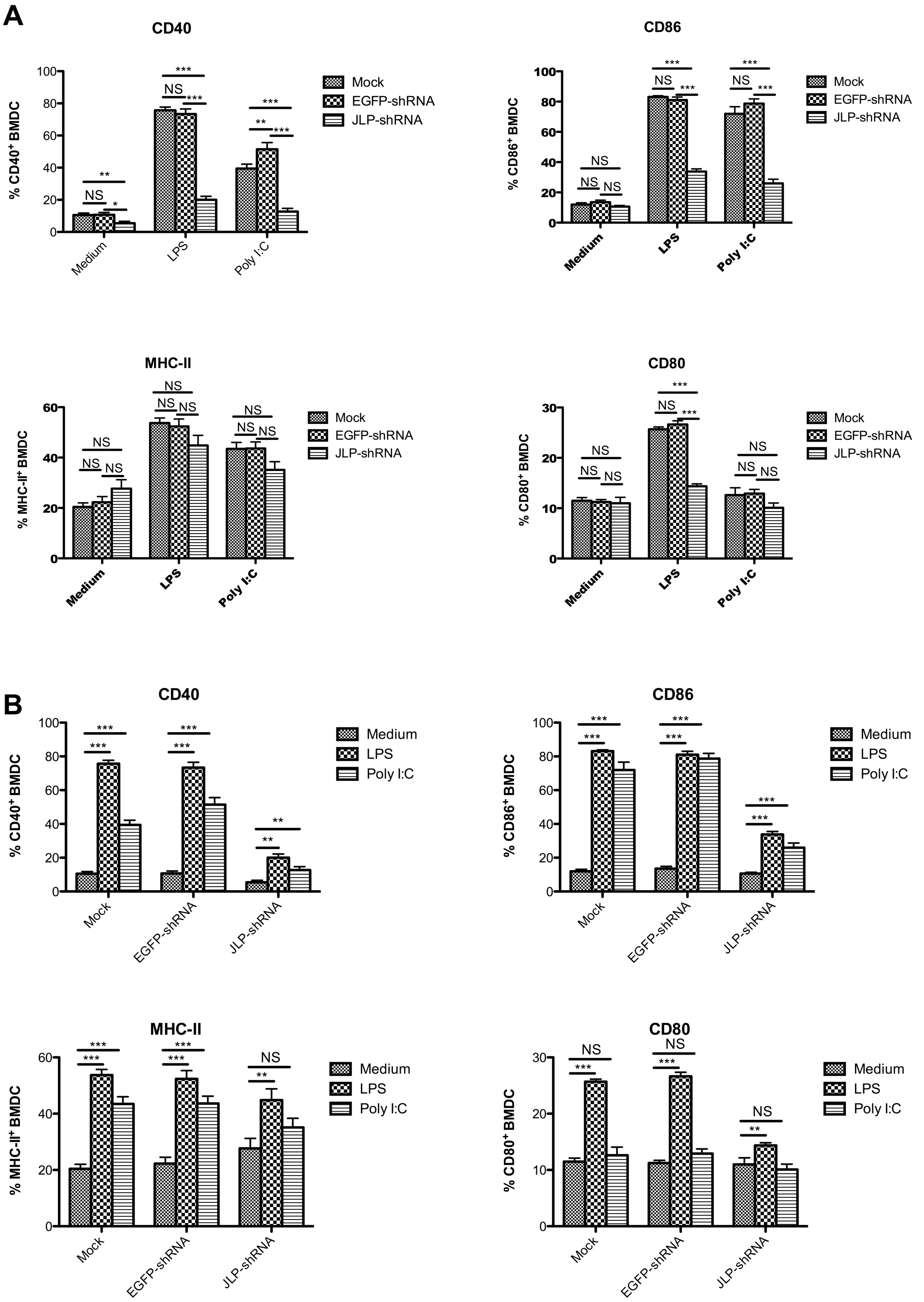
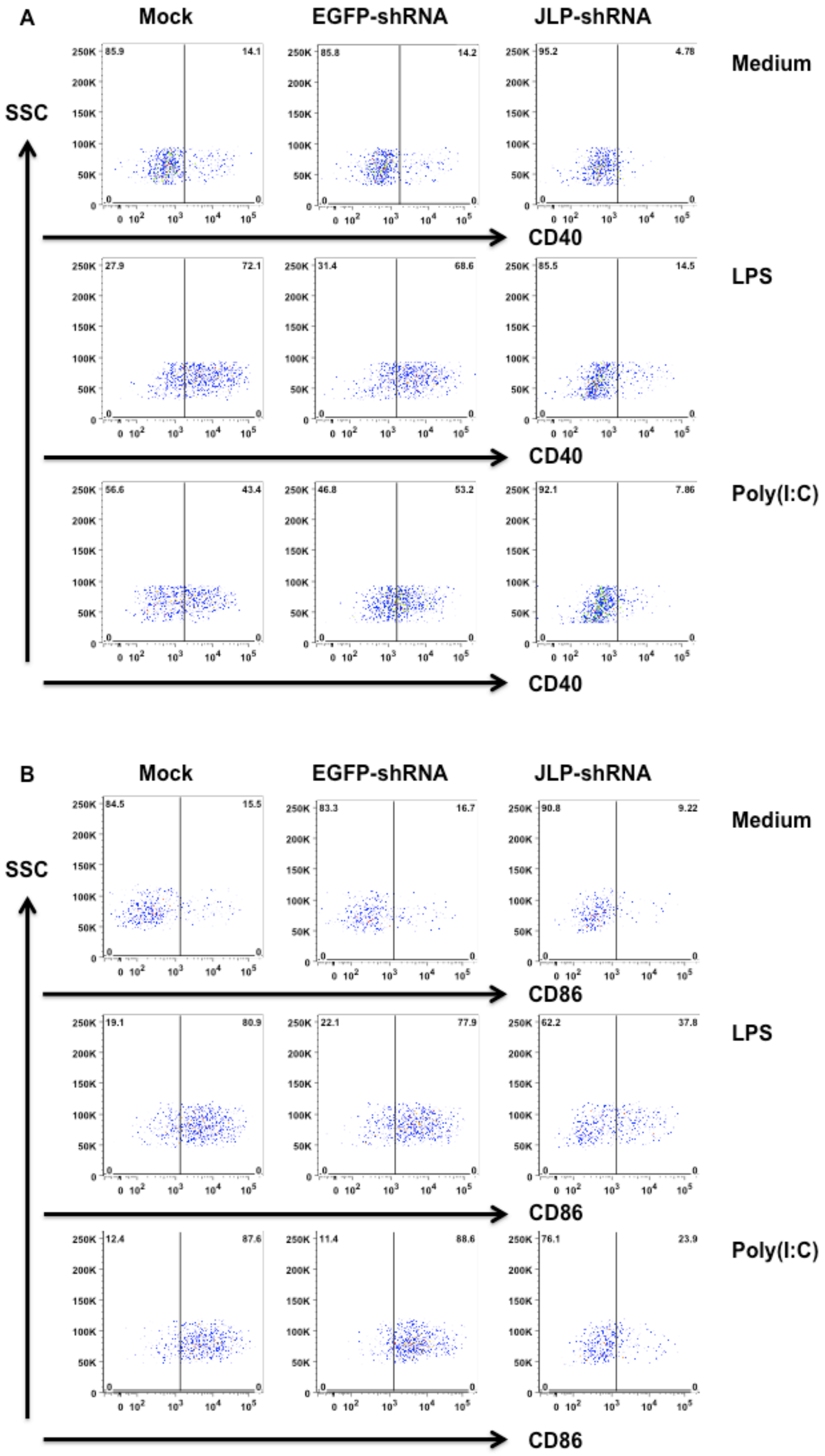


Figure 8: Percentage of CD40⁺, CD86⁺, MHC-II⁺ and CD80⁺ BMDC (A) and LPS- and poly(I:C)-induced up-regulation of surface molecules (B). BMDC were stimulated with either LPS (1μg/ml) or poly(I:C) (10μg/ml) for 24 hours. And surface CD40, CD80, CD86 and MHC-II were detected by performing FACS. (Results are the mean of n=12 treatments; * $p<0.05$, ** $p<0.01$, *** $p<0.001$)


A
Mock
EGFP-shRNA
JLP-shRNA
SSC
Medium
85.9
14.1
85.8
14.2
95.2
4.78
CD40
LPS
27.9
72.1
31.4
68.6
85.5
14.5
CD40
Poly(I:C)
56.6
43.4
46.8
53.2
92.1
7.86
CD40
250K
200K
150K
100K
50K
0
0 10^2 10^3 10^4 10^5
B
Mock
EGFP-shRNA
JLP-shRNA
SSC
Medium
84.5
15.5
83.3
16.7
90.8
9.22
CD86
LPS
19.1
80.9
22.1
77.9
62.2
37.8
CD86
Poly(I:C)
12.4
87.6
11.4
88.6
76.1
23.9
CD86

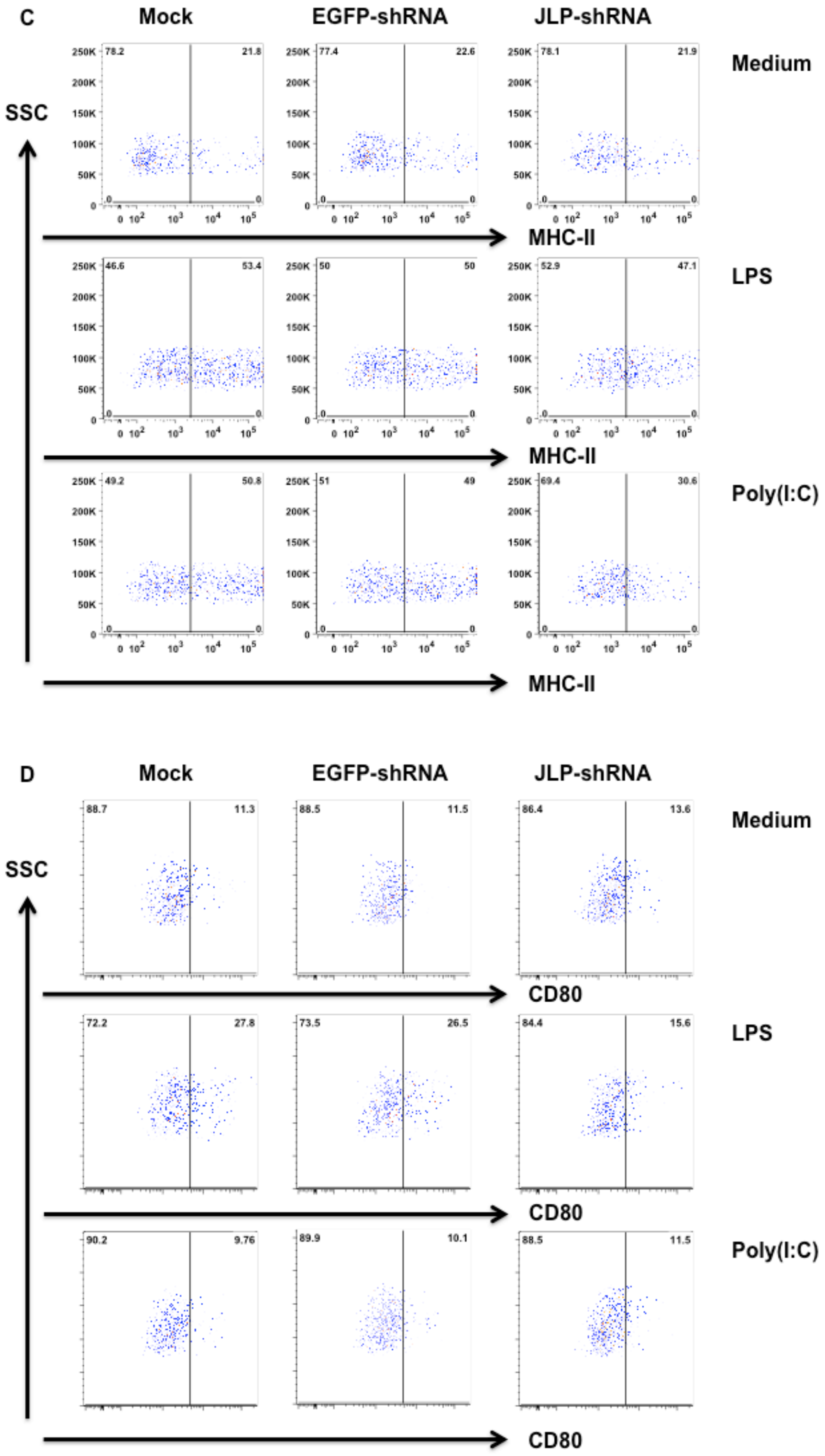


Figure 9: Representative data of up-regulation of (A)CD40, (B)CD86, (C)MHC-II and (D)CD80 on the surface of BMDC with or without JLP. BMDC were stimulated with either LPS (1μg/ml) or poly(I:C) (10μg/ml) for 24 hours. And surface CD40, CD80, CD86 and MHC-II were detected by performing FACS.

3.6 JLP in BMDC is involved in limiting IL-12 up-regulation upon LPS stimulation with and without CD40 ligation, whereas JLP does not affect IL-12 production in poly(I:C) stimulated BMDC

IL-12 produced by DC has been demonstrated to assists the polarization of T helper 1 (Th1) cells. IL-12 production can be up-regulated upon activation of DC via TLRs, and CD40 crosslinking can further enhance IL-12 secretion. Moreover MAPK signaling is involved in both TLR3- and TLR4-mediated stimulation in DC. Therefore, we reasoned that JLP silencing may lead to different consequences of IL-12 production in BMDC stimulated by LPS and poly(I:C) respectively.

In the supernatant of immature mock and EGFP-transduced BMDC, low level of IL-12 was detected. Furthermore IL-12 level of immature JLP-silenced BMDC was lower than immature mock and EGFP-transduced BMDC (Fig. 10A). Upon LPS treatment IL-12 level was dramatically increased in all three groups of BMDC (Fig. 10B), and poly(I:C) can slightly but not significantly increase IL-12 production (Fig. 10C). And stimulation via CD40 crosslinking post-maturation further elevated the IL-12 production level (Fig. 10B&10C). However, it was noteworthy that JLP-silenced BMDC secreted significantly higher level of IL-12 than mock and EGFP-transduced BMDC upon LPS-induced maturation as well as CD40 crosslinking (Fig. 10B). Upon poly(I:C) stimulation, JLP-silenced BMDC produced less IL-12 than mock and EGFP-transduced BMDC (Fig. 10C), which is consistent with the trend of IL-12 production in immature mock, EGFP-shRNA-transduced and JLP-silenced BMDC (Fig. 10A), and after CD40 crosslinking JLP-silenced BMDC produced the same level of IL-12 as mock and EGFP-transduced BMDC (Fig. 10C). Moreover, the up-regulation of IL-12 upon CD40 crosslinking was not impaired due to the lower level of CD40 on the surface of LPS- and poly(I:C)-stimulated JLP-silenced BMDC. These observations showed that: (1) in the absence of JLP, higher level of IL-12 was produced in the LPS-stimulated BMDC; (2) in poly(I:C) matured BMDC, JLP did not play any role in IL-12 production.

Previous work in our lab showed upon CD40 crosslinking, BMDC with reduced surface CD40 expression produce low level of IL-12. However, It is noteworthy that in this study though JLP-silenced BMDC displayed low surface level of CD40, but upon CD40 crosslinking either LPS- or poly(I:C)-stimulated JLP-silenced BMDC produced high level of IL-12. Therefore, we also speculated JLP played an inhibitory role in CD40-induced IL-12 production.

Conclusively, these data suggested that JLP plays a role in the down-regulation of IL-12 production in LPS-matured BMDC, but not in poly(I:C)-activated BMDC.

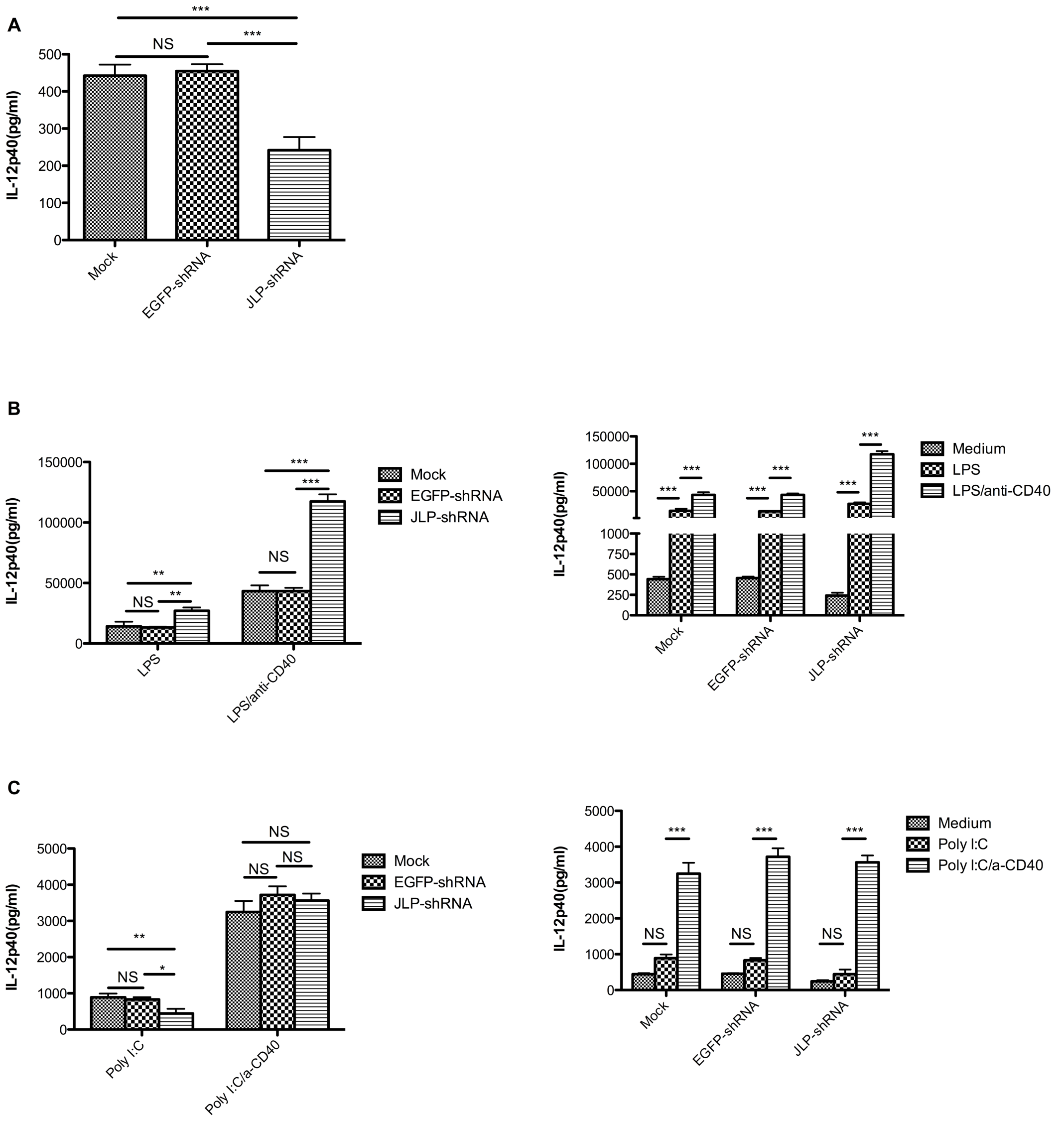


Figure 10: IL-12 production of JLP-silenced BMDC compared to mock and EGFP-shRNA-transduced BMDC. (A) IL-12 production in immature BMDC. IL-12 production and up-regulation (B) upon LPS stimulation with or without CD40 crosslinking and (C) upon poly(I:C) stimulation BMDC with or without CD40 crosslinking. Immature BMDC with and without JLP were cultured (0.05x10^6/well) in a total volume of 0.2ml in 96-well plates with GM-CSF (20ng/ml) and with and without LPS (1μg/ml) or poly(I:C) (10μg/ml) for 24 hours, then cells were cultured in fresh complete medium with and without anti-CD40 mAb (5μg/ml) for another 24 hours, after which supernatants were collected and kept frozen (-20°). IL-12 was quantified in supernatants from BMDC culture by enzyme-linked immunosorbent assay (ELISA). (Results are the mean of A: n=15, B: n=8, C: n=15 treatments; * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

3.7 JLP associates the up-regulation of IL-6 secretion upon poly(I:C) stimulation with and without CD40 ligation, whereas in LPS matured BMDC JLP does not affect IL-6 production

IL-6 produced by mature DC play a critical role in priming of naïve T cells. Studies have demonstrated that IL-6 stimulates T cell proliferation and enhances survival of naïve T cells [317-323]as well as directs differentiation of Th17 cells together with TGF-β [124]. Moreover IL-6 can induce INF-γ production by differentiating T cells [324, 325].

In our experiment, IL-6 was not detectable in the supernatant of immature BMDC (Fig. 11A), and IL-6 production was induced upon LPS and poly(I:C) activation in mock, EGFP-shRNA-transduced as well as JLP-silenced BMDC (Fig. 11B & 11C).

Upon poly(I:C) activation with or without CD40 crosslinking, JLP-silenced BMDC produced dramatically lower IL-6 level than mock and EGFP-shRNA-transduced BMDC (Fig. 11C). Moreover, ligation of CD40 highly up-regulated IL-6 production in poly(I:C) matured mock and EGFP-transduced BMDC rather than JLP-silenced BMDC (Fig. 11C). However, in LPS matured BMDC with or without CD40 ligation, no difference was observed in IL-6 produced by mock, EGFP-shRNA-transduced and JLP-silenced BMDC, though IL-6 production in the supernatant of LPS-activated EGFP-shRNA-transduced and JLP-silenced BMDC was not up-regulated significantly upon CD40 ligation (Fig. 11B). These results suggested without JLP, up-regulation of IL-6 upon poly(I:C) with or without CD40 crosslinking is impaired, on the contrary IL-6 production was not affected by the absence of JLP in LPS-activated BMDC.

According to the result, JLP is required for IL-6 up-regulation in poly(I:C)- but not LPS-activated BMDC with or without CD40 crosslinking.

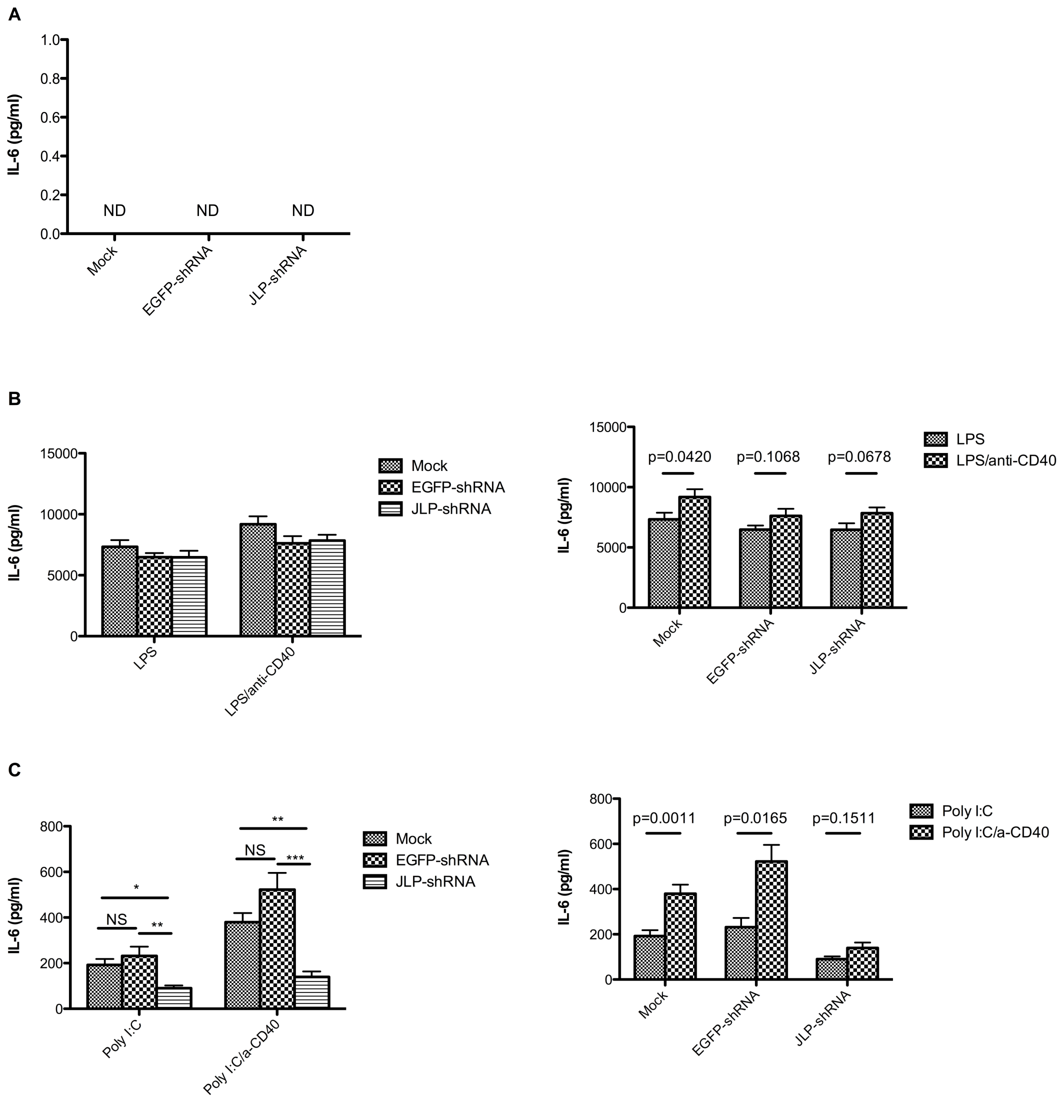


Figure 11: IL-6 secretion of JLP-silenced BMDC compared to mock and EGFP-shRNA-transduced BMDC. (A) IL-12 production in immature BMDC. IL-12 production and up-regulation (B) upon LPS stimulation with or without CD40 crosslinking and (C) upon poly(I:C) stimulation BMDC with or without CD40 crosslinking. Immature BMDC with and without JLP were cultured (0.05x10^6/well) in a total volume of 0.2ml in 96-well plates with GM-CSF (20ng/ml) and with and without LPS (1µg/ml) or poly(I:C) (10µg/ml) for 24 hours, then cells were cultured in fresh complete medium with and without anti-CD40 mAb (5µg/ml) for another 24 hours, after which supernatants were collected and kept frozen (-20°). IL-6 was quantified in supernatants from BMDC culture by enzyme-linked immunosorbent assay (ELISA). (Results are the mean of A: n=20, B: n=12, C: n=20; * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

3.8 JLP negatively regulates antigen presentation via MHC-II of both LPS and poly(I:C) stimulated BMDC

Antigen presentation is one of main functions of antigen presenting cells. Therefore we performed the antigen presentation assay to test if antigen presentation ability of BMDC was affected by JLP. We examine the antigen presentation ability via MHC-II of JLP-silenced BMDC using BO97.10 hybridoma T cell lines. BO97.10 recognizes OVA 323-339 episode, which binds to I-A^d major histocompatibility molecules class II molecules. We exposed BMDC to either OVA 323-339 peptide or ovalbumin protein (OVA) without stimulation or stimulated with LPS or poly(I:C), and then we co-cultured antigen bearing BMDC with BO97.10 cells. After 3 days supernatant was collected to examine the IL-2 secreted by BO97.10 cells.

Shown in Fig. 12A, loaded with OVA 323-339 peptide, IL-2 produced by BO97.10 cells co-cultured with immature and poly(I:C)-activated JLP-silenced BMDC were comparable with mock and EGFP-shRNA-transduced BMDC. However co-cultured with LPS-stimulated JLP-silenced BMDC, BO97.10 produced slightly higher level of IL-2. However, presentation of OVA peptides did not reflect the antigen processing in BMDC. Therefore we further examined antigen presentation via MHC-II with OVA pulsed BMDC (Fig. 12B). Significant elevation of IL-2 production was observed in co-culture of BO97.10 and immature, LPS- and poly(I:C)-activated JLP-silenced BMDC, thus JLP-silenced BMDC had higher antigen presentation efficiency via MHC-II molecules.

According to the result, antigen presentation via MHC-II was enhanced in immature LPS-and poly(I:C)-activated JLP-silenced BMDC. Therefore we concluded that JLP negatively regulates MHC-II antigen presentation.

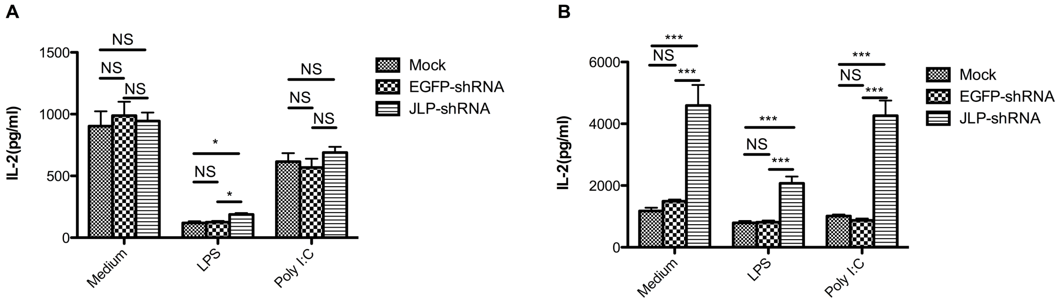


Figure 12: Antigen presentation ability of JLP-silenced BMDC via MHC-II was enhanced. Mock, EGFP-shRNA-transduced and JLP-silenced BMDC were pulsed with (A) OVA 323-339 peptide and (B) OVA protein and stimulated with LPS and poly(I:C) for 24 hours, and then antigen loading BMDC were co-cultured with BO97.10 cell line for 3 days. IL-2 in the supernatant was measured by ELISA. (Results are the mean ± S.E.M. of n=6 treatments; * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

We also examined antigen presentation via MHC-I in JLP-silenced BMDC using RF33.70 cell line specific for OVA 257-264 episode restricted to H-2K^b. Co-cultured with OVA 257-264 peptide bearing immature and poly(I:C)-stimulated JLP-silenced BMDC, IL-2 produced by RF33.70 was comparable to mock and EGFP-shRNA-transduced BMDC, whereas IL-2 secreted by RF33.70 co-cultured with LPS-stimulated rather than poly(I:C)-stimulated JLP-silenced BMDC was higher comparing to mock and EGFP-shRNA-transduced BMDC (Fig. 13). We also tested MHC-I antigen presentation with OVA-pulsed BMDC, but IL-2 production was not detectable. Therefore we could not draw a conclusion for antigen presentation ability of JLP-silenced BMDC via MHC-I molecules because this part of result was not intact.

Interestingly, in our experiment we observed IL-2 production by BO97.10 cells co-cultured with LPS-stimulated OVA 323-339 bearing BMDC was much lower than BO97.10 cells co-cultured with immature BMDC loaded with the same antigen (Fig. 12A). IL-2 produced by RF33.70 cells co-cultured with LPS stimulated OVA 257-264 bearing mock and EGFP-shRNA transduced BMDC slightly lower than RF33.70 cells co-cultured with immature OVA 257-264 bearing mock and EGFP-shRNA-transduced BMDC. A previous study has demonstrated that TNF-α-related apoptosis-inducing ligand (TRAIL) expressed on BMDC can mediate apoptosis of T hybridoma cells MF2.2D9 and RF33.70, and LPS can further enhance the cytotoxicity by up-regulating surface expression level of TRAIL in BMDC [326]. Therefore we speculated that the reason of the observation described above was that LPS stimulated BMDC expressed higher level of TRAIL than immature BMDC and accelerated apoptosis of BO97.10 and RF97.10 cells, which led to decreased number of IL-2 producing cells.

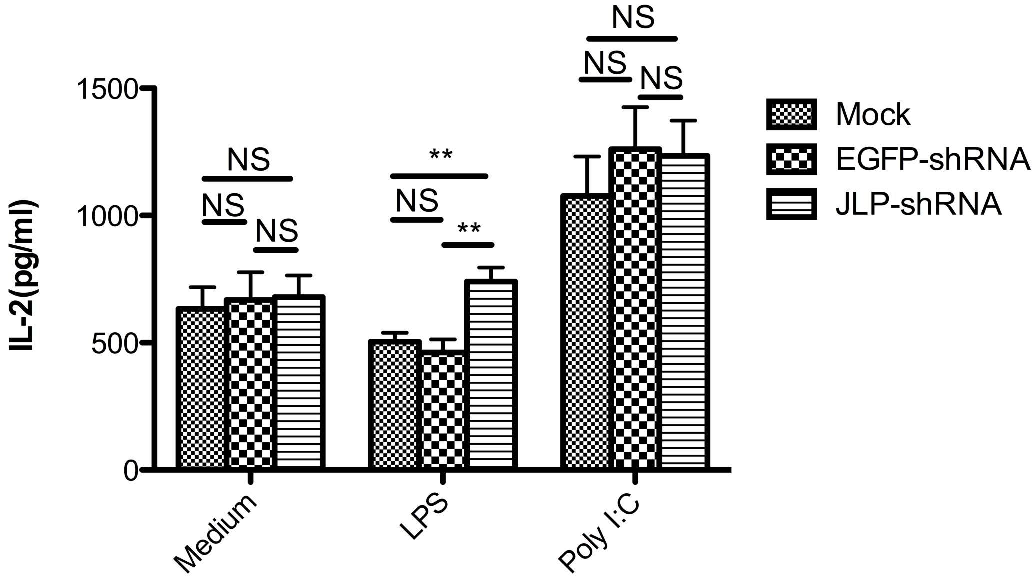


Figure 13: Antigen presentation via MHC-I in response to OVA 257-264 peptide. Mock, EGFP-shRNA-transduced and JLP-silenced BMDC were pulsed with OVA 257-264 peptide and stimulated with LPS and poly(I:C) for 24 hours, and then antigen loading BMDC were coclutured with RF33.70 cell line for 3 days. IL-2 in the supernatant was measured by ELISA. (Results are the mean ± S.E.M. of n=6 treatments; * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

3.9 JLP plays a role in promoting LPS-stimulated BMDC survival in the absence of GM-CSF, whereas it has no impact on lifespan of poly(I:C) stimulated BMDC

Recognition of PAMP via PRRs triggers DC maturation and the activated DC become potent APCs. In addition to up-regulation of surface MHC-II and co-stimulatory molecules expression as well as elevated cytokine secretion, survival of mature DC is another fundamental aspect that interfere T cell priming. Previous studies have demonstrated that activation of TLR9 by poly(I:C) and TLR4 by LPS inhibit apoptosis of mature DC after the withdraw of growth factors [90, 227]. On the contrary, double-stranded RNA induced TLR3 activation was reported to trigger apoptosis of pancreatic cell as well as various tumor cells [311-313], however the effect of TLR3 activation on DC survival is not well understood.

To understand the effect of JLP absence on survival of immature and mature DC, we performed apoptosis analysis with Annexin-V and 7-AAD staining on immature and LPS- as well as poly(I:C)-stimulated BMDC. The mock, EGFP-shRNA-transduced and JLP-silenced BMDC were stimulated with LPS or poly(I:C), and was further treated CD40 antibody because CD40 ligation was reported to enhance DC survival . The cell culture was collected 24 hours and 72 hours after CD40 ligation.

In immature mock, EGFP-shRNA-transduced and JLP-silenced BMDC, no significant difference was observed in percentage of viable cells, apoptotic cells or dead cells, as shown in the first column of Fig. 14A-C.

With activation of LPS, percentage of viable cells in mock and EGFP-shRNA-transduced at 24 and 72 hour were higher than those immature cells (Fig. 14), however at 24 and 72 hour the survival of LPS stimulated JLP-silenced BMDC was not enhanced comparing to immature JLP-silenced BMDC (Fig. 14) and control groups (15A). With ligation of CD40, percentage of viable cells in LPS-matured JLP-silenced BMDC was lower than mock and EGFP-shRNA-transduced BMDC (Fig. 15A). Meanwhile, 24 and 72 hour after LPS activation with or without CD40 ligation, we observed an elevated percentage of apoptotic cells in JLP-silenced BMDC comparing to the apoptotic cell percentage in mock and EGFP-shRNA-transduced BMDC (Fig. 15B). Furthermore, upon LPS activation with or without CD40 ligation, percentage of dead cells in JLP-silenced cells was higher than mock and EGFP-shRNA-transduced cells at 24 hour, whereas no significant difference was observed at 72 hour (Fig. 15C).

Poly(I:C) stimulation did not improve survival of the mock, EGFP-shRNA-transduced or JLP-silenced BMDC, even though a higher percentage of viable cells in poly(I:C)-activated EGFP-shRNA-transduced BMDC than immature EGFP-shRNA-transduced BMDC at 72 hour was observed(Fig. 14). Moreover, difference in percentage of viable, apoptotic or dead cells was observed in none of poly(I:C)-matured mock, EGFP-shRNA-transduced or JLP-silenced BMDC with or without CD40 crosslinking, neither at 24 hour nor 72 hour(Fig. 15A-C).

Based on these data we concluded that with the absence of JLP, the LPS-induced inhibition of DC apoptosis was impaired, whereas JLP had no effect on the lifespan of poly(I:C)-stimulated BMDC. Therefore, the result suggested a role of JLP in promoting the LPS-induced enhancement of DC survival.

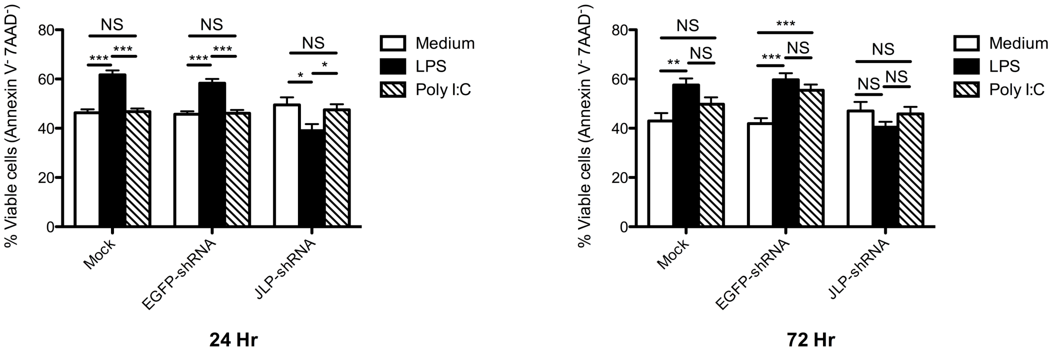


Figure 14: Percentage of viable cells in immature, LPS- and poly(I:C)-activated mock, EGFP-shRNA-transduced and JLP-silenced BMDC. Survival of mock and EGFP-shRNA-transduced BMDC were enhanced upon activation of LPS instead of poly(I:C), and JLP-silenced BMDC survival was not improved by neither LPS nor poly(I:C) stimulation. (24 Hr: n=12, 72 Hr: n=11; * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

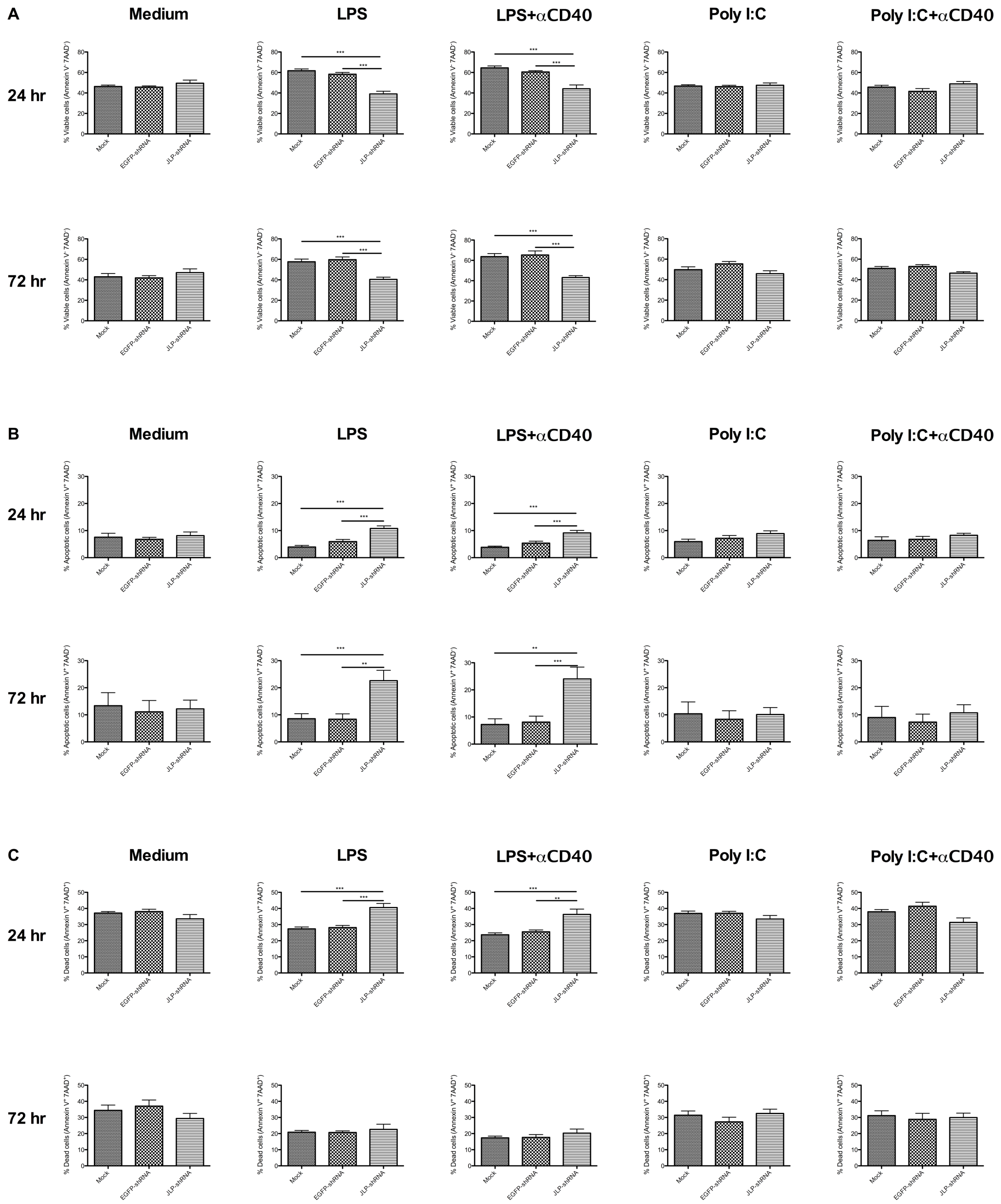


Figure 15: Percentage of (A) viable, (B) apoptotic and (C) dead cells of mock, EGFP-shRNA-transduced and JLP-silenced BMDC, which were immture, LPS- or poly(I:C)-activated with and without CD40 ligation. Immature BMDC with/without JLP were cultured (0.05×10^6/well) in a total volume of 0.2ml in 96-well plates with GM-CSF (20ng/ml) and with/without LPS (1μg/ml) or poly(I:C) (10μg/ml) for 24 hours, then cells were cultured in fresh complete medium with/without anti-CD40 mAb (5μg/ml). Cells were collected at 24 hour and 72 hour, and were stained with Annexin-V and 7-AAD. Cell viability detected by FACS. (24 Hr: n=12, 72 Hr: n=11; * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

Chapter 4 Discussion

As professional antigen presenting cells, DC are remarkable for antigen uptake and presentation as well as priming of naïve T lymphocytes. However, though immature DC in peripheral tissues are capable for antigen uptake through endocytosis, phagocytosis and macropinocytosis, immature DC induce anergic T-cell response. Therefore, functional transformation from immature DC to mature DC is a critical event for DC to become potent antigen presenting cells that acquire a potent capability to prime antigen specific T-cell response. Upon maturation, antigen uptake ability of DC decreases gradually, whereas DC start to migrate from peripheral tissues to lymph organs where they meet T cells, and the surface molecule expression such as MHC molecules and co-stimulatory molecules as well as cytokine production are up-regulated, which are essential for efficient T-cell priming.

DC maturation is induced by pattern recognition receptor-dependent pathogen recognition. When DC engage with invading pathogens, in addition to antigen uptake, recognition of microorganism via germline-encoded pattern recognition receptors occur simultaneously. So far, various types of PRRs have been identified, which can be divided into four families, Toll-like receptors (TLRs), C-type lectin receptors (CLRs), the Retinoic acid-inducible gene-I-like receptors (RLRs) and NOD-like receptors (NLRs). Each type of PRRs recognizes different pathogen-derived molecules, such as lipopolysaccharide (LPS), lipoproteins and nucleic acids. Binding with ligands, PRRs initiate signal transduction and eventually lead to activation of transcription factors, gene transcription and DC maturation, which are tightly regulated at different levels.

Signal transduction initiated by PRRs is the first level that regulates DC maturation. TLR is a well-characterized family of PRRs, and the signal cascades of TLRs have been divided into two general pathways according to the adaptor proteins involved: MyD88-dependent pathway and Trif-dependent pathway, in which MAPKs are involved. Nowadays, since the identification of scaffold proteins in distinct signaling pathways such as MAPKs, calcium signaling and immune receptor signaling, accumulating evidences suggest that scaffold proteins play an important role in signal transduction by using various mechanisms. Therefore, we speculated that scaffold proteins are also function as critical regulators in TLR signal cascades, which have an impact on DC maturation.

JNK-associated leucine zipper protein (JLP) is a newly identified scaffold protein that associate with JNK/p38 phosphorylation, and previous study also have shown JLP can act as Myc/Max-interacting protein and physically interact with $G_{\alpha12/13}$. We found in immature BMDC, a low level of JLP expression can be detected by western bolt, and stimulated with LPS and poly(I:C) respectively, expression of JLP in BMDC was up-regulated. Taken together that JNK and p38 participate the signal cascade of TLR3 and TLR4, and we hypothesized that JLP play a role in DC maturation induced by activation of TLR3 by poly(I:C) and TLR4 by LPS. Moreover, previous study indicated that different TLR ligands induce specific mature DC function properties [314-316], we further speculated that JLP may play a differential role in LPS- and poly(I:C)-activated BMDC.

To study the biological function in BMDC, we utilized the lentivial gene silencing system to silence the expression of JLP in BMDC and we detect the silencing effect by western blot. DC2.4 is a DC cell line in which JLP is abundantly expressed, and we found that JLP was successfully silenced in DC 2.4. Then we further detect the JLP silencing efficiency in primary BMDC, and JLP was also silenced in LPS- and poly(I:C)-activated BMDC. Therefore, we showed that lentivial gene silencing system was a efficient tool to silenced JLP expression in BMDC stably and consistently.

Antigen processing and presentation via MHC molecules, co-stimulation and inflammatory cytokine production are three aspects to define DC maturation and function. Moreover the lifespan of mature DC with absence of growth factor is also important for T-cell priming and homeostasis of antigen specific T cell response. Thus we studied the impact of JLP silencing on these DC functional aspects.

Down-regulated expression of surface CD40 and CD86 are observed in both poly(I:C)- and LPS-activated, JLP-silenced BMDC. This could be because of poor activation of transcription factors that relative to CD40 and CD86 expression. However, previous work in our lab showed total expression level of CD40 was not impaired in JLP-silenced BMDC activated by LPS, but surface level of CD40 was lower and internalization of surface CD40 upon ligation was inhibited compared to normal BMDC. In another word, the transportation of CD40 molecules between cell surface and cytosol was inhibited in LPS-stimulated JLP-silenced BMDC. Therefore, in poly(I:C)-stimulated BMDC, JLP may play the same role for CD40 transportation. NF-κB is responsible for up-regulated expression of both CD40 and CD86. Therefore transcription of CD86 gene may be intact in JLP-silenced BMDC. To confirm if down-regulated surface level of CD86 is because of impaired CD86 expression at gene transcription level or inhibited CD86 transportation from cytosol to plasma membrane, detection of total level of CD86 in JLP-silenced BMDC stimulated by poly(I:C) and LPS is necessary.

Previous study showed CD80 expression on surface of BALB/C mice-derived BMDC was up-regulated upon poly(I:C) stimulation[314]. However, in our system we consistently observed that poly(I:C) did not up-regulate CD80 on the surface of BMDC derived from C57BL/6 mice. Therefore, the data was not sufficient to support the opinion that JLP did not affect CD80 surface expression in poly(I:C)-stimulated BMDC.

Though higher IL-12 production in LPS-stimulated JLP-silenced BMDC and lower IL-6 production in poly(I:C)-stimulated BMDC were observed in our experiments, other cytokines produced by JLP-silenced BMDC are still to be tested, such as TNF-α and IL-1β, especially anti-inflammatory cytokine IL-10 which can direct differentiation of regulatory T cells. Cytokine production of APCs is crucial for the differentiation of antigen-specific T cells, which is termed as ‘signal 2’. Thus by regulating cytokine production of DC, JLP may further have an impact on the type of T cell response, which still needs to be further studied.

It is noteworthy that result of IL-12 and IL-6 production upon CD40 ligation in LPS- and poly(I:C)-activated JLP-silenced BMDC indicated that JLP may play a role in CD40-mediated IL-12 up-regulation. IL-12 up-regulation upon CD40 crosslinking was not impaired in JLP-silenced BMDC, though JLP-silenced BMDC expressed lower level of CD40 on the cell surface. Therefore, the effect of CD40-crosslinking on IL-12 up-regulation was enhanced in JLP-silenced BMDC. IL-12 plays a key role in differentiation of helper 1 T cell, thus JLP-silenced BMDC may have enhanced capability to induce Th1 response.

In this antigen presentation assay, we observed JLP-silenced BMDC loaded with OVA peptide 323-339 with or without poly(I:C) stimulation displayed a comparable antigen presentation ability as normal BMDC, whereas LPS-stimulated JLP-silenced BMDC displayed a stronger antigen presentation via MHC-II. We also performed antigen presentation assay using OVA whole protein, and we observed enhanced antigen presentation ability in JLP-silenced BMDC. So JLP may play a negative role in regulating antigen processing and presentation via MHC-II. However, this assay was not sensitive enough for antigen presentation via MHC-I, therefore if JLP affect antigen presentation through MHC-I molecules still need to be investigated by other methods.

The survival of JLP-silenced BMDC was also examined. By apoptosis assay, we observed that survival LPS-stimulated rather than poly(I:C)-stimulated JLP-silenced BMDC was impaired, which indicate that JLP plays a role in promoting LPS-stimulated JLP-silenced BMDC survival. Previous studies showed that ERK signaling was involved in LPS rescued DC from immediate apoptosis after withdraw of growth factors [90], and PI3K kinase and p38 SAP kinase signaling pathways were involved in survival of LPS-stimulated human monocyte-derived DC [327]. However the role of JLP in survival of LPS-stimulated DC is still undiscovered. Mitochondria are the organelles tightly related to cell apoptosis. Therefore examination of mitochondrial membrane potential of LPS-stimulated JLP-silenced BMDC might useful in further examination of a role of JLP in promoting LPS-stimulated BMDC survival.

Cell death ultimately leads to cell lysis, which cause release of cellular contents. And ELISA detects the cytokines secreted to supernatant. Thus impaired survival of LPS-stimulated JLP-silenced BMDC may have an influence on the results of ELISA, which may lead to false understanding of enhanced IL-12 production in LPS-stimulated JLP-silenced BMDC. However, the result of IL-6 production by LPS-stimulated JLP-silenced BMDC indicated that highly elevated level of IL-12 was not caused by apoptosis of JLP-silenced BMDC, because compared to mock and EGFP-shRNA-transduced BMDC, JLP-silenced BMDC stimulated by LPS produced comparable level of IL-6. Nevertheless, detection of quantities of mRNA of IL-12 and IL-6 is helpful for understanding the mechanism by which JLP regulates cytokine production.

In our study, current data indicated that JLP regulates CD40 and CD86 up-regulation during DC maturation, negatively regulates antigen presentation via MHC-II, and also regulates IL-12 production of LPS-activated BMDC and IL-6 production of poly(I:C)-stimulated BMDC. Moreover, JLP is also involved in promoting survival of LPS-stimulated BMDC with absence of growth factor.

Since JLP regulates DC function, we speculated further that JLP have an impact on T cell priming via DC. CD40 and CD86 up-regulation in JLP-silenced BMDC is inhibited, thus delivery of co-stimulatory signal to T cells are blocked, which may lead to T cell anergy, even though an enhancement of antigen presentation via MHC-II in JLP-silenced BMDC was observed. However, LPS-stimulated JLP-silenced BMDC produced higher IL-12, it may favor Th1 responses. And poly(I:C)-activated JLP-silenced BMDC produce lower amount of IL-6 and may impair Th17 differentiation. Therefore, taken together these evidences indicate JLP plays a role in by regulating DC maturation and functions, which further directs T cell responses, though the effect of JLP silencing in DC on antigen-specific T cell response still need to be further studied.

Chapter 5 Future direction

Previous studies on scaffold proteins have demonstrated that scaffold proteins utilize various mechanisms to control the cellular signal transduction. To further study the details how JLP regulates signal transduction initiated by activation of TLR3 and TLR4, we will further test the effect of JLP knockdown on activation of p38 MPAKs in LPS- and poly(I:C)-stimulated BMDC. NF-κB is one of transcription factors activated after TLR3 and TRL4 activation, which is responsible for co-stimulatory molecule expression and inflammatory cytokine expression. Therefore we will also test if activation of NF-κB in LPS- and poly(I:C)-activated JLP-silenced BMDC. Moreover, we will study the antigen-specific T cell response induced by JLP-silenced BMDC in vitro and in vivo using OVA-specific DO11.10 and OT-I transgenic mice. To define the T cell priming ability of JLP-silenced BMDC, we will focus on proliferation and cytokine production of OVA-specific $CD4^+$ and $CD8^+$ T cells as well as helper T cell subset differentiation. Therefore through these studies, we will acquire a better understanding of the mechanism of JLP regulating TLR3- and TLR4-mediated DC maturation and functions.

Consequently, we speculate that T cell response can be modified by using gene therapeutic methods to regulate JLP expression in DC or by utilizing synthesized small molecules to modulate JLP functions in BMDC. For this reason, meticulous studies on mechanism through which JLP regulates DC biological functions will shed light on development of novel therapies for immunological diseases, such as autoimmune diseases and allergy.

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