Analysis of Notch-driven regulation of Dendritic cell function

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ABSTRACT

An effective immune response against pathogens or cancer requires proper guidance of the immune system. Being the first responders in innate immunity, Dendritic Cells (DCs) provide essential and differentiation cues to T cells, guiding them down specific effector lineages. Cell-cell communication through Notch signaling is initiated by trans-binding of receptor and ligand on neighboring cells to guide various cell fate decisions. However, recently it has been shown that *cis*-interaction of Notch receptor and Delta ligand in mammalian cells can result in inhibition of Notch signaling. DCs are known to express both Notch ligands and receptors. We have previously shown that DCs differentially express Notch ligands in response to stimuli associated with specific T_H lineages. These findings suggest that relative abundance of Notch ligands to receptors may lead the cell to adopt a phenotype associated with signaling through either Notch ligand or receptor. Thereby, **we hypothesize that the Notch signaling directly regulates DC function and therefore indirectly modulates T-cell polarization**.

To observe Notch signaling associated with different states of DC activation, we measured expression of components of Notch signal transduction in response to the Toll-like receptor (TLR) ligand lipopolysaccharide (LPS) and *Leishmania* parasites. Activation of DCs with T_H1-directing LPS maintains the low Notch1 levels, but increases Notch2 expression. DCs infected with *Leishmania* remain immature, even if exposed to LPS subsequently, show higher Notch1 levels. Analysis of Notch1-deficient DCs, illustrated that Notch1 expression negatively regulates DC activation. Similarly, DCs

stimulated with T_H1-inducing agents maintain low Notch1 levels but have increased Delta4 expression. On the other hand, Notch2 expression positively regulates DC activation. Induced Notch2 expression leads to increased expression of co-stimulatory molecule CD86 and proinflammatory cytokine Interleukin 12 (IL-12). The increased Notch2 expression in DCs stimulated with LPS is, however, accompanied by a reduction in Jagged2 expression. Accordingly, Jagged2 is shown to negatively regulate IL-12 secretion. Such pattern of expression among Notch ligands and receptors (i.e. mutually exclusive expression of Notch1/Delta4 or Notch2/Jagged2) supports the evidence for *cis*-inhibition, a mechanism by which Notch receptors and ligands expressed on the same cell reciprocally inhibit one another. Such *cis*-interaction results in removal of the receptors and ligands from the cell surface and their subsequent proteolysis. All in all, our results propose that relative levels of Notch receptor and ligands can directly alter DC phenotype.

RÉSUMÉ

Une réponse immunitaire efficace contre des pathogènes ou des cellules cancéreuses requiert un bon encadrement de la part du système immunitaire. Les cellules dendritiques (DCs) sont les premières à intervenir. Elles transmettent des signaux essentiels aux cellules T qui permettent à celles-ci de se différencier en lignées effectrices spécifiques. La communication intercellulaire à partir de la signalisation via Notch, initiée par la liaison d'un récepteur et de son ligand situés sur des cellules voisines, joue un rôle important dans la détermination du sort de ces cellules. Cependant, il a récemment été remarqué qu'une liaison en cis de Notch et de son ligand Delta résultait, chez les cellules de mammifères, en une inhibition de la voie de signalisation par Notch. Les DCs expriment les récepteurs Notch ainsi que leurs ligands. Nous avons par le passé démontré que les DCs exprimaient de façon différentielle les ligands de Notch en réponse aux stimuli associés au lignées $T_{H.}$. Ces découvertes suggèrent que l'abondance relative du ligand par rapport au récepteur entraine la cellule à adopter un phénotype de cellule émettrice ou receveuse du signal. Ainsi, **nous croyons que la signalisation par Notch régule de façon directe les cellules dendritiques et, indirectement, qu'elle module la polarisation des cellules T**.

Afin d'examiner la signalisation par Notch associée aux différents états d'activation des DCs, nous avons mesuré l'expression de certains composants de la voie de signalisation de Notch en réponse à une stimulation au lipopolysaccharide (LPS) ou au parasite *Leishmania.* L'activation des DCs par le LPS, qui conduit à une réponse de type

T_H1, a résulté en un faible taux d'expression de Notch1 et à une augmentation de l'expression de Notch2. Les cellules infectées mais demeurées immatures même suite à l'exposition au LPS avaient, elles, une forte expression de Notch1. À l'aide d'un mutant déficient conditionnel, nous avons démontré que l'expression de Notch1 régule négativement l'activation des DCs. De plus, les DCs activées accroissent l'expression du ligand Delta4, associé à l'induction d'une réponse T_H1, tout en maintenant un faible niveau d'expression de Notch1. À l'inverse, l'expression de Notch2 régule positivement l'activation des DCs comme le démontre l'augmentation de l'expression de la molécule de co-stimulation CD86 ainsi que de la cytokine pro-inflammatoire Interleukine 12 (IL-12). Ce phénotype est également accompagné d'une réduction de l'expression de Jagged2, qui régule négativement la sécrétion d'IL-12. Cette observation est de plus appuyée par le phénotype observé lors de la stimulation au LPS : l'expression de Jagged2 diminue alors qu'augmente celle de Notch2. Ainsi, l'expression mutuellement exclusive de Notch1/Delta4 ou de Notch2/Jagged2 supporte l'hypothèse d'une inhibition en *cis*, un mécanisme par lequel les récepteurs Notch et leurs ligands exprimés sur une même cellule s'inhibent de façon réciproque. De telles interactions résultent en un retrait des récepteurs et des ligands de la membrane cellulaire suivi de leur protéolyse. Ainsi, nos résultats nous permettent de proposer que les niveaux d'expression des récepteurs Notch et de leurs ligands puissent directement altérer le phénotype des cellules dendritiques.

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LIST OF ABBREVIATIONS

AP-1	activator protein
APC	antigen presenting cells
BMDC	bone marrow derived DCs
ChIP	chromatin immunoprecipitation assay
CLP	common lymphoid progenitors
CLR	C-type lectin receptors
Dlk1	Delta-like 1
DNER	Delta/Notch-like EGF-related receptor
dnMAML	dominant negative MAML
ds	double-stranded
Dvi	Dishevelled
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
ERK1/2	extracellular signal-regulated kinase 1/2
GVHD	graft-versus-host disease
HDM	house dust mite
IKKγ	IκB kinase γ
IPS1	Interferon promoter stimulator 1
IRAK	IL-1R-associated kinase
LPS	lipopolysaccharide
LRRs	leucine-rich repeats

mAb	monoclonal antibody
MAGP	microfibril-associated glycoprotein family
MAP3K	mitogen-activated protein (MAP) kinase kinase kinase
MAPK	mitogen-activated kinase
MEL	murine erythroleukemia
MINT	Msx2-interacting nuclear target protein
MM	malignant mesothelioma
mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation primary response 88
MZ	marginal zone
NES	nuclear export signal
NFκB	nuclear factor κB
NICD	Notch intracellular domain
NIK	NFκB inducing kinase
NK	Natural Killer
NLR	NOD-like receptors
NLS	nuclear localization sequence
pAPC	professional APC
PI3K	phosphatidylinositol 3-kinase
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
poly[I:C]	polyinosinic-polycytidylic acid
PRR	pattern recognition receptors
PTEN	phosphatase and tensin homolog

qRT-PCR	quantitative real-time polymerase chain reaction
RGS19	G-protein signaling 19
RIP	receptor-interacting protein
RLR	Retinoic acid-inducible gene (RIG)-I-like receptor
SAP130	Sin3-associated polypeptide p130
SARM	Sterile-alpha Armadillo motif-containing protein
shRNA	short hairpin RNA
SOCS3	suppressor of cytokine signaling 3
ss	single-stranded
T-ALL	T-cell acute lymphoblastic leukemia
TBK1	TANK-binding kinase 1
T_H	helper T cells
TIR	Toll/IL-1R homology
TLR	Toll-like receptors
TRAF	Tumor necrosis factor (TNF) receptor-associated factors
TRAM	TIRAP/Mal TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFNβ

PREFACE

My thesis work is focused on the significance of Notch signaling among bone marrow derived Dendritic Cells (BMDCs) in relation to the development of adaptive immune responses. DCs subtypes are shown to follow different developmental pathways and convey different information based on their location and activation status. However, due to a lack of general consensus on the classification of DC subtypes, first our current understanding of DC subtypes will be briefly reviewed. DC response to pathogens is known to be transmitted to T cells through antigen presenting MHC molecules, co-stimulatory ligands, and cytokines. Along with this instructive signaling, DCs differentially express Notch ligands and receptors in response to various helper T cell (T_H cell)-inducing pathogens. Notch signaling has also been implicated to play a role in regulating inflammation of Antigen Presenting Cells (APCs). T_H polarization by external stimuli is achieved through coordination of various extracellular and intracellular singling events. In order to appreciate such interplay with Notch signaling, one should understand not only the mechanical make-up of this pathway but also its role during the development of the immune system, thus the second half of the introduction will review this topic accordingly. This will be followed by the rationale for my M.Sc. project.

1. INTRODUCTION

1.1 Pathogen recognition and innate immunity

Our immune system possesses the unique capacity to protect against a broad range of microbes, parasites, and cancer. However, pathogens possess different characteristics and harbor diverse mechanisms to induce their harmful effects. An effective immune response is specific against the pathogen, which is achieved by appropriate education of the adaptive immunity by the innate immune system. Prior to the discovery of Toll-like receptors, involved in recognizing microorganism and endogenous danger signals, the innate immunity was considered the unsophisticated and inflexible arm of the immune system; solely responsible for systemic immune responses and activation of the adaptive immune system, such as polarization of naïve helper T cells (T_H). The insight into such pathogen Pattern Recognition Receptors (PRRs) has now elucidated how our innate immune system participates in clearance of pathogens and shapes the adaptive immune response through secretion of cytokines and chemokines[1].

Antigen presenting cells (APCs), including immune cells, fibroblast, and epithelial cells, express PRRs that recognize exogenous foreign molecules and endogenous danger ligands[2]. Activation of signaling downstream PRR initiates an acute inflammatory response which triggers the early phase immune response or maintains tissue's normal function[2]. APCs uptake pathogens through recognition of conserved molecular sequences via PRRs and, then, present the digested antigens to the adaptive immune system in the context of MHC class II molecules[3]. On the other hand, MHC

class I molecules, expressed by all nucleated cells, are important in presentation of intracellular proteins and signaling cytotoxic T lymphocytes (CTLs) to virally infected cells[4]. The receptor repertoire of APCs identifies molecular patterns consisting of peptides, lipids, saccharides, nucleic acids[2]. The PRR families include transmembrane proteins such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), the cytoplasmic Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and NOD-like receptors (NLRs)[5].

The TLRs is one of the best characterized families of PRR, with 10 receptors discovered in humans and 12 in mice[5]. TLRs are characterized by N-terminal leucine-rich repeats (LRRs) and a transmembrane region followed by a cytoplasmic Toll/IL-1R homology (TIR) domain[6]. The ligands of TLRs can be classified into two groups of bacterial cell-wall components (mainly recognized by TLR2 and TLR4) and bacterial or viral nucleic acids[3,6]. Activation of TLRs by nucleic acids leads to production of type I IFNs in addition to proinflammatory cytokines[7]. TLR3 recognizes polyinosinic-polycytidylic acid (poly[I:C]), a viral double-stranded (ds) RNA analogue, and TLR9 recognizes bacterial and viral RNA abundant in unmethylated CpG[5,6]. TLRs can also dimerize to form new pockets for antigen interaction, for example triacylated lipoprotein is recognized by TLR1-TLR2 heterodimer[5]. TLR4 recognizes lipopolysaccharide (LPS) together with myeloid differentiation factor 2 (MD2) on the cell surface[8]. LPS is a component derived from the outer membrane of Gram-negative bacteria and is known to be a cause of septic shock[7,8]. The antigen specificity of TLRs may also change with the cell type, for example, while TLR7 binds to viral single-stranded (ss) RNA in some cell types, it recognizes bacterial RNA in plasmacytoid DCs[5,6,9.] Though not fully understood,

it is speculated that one mechanism by which TLRs can discriminate between self and non-self nucleic acids is through the localization of such receptors to endosomes, where self-nucleotides are rarely found[7]. Activation of TLR pathway leads to upregulation of a transcriptional profile dependent on the cell type and the TLRs involved[5-7]. Such difference in the signaling cascade is partly due to the TIR domain-containing adaptor proteins recruited, including myeloid differentiation primary response 88 (MyD88), TIR domain-containing adaptor inducing IFNβ (TRIF), TIRAP/Mal, TRIF-related adaptor molecule (TRAM), and sterile-alpha and Armadillo motif-containing protein (SARM)[5-7]. TLR signaling can be generalized into two types of MyD88- or TRIF-dependent pathways[7] (**Fig. 1**).

During the MyD88-dependent signaling, the IL-1R-associated kinase (IRAK4), a serine/threonine kinase, is recruited to the death domain of MyD88[6,7]. IRAK4 then activates other families of IRAK and induces their binding to the E3 ubiquitin ligase TNFR-associated factor 6 (TRAF6)[6,7]. In association with an E2 ubiquitin ligase complex, TRAF6 catalyzes formation of a lysine 63 (K63)-linked polyubiquitin chain on itself and IκB kinase γ (IKKγ)[6,7]. This initiates the activation of several mitogen-activated protein (MAP) kinase kinase kinases (MAP3Ks), which in turn activates nuclear factor κB (NFκB) pathway and activator protein 1 (AP-1) transcription factor, leading to production and regulation of proinflammatory cytokines, respectively[2,6,7]. TLR7 and TLR9 signaling induces type I IFNs in addition to other NFκB-dependent cytokines[7].

In addition to the MyD88-dependent pathway, if TLR4 is translocated to the endosome, TRIF is recruited instead[2,8]. Stimulation of TLR3 with dsRNA, also, triggers the TRIF-dependent pathway[7,8]. TRIF binds to receptor-interacting protein 1 (RIP1) and

TRAF6 to activate NFκB[7]. This interaction is functionally partially-redundant, as TRAF6 but not RIP1 recruitment is essential for NFκB activation in fibroblasts[10] while TRAF6 is

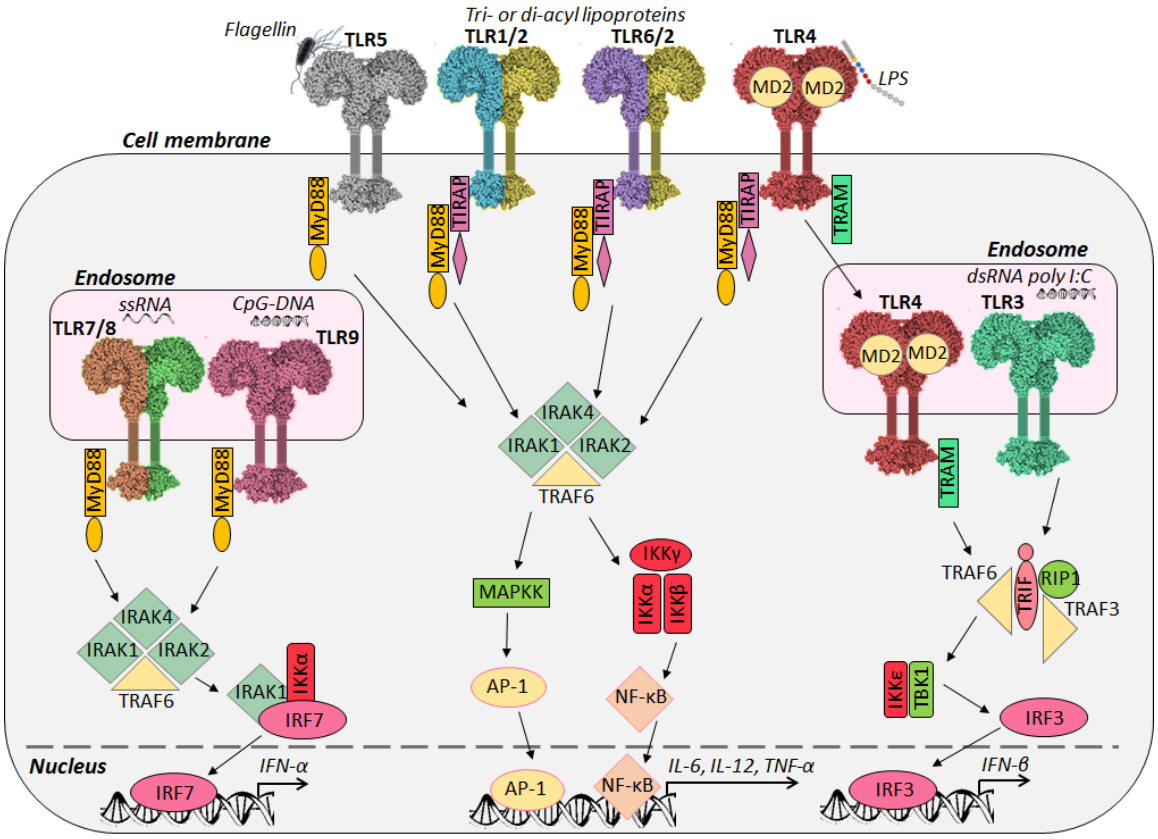

Fig. 1. The overview of TLR signaling. Ligand-induced dimerization of TLRs increases their specificity and sensitivity in recognition of microbial components. TLR5 homodimer binds to Flagellin while TLR4 homodimer can further enhance its recognition of endotoxin lipopolysaccharide (LPS) by binding to MD2 proteins. TLR2 may form a heterodimer with either TLR1 or TLR6 to recognize tri-acyl and di-acyl lipoproteins, respectively. In contrast to the aforementioned TLRs, which exist on the cell surface, TLR3, TLR7/8 and TLR9 are localized to endosomes where they recognize nucleotides derived from microbial agents. TLR4, on the other hand, can either remain on the plasma membrane or translocate to endosomes. Once the TLR pathway is triggered, it progresses downstream either via myeloid differentiation primary-response protein (MyD88)- or via TIR domain-containing adaptor protein inducing IFN-β (TRIF)-dependent pathways. Upon initiation of MyD88-dependent pathway, IL-1R-associated kinases (IRAKs) and the adaptor molecule TNF receptor-associated factors 6 (TRAF6) interact to activate mitogen-activated protein kinases (MAPKs) and IκB kinases (IKKs). Consequently, transcription factors, including activator protein 1 (AP-1), NF-κB, and Interferon-regulatory factor 7 (IRF7), are translocated to the nucleus. In the case of TRIF-dependent pathway, TRAF3 and TRAF6 are recruited to activate IRF3. As a result, the transcription of proinflammatory cytokines, via plasma membrane TLRs, and type 1 IFNs, via the endosomal TLRs, is induced. dsRNA, double-stranded RNA; MKK, MAP kinase kinase; RIP1, receptor-interacting protein 1; ssRNA, single-stranded RNA; TBK1, TANK-binding kinase 1; TRAM, translocating chain-associating membrane. (Figure adapted by author from Luke A. J. O'Neill, 2013).

not needed for NFκB activation in macrophages[7]. TRIF can also interact with TRAF3, which, in association with noncanonical IKKs, activates IRF3 and subsequently results in IFN-β production downstream of TLR3/4[7]. TRAF3 is also responsible for type I IFN production in MYD88-mediated TLR7/9 activation or even in TLR-independent responses to viral infection[7]. Despite the capability of TLRs to recognize viruses that are in the endosomal compartments, for an efficient antiviral response, cytoplasmic receptors are required to spot the virus where it replicates, i.e. inside the cell[5,7]. RLRs detect viral RNA and transmit their signal through a common adaptor protein, Interferon promoter stimulator-1 (IPS1)[5]. Short dsRNA and ssRNA are RLR ligands, which in parallel with TLRs, result in production of type I IFNs[5].

In addition to TLRs, NLRs are also capable of inducing antibacterial responses; however, unlike TLRs, NLRs recognize the bacteria in the cytoplasm[5]. Some NLRs are localized to inflammasomes which are presumably activated by danger signals[5,9]. In case of infection with microbes these NLRs are suspected to be indirectly activated through lysosomal destabilization, potassium efflux, or ROS generation[9]. CLRs are also receptors for endogenous ligands such as Sin3-associated polypeptide p130 (SAP130) released from damaged cells[11]. CLRs such as DC-SIGN and mannose receptors recognize pathogens, and along with many other innate receptors, take part in shaping adaptive immune response[9]. The diversity of all classes of PRRs is proving their importance in the innate immunity, however, our picture of this complex network is far from complete.

1.2 Dendritic cells, the central regulator of our immune system

Dendritic cells (DCs) are crucial players of the innate immune system, forming a remarkable cellular network. DCs, discovered by Ralph Steinman and Zanvil Cohn in 1970s, are professional APCs (pAPCs)[12,13] which upon an encounter with peripheral stimuli transmit the threat-specific information to other components of the immune system[14]. Unlike DCs, macrophages are inefficient in antigen presentation, however their enhanced phagocytic and catabolic activity makes them effective tissue-resident killers[15]. DCs, in addition to direct activation by pathogens, can orchestrate information coming from a range of immune cells such as T cells, NK cells, NK T cells, basophils, and mast cells[16]. The comprehensive ability of DCs to modulate immune system is evident in their wide range of types and shapes. Consequently, classification of DCs has been challenging and currently being described to distinguish each cell type, several DC categories have been described.

Conventionally, DCs are divided into plasmacytoid (pDCs) and classical DCs (cDCs), sharing the expression of integrin molecule CD11c with macrophages[16,17]. pDCs, identified from cDCs by expressing B220/CD45R but lacking CD11b surface markers. pDCs have narrow range of TLRs and have lower capacity to present antigens, while cDCs populate most of lymphoid and non-lymphoid organs and have enhanced ability to sense tissue injuries, capture peripheral cues, and present T cells with phagocytized antigens[17]. DCs are also classified based on their migratory capacity. Non-lymphoid cDCs are capable of migrating to T-cell zones of lymph nodes (LNs) loaded with tissue antigens[18]. LNs additionally contain lymphoid cDCs, classified by CD8α, and myeloid cDCs, classified by integrin CD11b expression. $CD8\alpha^+$ cDC are efficient in priming

$CD8^+$ T cells through histocompatibility molecule MHC class I, as opposed to $CD4^+$ cDCs that are more efficient in activating $CD4^+$ T cells through MHC class II[17,19,20]. In contrast to migratory non-lymphoid DCs arrived to LNs, lymphoid-resident cDCs are in an immature state at their steady state[17].

Even though peripheral cDCs are classified based on expression of integrin molecules CD103 and CD11b and lack of CD4 or CD8α, peripheral $CD103^+$ subsets functionally resemble lymphoid $CD8\alpha^+$ cDCs, while $CD11b^+$ cDCs resemble $CD4^+$ cDCs[16,17]. Thus, classification of DCs by their transcriptome profile may provide a more accurate means of functional classification rather phenotypic surface markers. Accordingly, studies in various transcription factor knockouts have revealed lineage specific molecules important for development and function different subsets of DCs[17]. For example, Zinc Finger And BTB Domain Containing 46 (Zbtb46), a negative regulator of DC activation, is expressed on lymphoid $CD8\alpha^+$ and peripheral $CD11b^+$ [21], while basic leucine zipper transcription factor, ATF-like 3 (BATF3) is required for development and maturation of $CD8\alpha^+$ cDCs[17,22] and Notch2 is essential to $CD11b^+$ cDC development[23]. $CD8\alpha^+$ and $CD103^+$ cDCs are shown to be important in mediating appropriate T-cell response against intracellular pathogens[24], while $CD11b^+$ and $CD4^+$ cDCs are responsible for extracellular pathogen immunity and autoimmune responses[16,25]. Furthermore, even though $CD8\alpha^+$ cDCs have the capacity to activate effector $CD8^+$ T cells[16], pDCs have an enhanced capability to modulate antiviral type 1 IFN response by priming $CD8^+$ T cells and NK cells[26]. Upon recognition of pathogen-associated microbial patterns (PAMPs), such as recognition of gram-positive bacteria by TLR2[1], DCs activate T-cells by providing three signals. First they present components of

the pathogen in the context of antigen presenting MHC molecule[16,17,27]. The second signal is provided through expression of co-stimulatory ligands CD80 and CD86, which primes T cells upon binding to CD28 or CTLA-4 receptors[16,17,27]. Cytokines secreted by DCs provide the differentiation cues necessary for polarization of T-cells into different lineages, crucial for effective clearance of pathogens[20]. DC encounter of intracellular pathogens such as viruses and parasites leads to secretion of cytokines such as IL-12 and TNF-α, mainly by $CD8\alpha^+$ and $CD103^+$ cDCs, and type I IFNs, predominantly by pDCs, which guide the differentiation of $CD4^+$ T_H1 and $CD8^+$ T cells[16,26,27]. More recently, it has been shown that DCs only exposed to *Leishmania* soluble antigens become activated and produce IL-12 but no TNF-α [28]. Further, these bystander cells mediate polarization of IL-2 and IFN-γ producing CD4+ T cells[28]. On the other hand, Helminth worms and allergens result in secretion of thymic stromal lymphopoietin (TSLP) by $CD11c^+$ cDCs and shape the less characterized T_H2 response[29,30]. More recently, several other T_H-lineages have been identified, for example, extracellular pathogens such as bacteria and zymosan cause secretion of cytokines such as IL-6, IL-23, and TGF-β, mainly by $CD11b^+$ and $CD4^+$ cDCs, which favors a T_H17 response[31].

While inflammatory DCs can activate either $CD4^+$ or $CD8^+$, or even $CD4^-CD8^-$ T, B, and NK cells to become immunogenic, immunosuppressed DCs presenting innocuous environmental antigens or producing pleiotropic anti-inflammatory factors, such as IL-10, can prevent, inhibit, or modulate T cell-mediated effector responses[32,33]. Tolerogenic DCs can induce immunological tolerance by activating one of several types of regulatory T cells (T_H3 and T_R1)[32]. Regulatory T cells (T_{REG}) are essential to restrain autoimmunity and chronic inflammation, on the other hand, such immunosuppression can assist

pathogens or tumors to evade the immune system[32-34]. For example, infection of DCs by *Mycobacterium tuberculosis*[35] or *Leishmania*[36] prevents mycobacteria- or LPS-induced DC maturation[37]. Infection of DCs or macrophages by *L. mexicana* was shown to inactivate MAPK activity and prevent NFκB and AP-1 translocation to the nucleus[38]. DCs infected by *L. mexicana* were shown to have reduced capacity to present antigents and to promote T_H1 polarization[38]. Similarly, splenic DCs infected by *L. infantum* were shown to polarize naïve $CD4^+$ T cells towards a nonprotectie T-bet^+IFN-γ^+IL-10^+ phenotype[39]. Consequently, the adpotive transfer of such parasite Ag-specific T cells facilitated viceral leishmanasis[39]. Moreover, several studies suggest that *M. bovis* harbors DC-SIGN receptor to infect DCs[40] and, consequently, inhibit cytokine production through upregulation of Notch1 expression[35,38]. Similarly, in a clinical setting, while adoptive transfer of T_{REG}s may resolve autoimmune disorders, inhibition of tolerance can improve anti-tumor immune responses[41]. Therefore, the understanding of the role of DCs in mediating inflammatory responses through regulation of T cell polarization is helpful in immunotherapies involving DC-based vaccination.

Today we know that polarization of T cells is highly dynamic and plastic[42]. Depending on the microenvironment helper T cells encounter, they can re-differentiate to other lineages[43]. Inflammatory microenvironments are shaped by local secretion of cytokines and cell-cell contacts. Such processes are tightly regulated and, hence, imbalances in T_H cell polarization can lead minor to lethal pathologies. For example, resistance to *tuberculosis* and *Leishmaniasis* infections require an appropriate T_H1 response[44,45]. However, inflammatory cytokines were shown to be insufficient for full activation of DCs, which is necessary for directing T_H cell polarization[46]. Similarly, mice

models deficient for components of cytokine signaling machinery reveal that such T cell immune response are not entirely dependent on cytokines. For example, in the absence of signal transducer and activation of transcription 6 (STAT6), necessary for IL-4 receptor signaling, T_H2 cells differentiation is not decreased during helminth infection [47]. Similarly, certain viral T_H1 cell responses, such as those generated against choriomeningitis and hepatitis, are fully independent of IL-12 [48]. Moreover, IL-12-deficient mice infected with intracellular pathogens did not default to a T_H2 profile and generated substantial numbers of IFN-γ producing $CD4^+$ T cells[49]. As supported by these findings, considering the dependence of T_H cell polarization on the microenvironment context and cell-cell contact, it is evident that cytokine-induced T-cell lineage commitment is only one arm of such sensitively orchestrated machinery and other membrane-bound factors also have a decisive role.

Notch transmembrane proteins are extensively involved in cell-cell communication, cell-fate decision, and cell survival [50]. Notch functions range from neuronal development and angiogenesis to expansion of hematopoietic stem cell and T cell development[50,51]. Taking into account the implications of Notch signaling in T cell expansion and pathogen-induced Notch remodeling in DCs[52,53,54] it is likely that Notch signaling directly regulates DC phenotype and indirectly participates in T cell polarization.

1.3 Notch signaling, simple but flexible

During development of structures with arranged layout and patterns, proper cell-cell communication is necessary to transmit information concerning the dynamic environment in which cells are differentiating and reorganizing. Evolutionary conserved pathways such as Wnt, Sonic hedgehog (Shh) and Notch are vital players of such developmental processes in embryogenesis[55]. In 1917, Notch signalling was first described when anomalies in *Drosophila* wings (notched) was linked to *Notch* partial loss of function[56]. Since then, observations in lethal Notch knockouts in mice and developmental anomalies and cancer associated with deregulated Notch signalling in humans have stressed the importance of precise Notch signalling[50, 57]. This highly evolutionary conserved transmembrane protein, present in multicellular metazoan organisms, participates in a linear pathway that can modulate the genetic profile of the cell directly. Notch signaling has been recently extensively reviewed[50, 58, 59, 60] and is briefly summarized below (**Fig. 2**).

In mammalian cells there are four Notch receptors (Notch1 to 4) and five Jagged and Delta-like Notch ligands (Jag1 and 2 and Dll1, 3, and 4)[50]. Both Notch ligands and receptors are multi-domain single-transmembrane proteins[50]. The receptors contain multiple extracellular epidermal-growth-factor-like (EGF-L) repeats that contains disulphide bonds forming β-strands, followed by three cytosine rich Lin/Notch repeats (LNR), also referred to as Delta/Serrate/Lin (DSL) domain, which bind to ligands[40,58]. The heterdimerization domain spans through the plasma membrane and consists of non-covalent linkage of extracellular and intracellular portions[50,58]. The transmembrane domain includes the membrane-proximal RBP-J-associated molecules (RAM) and

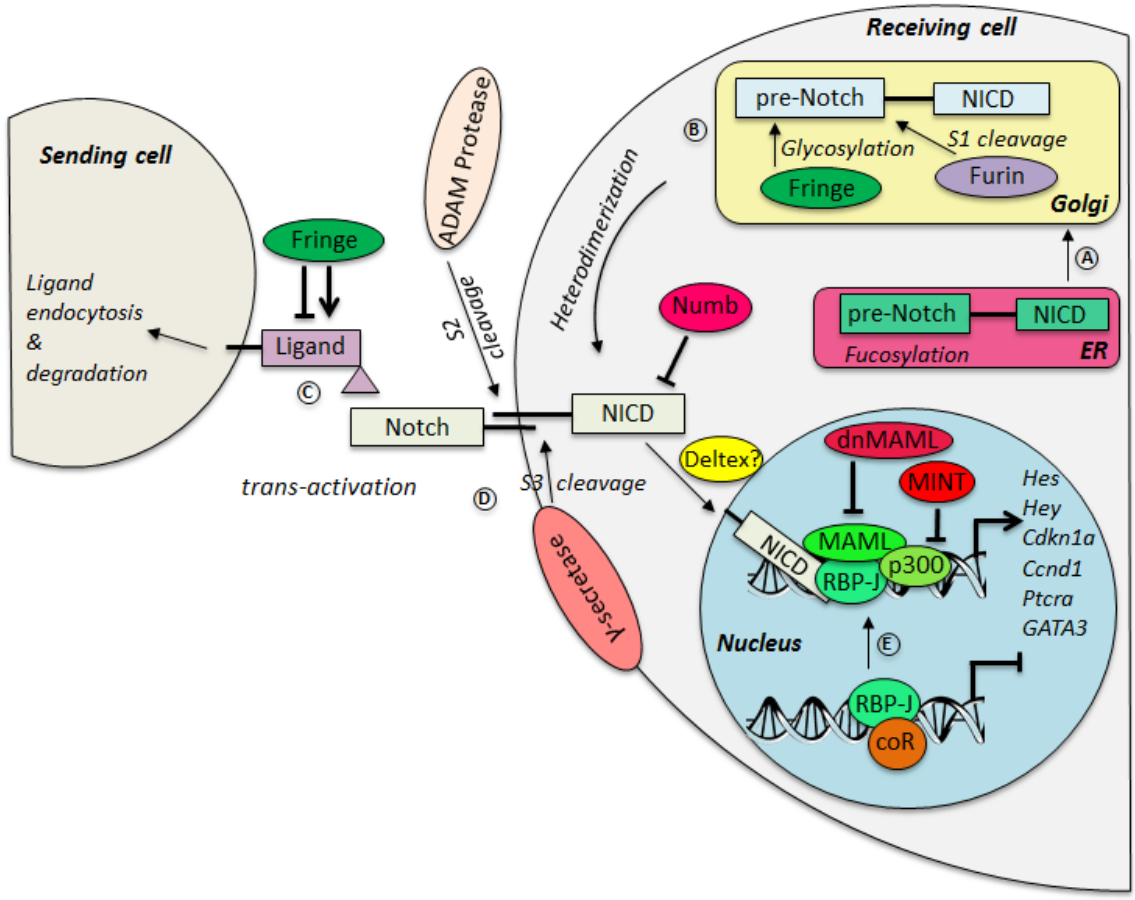

Fig. 2. The Notch Pathway, simple but flexible. The canonical Notch signaling is unique in that it requires cell-cell contact and results in direct communication between adjacent cells. There are five mammalian Notch ligands (Delta1-3 and Jagged1-2) and four Notch receptors (Notch1-4). **A)** The intracellular and extracellular domains of Notch receptors are first synthesized as one protein (pre-Notch) and transferred to the endoplasmic reticulum (ER) for addition of *O*-frucose, which serves as a substrate for the subsequent glycosylation events in the Golgi apparatus. **B)** Glycosylation of serine and threonine residues on the EGF-L repeats is catalyzed by members of the Fringe family. Furin convertase cleaves pre-Notch at S1 into extracellular and intracellular domains that are non-covalently linked. Notch heterodimer is then transferred to the plasma membrane. **C)** The glycosylation events carried out earlier by Fringe in the Golgi apparatus determine the responsiveness of Notch to Delta and Jagged ligands. The ligand downstream signaling events are less characterized. **D)** The *trans*-interaction of ligands and Notch triggers two consecutive proteolytic cleavages carried out by ADAM metalloprotease family and γ-secretase complex at S2 and S3, respectively. **E)** Consequently, the intracellular domain (NICD) is liberated and localized to the nucleus where it displaces co-repressors (coR) on RBP-J and recruits co-activators, including p300 and mastermind-like protein (MAML). As a result, several downstream targets of Notch, such as *Hes* (Hairy/enahncer of split), *Ptrca* (pre-T-cell-receptor α), *Ccnd1* (Cyclin D1), *Cdkn1a* (cyclin-dependent kinase inhibitor α), and *GATA3* (GATA binding protein 3), are transcribed. Regulators of Notch, including Numb, Deltex, MINT (MSX2-interacting nuclear target), and dominant-negative MAML (dnMAML), further modulate the activity of this pathway. Numb suppresses Notch signaling by preventing translocation of NICD to the nucleus and targeting it for degradation. Deltex, on the other hand, can either promote or inhibit Notch signaling depending on the cellular context. Alternatively, Deltex can mediate Notch signaling independently of RBP-J. Co-repressors dnMAML and MINT inhibit Notch signaling at the level of transcriptional complex assembly. (Figure adapted by author from Clemens Grabher, 2006).

(ANK) domains that are involved in interactions with several cytosolic and nuclear proteins[50,58]. There are also two nuclear-localization sequences (NLSs) spanning the ANK repeats[50,58]. Just before the *C*-terminal PEST (proline-, glutamate-, serine- and threonine-rich) domain there is a transactivation domain (TAD) mediating transcription, however Notch3 and Notch4 lack this domain[50,58].

The relative strength of Notch signaling can be modulated by post-translational modifications of Notch receptors[50,58]. The EGF-L repeats are first modified by *O*-glucose or *O*-fructose additions, which mediates further glycosylic extensions[50,58]. For example, addition of N-acetylglucosamine to *O*-fructose by Lunatic Fringe enhances activation of Notch by Dll4; loss of function of which skews T_H2 polarization[61]. *O*-glucose, in contrast, seems not to be involved in ligand interaction but, instead, with Notch cleavage by proteases, which is a necessary step for Notch signal transduction[50,58]. Co-expression of Fringe with Delta or Serrate (Jagged) ligands has been shown to effect tissue organization and boundaries and cell fate decisions in *Drosophila* wing and mouse and human spinal cord[50]. While canonical Notch ligands are responsible for the majority Notch signaling, a diverse group of structurally unrelated ligands can also modulate Notch signaling[50,58]. These noncanonical Notch ligands can be activating, inhibiting, or inert and contribute to the pleiotropic effects of Notch signaling[58].

1.3.1 Canonical Notch signaling

The classical Notch signaling is initiated upon binding of Notch receptor and ligand of two adjacent cells[50,58]. Once the DSL domain of the receptor is ligated, it undergoes a

conformational change that unfolds the negative regulator region (NRR) which exposes site S2 (12-13 amino acids outside of the transmembrane domain) for cleavage by a metalloprotease of the ADAM family[50,58]. However, some reports indicate this cleavage may also take place in absence of ligation[62]. The resultant generation of the Notch extracellular truncate (NEXT) facilitates the next cleavage at the intramembrane site S3 by a γ-secretase multienzyme complex[50,58]. Dependent on the complex's presenilin content and the activity of its modulator proteins, substrates are preferably cleaved[50,58]. Additionally, γ-secretase complex heterogeneously cleavages Notch, which, in turn, modulates duration of Notch signaling by generating Notch intracellular domains (NICDs) with various half-lives[58]. The liberated NICD is, then, translocated into the nucleus where it participates in the formation of a short-lived transcriptional activation complex[50,58]. This complex constitutes the DNA-binding transcription factors of recombination signal binding protein for immunoglobulin kJ region (RBP-J) family (also known as CSL in humans, suppressor of hairless in *Drosophila melanogaster*, and LAG-1 in *Caenorhabditis elegans*), the NICD and co-activator proteins of the Mastermind (MAML) family and p300[50,58]. The genes downstream of Notch signaling are numerous and specific to cell type, cycle, and lineage progression[50,58]. However, the genes *Hes* (mammalian hairy and Enhancer-of-split homologues) and *Hey* (Hairy/enhancer-of-split related with YRPW motif) of bHLH transcription factor family are the traditionally studied immediate targets[50,58]. Other documented genes include *Cdkn1a* (cyclin-dependent kinase inhibitor), *Ccnd1* (Cyclin D1), *Ptcra* (pre T cell antigen receptor alpha), *GATA3* (GATA binding protein 3), and *c-myc*[50,58]. Prior to NICD participation as a transcription factor, co-repressors in association with RBP-J repress transcription of

genes with CGTGGGAA motifs in their promoter[50,58]. The interaction dynamics between NICD and RBP-J varies with the Notch receptor involved, further contributing to generation of diverse transcriptomes[63].

During canonical Notch signaling each receptor is consumed to activate a linear transduction pathway, as opposed to many other pathways[50,58]. Thereby, the number of receptors expressed and activated is a key determinant of strength of Notch signaling[50,58]. Accordingly, even an incomplete knockout of Notch ligand Delta4 is lethal in mice. Haploinsufficiency of human Notch2 or Jagged1 can cause mild to severe heart and liver diseases[64]. While MAML-1 deficiency leads to partial disruption of Notch signaling, mice for both MAML-1 and -3 develop several cardiovascular and respiratory defects which leads to an early death during the organogenic period[65].

Several auxiliary intramembrane proteins regulate Notch signal transduction as well (Fig. 2). Numb is an endocytic adapter protein that antagonizes Notch signaling by recruiting E3 ubiquitin ligase itchy (Itch) to facilitate receptor degradation[66]. Moreover, trafficking of endocytosed NICD to lysosomal compartments is also regulated[50]. Similar to Notch extracellular domain, NICD is subject to several post-translational modifications[50,58]. For example, glycogen synthase 3β (GSK3β) phosphorylates *C*-terminus of ANK repeats, enhancing expression of *Hes1* by NICD1 but destabilizing NICD2[50]. Other modifications such as ubiquitylation, acetylation and hydroxylation of NICD are less understood, but recent evidence suggest half-lives of Notch proteins are differently affected by such post-translational events[50].

1.3.2 *Cis*-inhibition

Early studies in *Drosophila* revealed another form of ligand-receptor interaction that takes place on the same cell surface[67]. The clue for such interaction was disclosed when increased ligand expression led to enhanced receptor loss-of-function, and vice versa. During *Drosophila* wing development, overexpression of delta resulted in increased Notch signaling in adjacent cells while Notch activity was eradicated in *cis* [68]. Such Notch inhibition was also observed in vertebrates. For example, mouse Notch1 inhibited postmitotic neurite outgrowth, as supported by overexpression of constitutively active NICD1 [69] . When these neuroblastoma cells were co-cultured with fibroblasts overexpressing Dll1, neurites shortened due to *trans*-activation of Notch1[69]. However, co-transfection of neuroblastoma cells with Dll1 resulted in extensive postmitotic neurite outgrowth, a phenotype similar to that observed in co-transfection with Notch inhibitor, Numb[60,69]. Dll3 is a divergent member of DSL Notch ligand family in mammalian cells that was shown to lack the ability to *trans*-activate Notch but able to participate in *cis*-inhibition by targeting Notch1 for lysosomal degradation[70].

Co-immunoprecipitation experiments of chick Notch ligands, Delta1 and Serrate1, and mouse Notch1 suggested that *cis*-interaction is independent of the intracellular region but involves EGF repeats of receptors and DSL domain of ligands[71]. Furthermore, in contrast to catalytic studies, titration-based studies indicated that while levels of *trans*-Delta determined the amplitude of Notch signaling, the threshold of *cis*-Delta concentration under which Notch remained active was independent of *trans*-Delta levels [72]. One major question about *cis*-inhibition was whether the *cis*-bound ligand retained its ability to simultaneously *trans*-activate Notch[60] (**Fig. 3**). The same study

showed that Notch and its ligands reciprocally inhibited each other to dictate a mutually exclusive phenotype of "signal-sending" or "signal-receiving" [72]. These observations, collectively, suggest that several multi-layered regulatory mechanisms manage Notch signaling in level, duration and spatial distribution, aimed to establish a pathway evolved into multiple contexts.

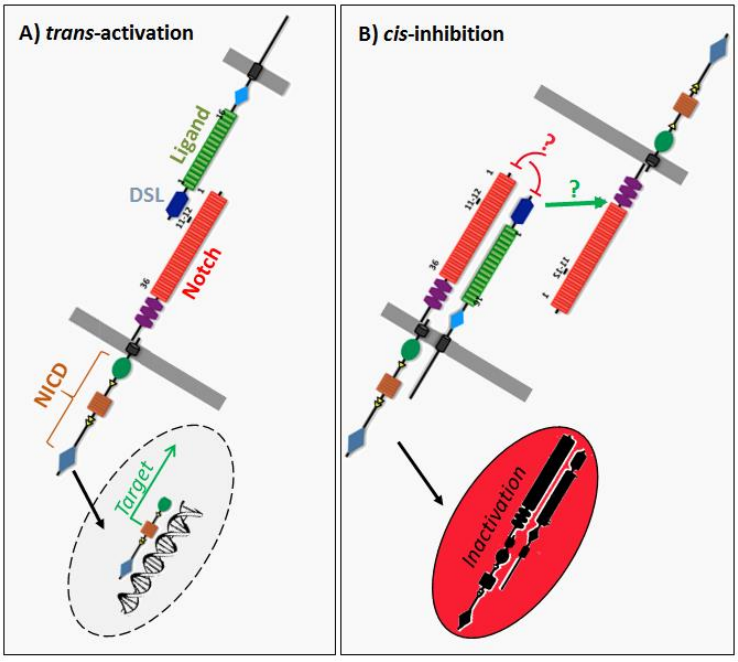

Fig. 3. The possible interactions between Notch receptors and ligands. The co-expression of both Notch and its ligands makes two types of interaction possible between these molecules. **A)** Interaction of Notch (red) with its ligand (green) on the adjacent signal-sending cell results in *trans*-activation of Notch and the subsequent translocation of Notch intracellular domain (NICD) into the nucleus, where it activates transcription of its target genes. **B)** The presence of both the receptor and ligand on the same membrane results in *cis*-inhibition by the ligand and, consequently, internalization of the complex. The detailed mechanism of *cis*-inhibition is yet to be elucidated. It is not known if such inhibition is also inducible by the receptor or whether the ligand participating in *cis*-interaction is simultaneously capable of trans-activating Notch. DSL, Delta/Serrate/LAG-2.

1.3.3 Non-canonical Notch signaling

Unlike the canonical ligands, the DSL domain responsible for the activation of Notch is absent in noncanonical ligands[50,73]. These ligands are structurally unrelated and include integral and glycosylphsphatidylinositol (GPI)-linked membrane and soluble proteins[73]. One of the non-canonical ligands, best known for its inhibitory role in adipocyte generation, is Delta-like 1 (Dlk1). This protein is structurally very similar to Delta

ligands but lacks the DSL domain and suppresses Notch signaling in *cis*[73]. Dlk1 is expressed at high levels in the fetal thymus and early B cell lineages, and shown to be important for normal lymphocyte development[74,75,76]. Similar to Dlk1, Delta/Notch-like EGF-related receptor (DNER) is from the integral family, but it activates Notch in *trans*[73] instead and carries the development of glial cells at later stages of differentiation[77]. While the membrane-form of DNER is shown to act through RBP-J, its soluble form is implicated to utilize E3 ubiquitin ligase Deltex instead, as do GPI-linked neural cell adhesion molecules, contactin-1 and -6[73]. Both contactins activate γ-secretase-dependent Notch signaling to induce oligodendrocyte differentiation[78]. Another group of non-DSL proteins that can activate Notch signaling are the soluble microfibril-associated glycoprotein family, MAGP-1 and -2[73]. Both MAGP proteins induce γ-secretase-dependent generation of NICD, however, MAGP-2 does not require ADAM protease and can cause nonenzymatic dissociation of Notch in *cis*[79].

Compared with the canonical ligands, which require binding to EFG repeats of Notch to activate signaling, non-canonical ligands do not seem to share a common binding site[73] but have intrinsic signaling regardless of their interaction with Notch[80]. While non-canonical ligands may contribute to pleiotropic outcomes of Notch signaling, they do not appear to be crucial for embryonic development as DSL ligands are[73], but, instead, they may act as modulators of canonical Notch signaling through molecular mechanisms that remain to be elucidated[80].

1.4 Crossing paths with Notch

In concordance with recent appreciations for non-canonical Notch pathway, consequences of Notch interaction with other signaling pathways such as Wnt, TGFβ, PI3K/Akt, and NFκB are proving to be significant.

1.4.1 Wnt & TGFβ pathways

Wnt pathway, an important regulator of cell fate determination, is activated upon binding of glycoprotein Wnt to Frizzled receptors that leads to Dishevelled (Dvi)-mediated liberation of β-catenin, an integral E-cadherin adaptor protein, from a regulatory APC/Axin/GSK-3β complex[81]. The subsequent translocation of β-catenin to nucleus activates transcription of genes such as *c-Myc* and *Cyclin D1*[81]. However, Wnt was also shown to upregulate transcription of Notch2 and Dll4 in colorectal cancer cells and during vascular remodeling, respectively[82]. Several components of Wnt have also been shown to physically interact with NICD and MAML, further tuning Notch pathway [83,84].

NICD may also interact with TGFβ-activated SMAD, a homolog of Drosophila mothers against decapentaplegic (MAD)[85]. TGFβ pathway enhances Notch activity by presumably incorporating phosphorylated SMAD into NICD/RBP-J complex [86]. In smooth muscles, SMAD2 and 3 were shown to interact with NICD4 but not NICD1 or 2 [87]. In T cells, however, formation of NICD1/RBP-J/SMAD complex facilitates transcription of forkhead box P3 (*Foxp3*) and *Il9*, which is necessary for induction of T_{REG} and T_H9, respectively [88,89].

1.5.2 PI3K/Akt pathway

The pleiotropic effect of Notch signaling in the immune development and metabolism can be attributed predominantly to its capacity in regulating PI3K/Akt and NFκB pathways[59]. First indication of Notch involvement with PI3K/Akt pathway was observed when Notch inhibitors induced cell cycle arrest and apoptosis in some T-cell acute lymphoblastic leukemia (T-ALL) cell lines, but not in those with homozygous loss of the tumor suppressor gene phosphatase and tensin homolog (*PTEN*)[90]. The Akt pathway is triggered when stimuli such as growth factors or cytokines bind to their cognate receptor and induce PI3K activation [91] . Phosphorylation of PI3K causes generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) metabolites and subsequent translocation of Akt to the plasma membrane, where it gets activated by dual phosphorylation[92]. A number of phosphatases, including PTEN, buffer the Akt activity by inactivating PI3K[90]. *PTEN* expression, in turn, is indirectly regulated by Notch pathway, through Hes1-induced inhibition[93]. Another proposed mechanism of Notch-mediated Akt activation is through regulation of G-protein signaling 19 (RGS19)[94]; a protein known to modulate chemokine responses in DCs. Recently, RGS19 is also suggested to participate in cell differentiation and proliferation events by interacting with Wnt, JAK, and Akt pathways[94]. In T-ALL cell lines, RGS19 is shown to induce PTEN and Akt phosphorylation[95]. The molecular link to Notch was unfolded when γ-secretase inhibitors suppressed TLR-induced phosphorylation of RGS19 in macrophages[94].

1.5.3 NFκB pathway

NFκB proteins (p50, p65, c-Rel, p52, and RelB) act mainly as dimeric transcription factors that regulate critical genes involved in a broad range of biological processes, including immunity, stress response, apoptosis, and differentiation[96]. NFκB proteins can participate in such processes through three different pathways: canonical, non-canonical, and alternative pathway[96]. The canonical NFκB pathway is triggered when growth hormones, proinflammatory cytokines, LPS, or TCR ligation activates of IKK complex (comprised of IKK-α, -β, and -γ subunits)[96]. Inhibitory IκB proteins (IκB-α, -β, and –γ), normally bound to NFκB subunits, become phosphorylated by IKK proteins and, subsequently, polyubiquitinated for proteasomal degradation[96]. The resultant liberation of NFκB dimers unmasks their nuclear localization sequence (NLS), allowing their translocation to the nucleus. IκB-α also contains a nuclear export signal (NES), guiding the translocation of NFκB back to the cytoplasm when they are physically interacting[96]. The non-canonical pathway is triggered by ligands such as CD40L and lymphotoxin once NFκB inducing kinase (NIK) is activated[96]. Then, NIK phosphorylates IKKα homodimers which causes partial degradation of p100, p50 precursor, leading to the generation of a unique assembly of NFκB dimer with distinct gene expression pattern[96]. The alternative pathway is activated by hypoxia, oxidizing radicals, or UV radiation, causing IκB-α IKK-independent phosphorylation and subsequent degradation[96].

Notch1 was shown to upregulate expression of NFκB subunits and IκB-α in hematopoietic precursors, controlling LPS-induced proliferation of B cells and GM-CSF-induced differentiation of DCs[97]. Reciprocally, NFκB was shown to upregulate Jag1, Hes-5, and Deltex1 expression in B cells, presumably cooperating with Notch2 in MZ B

cell development [98]. Co-transfection experiments in Jurkat cells showed that the intracellular domain of Notch1 regulates NFκB in a dose-dependent manner, with low amounts of Notch1 stimulating NFκB transcriptional activity and higher amounts of Notch1 inhibiting IκB-dependent p50 transactivation[96]. Moreover, the domains of NICD1 responsible for binding to p50 and RBP-J overlap at the N terminus, which creates a competition between Notch and NFκB transcriptional activity[96]. Likewise, Notch1 was also immunoprecipitated with the chromatin region of *Ifng* despite the lack of RBP-J binding site in T cells[99]. It was discovered that during the late wave NFκB activation, sufficient Notch1 signaling enhances NFκB transcriptional activity by outcompeting IκB-α in the nucleus and preventing nuclear export of p50/c-Rel complex[99]. Furthermore, non-canonical Notch pathway in T-cell acute lymphoblastic leukemia (T-ALL) was shown to augment NFκB pathway by enhancing IKKa kinase activity[100]. Additionally, canonical and soluble Notch ligands are shown to induce rapid activation of NFκB once their bound to their cognate receptors. In the cytoplasm, Notch can physically interact with IKK proteins and enhancing their IκB kinase activity[96]. While Notch1 is shown to induce survival of murine erythroleukemia (MEL) cells through this mechanism [101], Notch3 seems to be also capable of activating NFκB non-canonical pathway independently of NIK[96]. In T lymphoma cells, overexpression of NICD3 was shown to bind to IKKα homodimer and induce p100 processing necessary for p52/RelB heterodimer generation[102]. These observations sketch a complex picture of Notch-NFκB interaction that needs further clarifications. Nevertheless, these findings strongly indicate that conventional methods of investigating Notch signaling are not sufficient and approaches capable of targeting various arms of this pathway need to be developed.

Considering the numerous feed-back loops present between Notch and other molecular pathways, those manipulating various members of Notch pathway are judicious to use tools that mimic physiological conditions.

1.5 Notch in the immune system development

The Notch signaling pathway regulates many aspects of embryonic development and homeostasis in several adult organ systems[47]. However, the appreciation for the regulatory role of Notch in immune cell development and function is relatively recent[56]. Within the hematopoietic system, Notch signaling is best characterized for its role in modulating lymphocyte development and guiding T cell maturation[56]. Our current understanding of Notch signaling in the development of immune system is briefly summarized below.

1.5.1 B cell development

B and T cells are generated from common lymphoid progenitors (CLPs) that differentiate from hematopoietic stem cells in the bone marrow[103]. While CLPs that migrate to thymus differentiate into T cells, those that remain in the bone marrow develop into immature B cells after going through pro-B and pre-B stages[103]. Retroviral expression of NICD1 in bone marrow progenitors suppresses early B cell development and causes ectopic generation of T cells[104]. Even though Jag1 is expressed in the stromal cells of the bone

marrow, potentially activating Notch1, no developing T cells are normally found in the bone marrow[104]. Therefore, there may be some modulators of Notch expressed in bone marrow that suppress Notch signaling. In accordance with these findings, overexpression of the transcription factor Hes1, downstream target of Notch, was shown to suppress B cell development in the bone marrow[105].

Once immature B cells have undergone successful DNA recombination in variable exons of immunoglobulin (Ig) gene loci[103], they migrate from the bone marrow into secondary lymphoid structures where they develop into follicular and marginal zone B cells[106]. In spleen, follicular B cells comprise the majority of circulating B cells and participate in T cell mediated responses, whereas marginal zone (MZ) B cells participate in fast T cell-independent antibody responses that is tailored to activation of NKT cells[103]. Development of MZ B cells was shown to be dependent on Dll1-medicated Notch2 signaling[107]. Though modulation of various components of Notch signaling, including RBP-J, supported the requirement of Notch singling in MZ B cell development, Hes1 was shown not to be involved[108]. Moreover, deletion of a specific negative regulator of Notch/RBP-J signalling, Msx2-interacting nuclear target protein (MINT), expressed at higher levels in follicular than MZ B cells, caused a decrease in the absolute number of follicular B cells but an increase in that of MZ B cells[109]. These findings suggest that Notch signaling is important for maintenance of MZ B cells. The MZ is the major entry site of antigens and lymphocytes into spleen, and since MZ B cells serve as the first line of defence against blood-borne bacteria[103,104], it is with no surprise that RBP-J conditional knockout mice are more susceptible to *Streptococcus pneumoniae* infection[110].

1.5.2 T cell development

Gain-of-function and loss-of-function studies showed that Notch1 signaling is essential for T-cell development[51, 111]. As Notch1-deficient mice were embryonically lethal, inducible inactivation of Notch1 was shown to institute ectopic development of B-cells and conventional and plasmacytoid DCs in the thymus[111], while inducible inactivation of Notch2 did not alter T cell development[106]. The commitment of early lymphoid progenitors to T cells is presumably supported by the Notch ligands expressed in the thymic epithelial microenvironment[112]. In agreement with this hypothesis, *in vitro* ectopic expression of Dll1 on OP9 BM stromal cell lines provides the support crucial for differentiation of fetal live progenitors into T cells and their subsequent expansion[113].

Once early thymocyte progenitors are committed to T cell lineage, they begin to adopt either αβ or γδ T cell fate[106]. While thymocytes expressing a T cell receptor (TCR) composed of an α and a β chain are abundant, γδ T cells are less common and are mainly found in the gut mucosa[114]. Heterogeneous deletion of Notch1 in bone marrow precursors or conditional knockout of Notch1 in early immature thymocytes lead to impaired development of αβ T cells and accumulation of γδ T cells[106]. Unlike αβ T cells, γδ T cells do not require antigen processing and their TCR is functionally akin to PRR in recognizing antigens directly and represent an evolutionary precursor of αβ TCR[115,116]. Consequently, γδ T cells are not MHC restricted and do not depend on CD4 or CD8 co-receptors[116]. On the other hand, αβ thymocytes undergo a series differentiation steps to eventually take on one of the $CD4^+$ or $CD8^+$ lineages[109]. These consecutive steps involve $CD4^-CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive, and finally $CD4^+$ or $CD8^+$ single positive (SP) T cells[92]. It is worthy to note that Notch1 expression levels change as

pre-T cells go through the aforementioned steps[109,116]. The survival of pre-T cells progressing through the DN stage was shown to be dependent on Notch1 signaling[113,116]. The Notch1-deficient thymocytes derived in the presence of OP9-Dll1cells showed reduced glucose metabolism and augmented cellular atrophy[113]. Furthermore, Notch-mediated maintenance of a trophic glycolytic rate in pre-T cells, but not their proliferation, is achieved through activation of the serine-threonine kinase Akt[116]. Induction of Akt phosphorylation, as a result of phosphatidylinositol 3-kinase (PI3K) production, is well documented in T cells having their TCR and CD28 co-receptor engaged[117]. Akt not only increases glucose uptake but also activates mammalian target of rapamycin (mTOR) to indirectly module several metabolic pathways[117]. Activity of mTOR signaling is required for sufficient effector function of $CD4^+$ and $CD8^+$ T cells[117]. However, the intrathymic deletion of Notch1 at later stages of development, after the pre-TCR checkpoint and DP stage, did not disturb $CD4^+$ or $CD8^+$ SP T cell differentiation[59]. The $CD4^+$ T cell polarization, nonetheless, skewed towards T_H1 even in the presence of T_H2 promoting cytokine IL-4[59]. These findings, together with the reduced $CD4^+$ T cell proliferation observed in the absence of RBP-J[104], suggest that the sudden shut down of Notch1 expression during the $CD4^+CD8^+$ DP stage is to facilitate positive and negative selection as Notch is involved in cell-linage commitment and survival[59].

1.5.3 T_H cell polarization

Despite the recognition of established models of cytokine-induced T_H polarization, several studies suggest that lineage commitment may happen prior to cytokine selection of a certain helper lineage. The first provocative implication of Notch participation in

APC-medicated T_H polarization showed TLR-dependent regulation of Notch ligand expression in bone marrow derived DCs (BMDCs) and macrophages[52]. Expression of Delta ligands favored polarization of IFN-γ producing T_H1 cells[118,119], whereas Jagged ligands were proposed to be involved in T_H2 or T_{REG} differentiation[52, 120]. Several approaches such as prevention of Notch signal transduction through γ-secretase complex inhibition to ectopic expression of Notch receptors were designed to study the role of Notch receptors in T_H cell polarization. Event though, there had been numerous studies *in vitro* and *in vivo*, there was no conclusive evidence for the role of Notch signaling in T_H differentiation and function. For instance, studies in mouse models of experimental autoimmune encephalomyelitis (EAE) suggested that Notch1, activated by Delta, forms complexes with RBJ-binding domain upstream of *Tbx21* promoter, inducing expression of T_H1 promoting transcription factor Tbet[121]. Further, through the canonical pathway, blockage of Notch by inhibition of γ-secretase prevented *Tbx21*-induced T_H1 polarization[118]. Moreover, administration of γ-secretase abrogated relapses of EAE[122]. However, another study showed that introduction of NICD1 resulted in $CD4^+$ T cell production of IL-4 cytokine[123]. The study further revealed that Notch1 interacts with RBJ-binding domain of the master regulator of T_H2 responses, *GATA3*, and drives expression of *exon1a*[123]. One possible explanation for such discrepancy could be the use of γ-secretase inhibitors, which not only blocks signaling through *all* Notch receptors but also several other pathways. Additionally, mice deficient for dominant negative MAML1 or RBP-J expression are able to mount an effective T_H1 response and clear *L. major* infection, respectively[124]. These findings imply that while T_H2 response results from the

classical Notch pathway, T_H1 responses may employ the non-canonical Notch in interaction with NFκB pathway.

1.5.4 DC and macrophage development

Unlike the established essential role of canonical Notch signaling in lymphocyte development, its role in myelopoiesis is yet to be elucidated as there are many discrepant reports on the role of Notch in differentiation of professional APCs (pAPCs)[52]. Despite the numerous implications of Notch signaling in various stages of DC development and maturation, *in vitro* studies have failed to arrive to an agreement on its importance. For example, several reports have either shown Dll1 to favor[125] or inhibit[126] DC development from hematopoietic precursors. Such conflicting results can arise when different studies employ inconsistent approaches. The subtypes of hematopoietic progenitors used, the extension of Notch signaling induced, and the method of Notch suppression employed can all contribute to side effects that do not correspond to physiological condition[52]. For example, Notch1 anti-sense mice were shown to have limited DC differentiation[126]. Further, embryonic stem cells lacking Notch1 had impaired capacity to generate DCs[126]. Alternatively, a Notch1 loss-of-function study *in vivo* showed that despite the failure of bone marrow precursors to generate T cells, none of the myeloid lineages were affected[127]. Even though the earlier findings suggest that Notch1 is dispensable for DC development, the possibility of redundant signaling through other Notch proteins cannot be dismissed. The issue of Notch redundancy can be partially compensated in the nucleus by RBP-J loss-of-function, disrupting canonical signaling all together[128]. Interestingly,

conditional deletion of RBP-J in bone marrow did not diminish DC lineage commitment, but reduced the total number and proinflammatory capacity of splenic CD8α$^-$ DCs[128]. Notably, similar to MZ B cells, splenic CD8α$^-$ DCs in the MZ are located in vicinity of Dll1-expressing stromal cells[128]. *In vitro* studies have shown that in contrast to Jag1, expression of Dll1 on splenic stromal cells promotes DC maturation via activation of canonical Wnt pathway[129]. Similarly, monocyte precursors from blood exposed to immobilized Dll1 differentiated to DCs with characteristics akin to that of immature DCs cultured in presence of GM-CSF and IL-4[125]. Moreover, a combination of antibodies against Dll1 and any other Notch ligand mimicked the CD8α$^-$ DC loss in RBP-J$^{-/-}$ mice[130]. Recent studies show that Notch2 signaling is an essential component of differentiation of tissue-specific DCs[23]. Notch2-deletion impaired the development of only a fraction CD11b$^+$ DCs, which functionally resemble CD4$^+$ DCs in the spleen and induce Th17 polarization in the intestinal laminal propria[23]. Conversely, the Notch2-independent subsets of intestinal CD11b$^+$ DCs closely resemble macrophages and secret large amounts of cytokines[131]. These findings reveal the heterogeneity of CD11b$^+$ population and further question the accuracy of our current model of DC subclasses and the significance of functional differences amongst them.

The role of Notch in pDC development is also poorly understood and not clear. Though lack of Notch1 did not diminish pDC development, RBP-J-deficient mice showed increased levels of pDCs, suggesting that Notch may play an inhibitory role in these subsets of DCs[132]. In contrast, activation of Notch1 signaling by Dll1-expressing stromal cells showed increased pDC development[132]. It is likely that in the absence of

RBP-J in knockout mice, noncanonical RBP-J-independent pathways with opposite effects are induced[133].

The role of Notch signaling in pAPCs becomes more complex as they express both Notch ligands and receptors, questioning the directionality of Notch signaling (i.e. inducing versus receiving) at various instances. Expression profile of these proteins changes in pAPCs to various degrees with different pathogen encounters[52]. For instance, Delta ligand levels increase as macrophages and DCs are stimulated with T_H1 inducing agents[45]. Notch patterns, on the other hand, have not been consistently shown to correlate with activation, as there has been conflicting reports indicating upregulation of Notch1 in response to both inflammatory agents LPS[134] and CpG[135] and the immunosuppressive parasite *M. bovis*[35]. The recent studies suggest that Notch mediates its influence on the functional capacity of pAPCs primarily through interactions with other signaling pathways, including Wnt, TGFβ, Akt, and NFκB. For example, TLR-induced upregulation of Notch1 was shown to be necessary for Akt-mediated macrophage survival. Inhibition of Notch signaling using γ-secretase inhibitors reduced Akt phosphorylation and, consequently, decreased cell populations in G1 and S phase[136]. Moreover, the LPS-induced Notch1 expression in macrophages was shown to promote M1 (inflammatory) over M2 (immunosuppressive) polarization, the evidence for which is supported in tumor associated macrophages that resemble a M2 phenotype[137]. Through interruption of Notch signaling in LPS or TNF-α stimulated macrophages production of IL-12 decreased while that of IL-10 increased[137]. The M1 phenotype was, however, restored when suppressor of cytokine signaling 3 (SOCS3) expression was induced, suggesting that SOCS3 acts downstream of Notch1[137]. SOCS proteins modulate the

immune response by acting in a negative-feedback loop to inhibit cytokines that signal though JAK/STAT pathway, with SOCS3 specifically inhibiting IL-6 and IFN-γ[138]. Chromatin immunoprecipitation assays (ChIP) in macrophages demonstrated that RBP-J and NFκB interact with *Socs3* promoter upon induction of Notch1 signaling during *M. bovis* infection[35]. On the contrary, in another study, both Notch1 and 2 were reported to be immunosuppressive and inhibit NFκB transcription activity[139]. Induction of constitutively active forms of Notch was shown to decrease phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in the mitogen-activated kinase (MAPK) cascade[139]. Consequently, Notch1 and 2 were shown to inhibit production of TLR4-triggered IL-6 and TNF-α cytokines while enhancing that of IL-10[139]. These findings oppose several other indications in the literature that Notch-induction augments NFκB activity and thereby enhances the inflammatory capacity of macrophages[96.134]. Similarly, concurrent stimulation of bone marrow derived DCs with TLR and Notch agonists resulted in enhanced production of IL-10 and IL-2 and decreased production of IL-12 cytokine[140]. Furthermore, Notch-induced production of IL-10 was shown to be mediated through PI3K and to be independent of γ–secretase activity[140]. Such discrepancies in the literature are not so surprising once we take into account several technical issues that are present in most of Notch studies. Firstly, overexpression of constitutively active forms of Notch may not participate in molecular mechanisms that regulate Notch signaling, such as *cis*-inhibition or ADAM-mediated cleavage. Secondly, the popular method of Notch suppression by γ–secretase inhibition, not only prevents intracellular liberation of all Notch receptors but also affects several other pathways required for normal cell-cell interaction, cell growth, and cell survival. Nonetheless,

majority of the reports highlight the complexity of the system and point out the importance of non-canonical Notch pathways in regulating appropriate immune function. Therefore, development of tools that can target specific molecular interaction more accurately is crucial for our better understanding of such a complex system.

1.6 Rationale and goal of the M.Sc. project

T cell polarization is highly plastic and regulated by the microenvironment context, set by the local secretion of cytokines and metabolites and cell-cell physical contact. Growing body of evidence in the literature suggests that one of such extracellular signals, induced by APCs, is received through the Notch receptors expressed on T cells. The intensity and type of Notch proteins activated can determine the fate of several other pathways and downstream genes transcribed; subsequently shaping the intracellular profile generated within T cells[59].

The differentiation cues provided to the adaptive immune system can also be shaped through the induction of specific Notch profiles in APCs by different pathogens. Interestingly, DCs and macrophages express both Notch receptors and ligands that are differentially regulated by the T_H-inducing pathogen they encounter[52]. For example, LPS-stimulated macrophages upregulate Dll4 expression, which is abrogated with MyD88 deletion[52]. Recent studies show that modulation of either the Notch receptors or ligands in APCs can alter several intracellular molecular processes and, subsequently, the local extracellular microenvironment[53,59,134,,135]. In macrophages, upregulation of Notch1 is

affiliated with enhanced NFκB activity and inflammatory capacity[134,156], while some reports show the contrary[35,139]. Thereby, **we hypothesize that Notch family proteins in DCs may regulate the T cell function directly by engaging the cognate receptors T cells express, and indirectly by shaping activity of DCs through modulation of the cytokine production**.

Despite the correlation between most Notch family proteins and various T_H lineages, a causative relationship is not yet established. For instances, even though Jag2 expression is upregulated in DCs stimulated by T_H2-inducing agents, Jag2 is shown to be dispensable to T_H2 polarization[150,151]. On the other hand, modulations of some Notch ligands and receptors in APCs and T cells are reported to alter T_H cell polarization. For example, expression of Delta ligands on macrophages is shown to augment T_H1 polarization, however, details and mechanisms of such regulations are barely cleared[117,147,149]. We aimed to study this phenomenon by observing the signaling state of DCs when a certain Notch profile is imposed. Therefore, our objectives were to first establish *in vitro* environments in which DCs became either activated or immunosuppressed in order to quantify the expression of Notch family proteins (by qRT-PCR and Flow Cytometry) in relation to variable conditions. Further, we investigated the activation phenotype of bone marrow derived DCs deficient for Notch1 or Notch2, or induced for Jag2 expression. We used Flow Cytometry to analyze expression of activation markers and ELISA to measure the secretion of cytokines. Since DCs express both ligands and receptors, as we observed of the relationship among various Notch proteins, we were attentive in finding evidence for the presence of *cis*-inhibition regulatory mechanisms.

2. MATERIALS & METHODS

2.1 Mice and reagents

Female wild-type C57BL/6 were purchased from The Charles Rivers Laboratory. Floxed Notch1 or Notch2 mice (with loxP sites placed flanking exon1 of the targeted gene) were provided by Nathalie Labreque (Université de Montréal, Montréal, QC). Animals were maintained in a specific pathogen-free environment. All experiments were conducted following the guidelines of the Canadian Council of Animal Care, as approved by the animal care committee of McGill University. Conjugated antibodies (Abs) used to detect the following proteins in FACS analyses: CD11c, CD80, CD86, CD40, MHC class I and II, Thy1.1, hCD8, and Notch1 (purchased from Pharmingen) and Jagged2, NICD1 and Notch2 (purchased from eBioscience).

2.2 Parasites and DC culture

L. major, L. mexicana, and *L. braziliensis* parasites grown until the stationary phase at 25°C in SDM-79 (Schneider's Drosophila medium) supplemented with 5 µg/ml hemin and 10% heat-inactivated FBS (HyClone). Bone marrow-derived DC were cultured, as previously described. Briefly, bone marrow DC precursors were differentiated in the presence of GM-CSF (20 ng/ml) in complete DC media (CDCM; RPMI containing 10% FCS, 100 U/ml pen/strep, 0.05 mM β-ME and 2 mM L-Glu). On day 8–10 of culture, DCs were harvested and resuspended in CDCM containing 5ng/ml GM-CSF. DCs were

then either activated with 100ng/mL LPS, 10μg/mL CpG, or 50μg/mL HDM or infected with stationary-phase promastigotes at DC to parasite ration of 1:10. After 12 to 24 hours, cells were washed three times to eliminate extracellular parasites and, if required depending on the assay, stimulated with LPS. After the completion of the incubation period 37°C, DCs were harvested and analyzed by flow cytometry.

2.3 eFluor450 labeling of parasites and flow cytometric analysis of DCs

Parasite labeling with cell proliferation dye eFlour450 (eBioscience) was performed prior to their co-culture with DCs. Briefly, parasites were washed twice in PBS and resuspended at 1×10^7/ml with 40 μM eFluor450, and incubated at room temperature for 20 min in the dark. Parasites were then washed three times in SDM (supplemented with 10% FBS), and resuspended in CDCM. For flow cytometry, DCs were harvested and blocked with anti-mouse CD16/CD32 (eBioscience) and goat anti-Armenian hamster IgG (Jackson Immuno Research Lab) for 20 min. Then, for surface markers, the cells were stained with fluorochrome-conjugated Abs for 30 min and fixed by using 2% formaldehyde. For intracellular analysis of cytokine production, 1U/mL Brefeldin A was added to cells 3 hours prior to their harvest. The cells were then fixed for 20 min, permeabilized with buffer from eBioscience, and stained for 30 min at 4°C using fluorochrome-conjugated Abs against IL-12 p40 or isotype control Abs. Samples were acquired on a FACSFortessa flow cytometer (BD Pharmingen), and analysis was performed using FlowJo software (Tree Star). Analysis gates were based on live $CD11c^+$ cells, as indicated in the figure legends. In order to isolate RNA from infected

and bystander DC population separately, the cells were sorted based on eFlour450 staining by BD FACSARIAII cell sorter.

2.4 Vector constructs and retroviral transduction

Jagged2 cDNA, cloned upstream of the IRES in the MSCV-IRES-Thy1.1 retroviral vector. The Cre cDNA, cloned upstream of the IRES in the MSCV-IRES-hCD8, was provided by R. Jones. Notch reporter cDNA which contained the promote, the RBP-J binding site, the histone H2B fusion protein coding sequence, and citrine or mcherry florescent protein coding sequences was provided by M. Elowitz. Mlu restriction sites were introduced at each end of the Notch reporter by PCR. The product was then cloned upstream of IRES at the MluI restriction site of the MSCV-IRES-Thy1.1 retroviral vector. Virus was produced in 293T cells by co-transfection of the retroviral construct with a helper plasmid containing the *gag*, *pol*, and *env* genes. Viral supernatants were collected after two days and supplemented with 0.05mM β-ME, 2mM L-Glu and polybrene (8μg/ml). Bone marrow cells were removed two days after the culture was initiated for spin-infections (90 min, 2500 rpm, 30 °C). After the spin, viral supernatants were replaced with CDCM containing GM-CSF (20 ng/ml). DCs were stained with positive markers for transduction, Thy1.1-APC.Cy7 or hCD8-APC.H7, prior to Flow Cytometry.

2.5 qRT-PCR

RNA was isolated using either Trizol (Invitrogen) and treated with DNase (TURBO DNA-free, Ambion). For real-time analysis of gene expression, cDNAs were synthesized using Oligo-dT primers and Superscript II polymerase (Invitrogen). Real-time PCR analysis was performed using SYBR green and run on a CFX96 Real-Time PCR system (Bio-Rad). Relative expression of Delta4 to HPRT was calculated using the 2-ΔΔCt method. Dissociation curves were generated to verify the presence of a single amplicon. Real-time Primers for Delta4 and HPRT were designed by Primer3 and PrimerBlast. Primer sequences; HPRT: Fw. 5' AGG ACC TCT CGA AGT GTT GG 3', Rev. 5' GGC TTT GTA TTT GGC TTT TCC 3', and Delta4: Fw. 5’ GGA ACC TTC TCA CTC AAC ATC CAA G 3’, Rev. 5’ CCA AAT CTT ACC CAC AGC AAG AGA G 3’.

2.6 ELISA

Cell culture supernatants from DC cultured in medium, RPMI1640, in combination with LPS or CpG were collected after 6 hours and were quantified for IL-12p70 secretion by enzyme-linked immunosorbent assay (ELISA) kits (eBioscience) according to the manufacturer’s instructions.

2.7 Statistics

Statistical analysis was performed using two-tailed Student’s *t* test. Differences were considered significant at $p < 0.05$.

3. RESULTS

3.1 *L. mexicana* parasite infects DCs and prevents activation

To establish positive and negative regulators of DC activation we first examined whether *Leishmania* infection can immunosuppress DCs and prevent subsequent activation by LPS. Bone marrow derived DCs were co-cultured with stationary phase promastigote *L. mexicana* and after 16 hours we observed (by optical microscopy) that ~100% of DCs were infected. We, then, stimulated the DCs, including those infected, with 100ng/mL of LPS for 24 hours and examined their activation status by analyzing the expression of activation markers (MHC class I and II, CD80, CD86, and CD40). We observed that while LPS stimulation induced upregulation of all activation markers, *L. mexicana* infection not only maintained the immature state in DCs but also obstructed later activation by LPS (**Fig. 4**).

3.2 Bystander DCs, but not infected DCs, up-regulate class II and co-stimulatory molecules

Since not all DCs become infected when co-cultured with *Leishmania* parasites for shorter periods of time, we next wanted to investigate the phenotype associated specifically with DCs that were infected as well as those that remained uninfected. Therefore, we infected the DCs with *L. braziliensis* parasites that were labeled with eFluor450 dye for 12 hours, which allowed us to separate the activation phenotype

associated with bystander (eFluor450 dim) from infected (eFluor450 bright) DC populations within the same culture (**Fig. 5A**). Infected DCs showed slightly lower expression levels of MHCII and co-stimulatory molecules CD80 and CD86 than the uninfected control DCs (**Fig. 5B**). The bystander population, in contrast, expressed higher levels of MHCII, CD80, and CD86.

3.3 Notch1 inversely and Notch2 positively correlate with DC activation

Notch signaling in T cells has been shown to be a key player in maturation of naïve T cells and their polarization into different helper lineages[59]. The professional APCs provide the differentiation cues deemed necessary for T cells, one of which is induced by Notch ligands the APCs express[59]. However, it is not clear what the role of Notch receptors are in DCs; whether they dictate how DCs respond to ligands present in their local microenvironment. To examine the behavior of Notch signaling within the DC population, we investigated the expression of Notch proteins as DCs encounter pathogens. Since *Leishmania* subverts host normal function through modulation of several molecular pathways, mainly by its master protease GP63[141], we postulated whether Notch signaling pathway is, too, one of the parasite's targets. We, then, looked whether the two Notch receptors with a TAD domain, Notch1 and 2, are differentially expressed at the membrane of DCs when they are immunosuppressed or activated. We used flow cytometry to investigate the expression of these transmembrane proteins in $CD11c^+$ DCs that were either activated with 100ng/mL of LPS or infected with stationary phase promastigote *Leishmania*.

When compared with control DCs, cells that were infected by either cutaneous *L. major* or mucocutaneous *L. braziliensis* became Notch1^{+}NICD1^{+}, whereas LPS stimulated DCs showed no change in Notch1 expression (**Fig. 6A**). Since Notch1 and NICD1 antibodies stain different domains of the same protein, cells expressing full-length Notch stained positive for both Notch1 and NICD1. Consistent with our finding, Notch1 protein possesses GP63 putative cleavage sites just downstream of its transmembrane domain, where γ-secretase cleaves. We then investigated the surface expression of Notch1 in activated *versus* resting DCs. Co-stimulatory molecules CD80 and CD86, necessary for T-cell priming, are upregulated with DC activation[142]. When compared with LPS, Notch1 expression increased only in DCs that had maintained low levels of CD80/86 (**Fig. 6B** and **6C**), which suggested that DCs maintain low levels of Notch1 with activation.

Notch2 expression on the surface of DCs was also measured through flow cytometry. When DCs were co-cultured with L. *braziliensis* at 1:10 ratio for 12 hours, a heterogeneous population of infected and bystander (non-infected) DCs formed[28]. In order to distinguish between the two populations, parasites were stained with 40uM of eFluor450 proliferation dye, prior to infection. DCs that were not infected did not stain for the parasite, thus segregated from the infected DCs on eFluor450 (**Fig 7A**). In contrast to the infected DCs, bystander cells had increased CD80/86 and MHCII expression[71]. Bystander cells also showed a significant increase in Notch2 expression when compared with control and infected cells (**Fig. 7B**). Based on the previous observations of MHCII expression levels in LPS-activated DCs (**Fig. 4**) the positive gate for the MHCII/Notch2 dot plot was set. Similarly, LPS stimulated DCs increased Notch2 expression (**Fig 7C**).

These results show that unlike Notch1, Notch2 expression positively correlates with DC activation.

3.4 Notch1 negatively and Notch2 positively regulate DC activation

To investigate whether the correlation between Notch expression and DC activation has a functional significance, we examined the expression of MHCII and co-stimulatory molecules in DCs with suppressed Notch signaling. Since γ-secretase inhibitors cannot discriminate against Notch proteins, in order to study Notch signaling by each receptor separately, we used BMDCs deficient for either Notch1 or Notch2. As Notch1-deficient mice are embryonically lethal and Notch2-deficient mice have impaired hematopoietic cell expansion and DC development[143], we first derived DCs, analogues to WT, from the bone marrow of floxed Notch1 or Notch2 mice. The DCs were then deleted for the Notch receptors by retroviral transduction of Cre-recombinase. The DCs were either transduced with control or Cre-recombinase containing vector on day 2 of the culture. The transduction efficiency was measured to be 60-80% on day 9-11 by vector hCD8 marker expression (**Fig. 8A**).

When compared with Notch1 floxed ($N1^{fl/fl}$), Notch1-deficient ($N1^{-/-}$) DCs showed an increase in Notch2, CD86, and MHCII expression (**Fig. 8B**, **8C**, and **9B**), indicating that Notch1 expression is necessary for maintenance of a resting state in DCs. Notch2-deficient ($N2^{-/-}$) DCs, on the other hand, had impaired CD86 and MHCII expression (**Fig. 9A** and **9B**), suggesting that Notch2 positively regulates DC activation. These results show for the first time that Notch1 and Notch2 have opposing effects in

modulating DC function. Since Notch2 expression is lost in $N2^{-/-}$ DCs, all cells were within the negative gates of the right panel (**Fig. 9A**). The positive correlation between CD86 and Notch and the upregulation of Notch2 expression by Notch1 deletion further support the evidence that Notch2 expression is associated with DC activation (**Fig. 8C**).

3.5 Notch2 positively regulates IL-12 production

The relationship between TLR-mediated cytokine production and Notch signaling seems to be far more complex, as several studies hint various mechanisms of regulation dependent on the experimental techniques used. TLR and Notch signaling appear to work synergically to both enhance and contain acute inflammatory responses[144]. Sufficient TLR-mediated IL-12 and IL-6 production is shown to be RBP-J-dependent with canonical Notch signaling cooperating with TLR-induced IKK- and p38- mediated signals to express Hes1 and Hey1, which negatively feed back onto the production of IL-6 and IL-12[145]. Moreover, IFN-γ treatment appears to override the negative regulatory mechanism of Notch by suppressing NICD2 production[145].

IL-12 production by DCs is considered essential for induction T_H1 polarization of $CD4^+$ T cells. Since IL-12 production peaks and then gradually decreases as DC stimulation persists, we investigated the behavior of Notch2 expression in response to T_H1 inducing stimuli, such as LPS or CpG, over the course of 24 hours. When DCs quiesce, MHCII expression withdraws and IL-12 production significantly drops. We observed that the pattern of Notch2 expression correlates with MHCII retraction (**Fig. 10A**), suggesting that Notch2 may play a role in regulating IL-12 synthesis. In agreement

with these findings, flow cytometry and ELISA assays of $N2^{-/-}$ DCs treated with LPS or CpG showed reduced IL-12p40 production after 16 hours and IL-12p70 secretion after 6 hours, respectively, (**Fig. 10B** and **10C**).

3.6 Expression of Delta4 inversely correlates with Notch1

Notch ligands in macrophages and DCs have been shown to be differentially expressed upon their encounter with pathogens and to induce guided polarization of T_H cells. There is evidence that blocking signal induction by Dll4 reduces T_H1 and T_H17 responses in a mouse model of EAE[146]. Consistently, stimulation of cDCs with LPS (TLR4), CpG (TLR9), and respiratory syncytial virus (RSV; RIG1 and TLR) induced MyD88-dependent Dll4 expression[147]. Additionally, pDCs are shown to constitutively express high-levels of Dll4 and enhance T_H1-mediated production of IL-10 in order to contain the acute proinflammatory response[148]. Interestingly, while Delta can *trans*-activate Notch1 on the neighboring cells, it is proposed to *cis*-inhibit Notch1 and induce its internalization[68]. The *cis*-response to Delta is suggested to be sharp and happen at a fixed threshold to generate a mutually exclusive signaling state[71]. However data supporting this relationship among DCs are lacking.

Considering our data showing increased Notch1 with infection and lower levels in the bystander cells (**Fig. 6**), we propose that Dll4 expression is downregulated in infected DCs while induced in the bystander population. As controls, we stimulated the DCs with LPS (T_H1 inducer) and HDM (T_H2 inducer) for 16 hours. The quantitative levels of Dll4 mRNA were measured against HPRT. When compared with unstimulated control DCs,

Dll4 levels were upregulated with LPS stimulation while there was no significant change after HDM treatment (**Fig. 11A** and **11B**). Moreover, Dll4 expression was maintained at resting levels with infection while it was induced in the bystander population (**Fig. 11C**).

3.7 Jagged2 negatively regulates DC activation

In addition to Delta ligands, even though controversial, there is evidence that Jagged plays a role regulating T_H cell polarization. For example, helmith parasites, soluble *Schistosoma mansoni* egg antigens (SEA), and cholerae toxins (CT), which are potent inducers of T_H2 response, are shown to upregulate Jag2 expression on DCs[149]. While some reports indicate Jag2 as an enhancer of T_H2-type cytokine production, suppression of Jag2 in DCs failed to inhibit their ability to induce a T_H2 response *in vivo*[149,150]. Jag1, on the other hand, is not consistently shown to be correlated with DC activation. Unlike Jag2, Jag1 is expressed moderately on immature DCs[149]. Despite the documented correlation between Jag2 expression and T_H2-stimlants, there is no evidence for the role of Jag2 in DCs. Thereby, first to determine whether Jag2 is differentially expressed with DC activation, we stimulated the cells with LPS or infected them with *L. braziliensis* for 12 hours. We looked at Jag2 in DCs with resting or increased CD80 expression. Although, increased Jag2 expression with CpG stimulation of splenic and BMDCs was reported[151], we showed that activated $CD80^{high}$ DCs in LPS stimulated or *L. braziliensis* bystander cells had decreased Jag2 expression (**Fig. 12**). The LPS-stimulated DCs showed the presence of two populations of $CD80^{high}Jag2^{low}$ and $CD80^{low}Jag2^{high}$. The percentage of $CD80^{low}Jag2^{high}$ population increased to 76% when DCs were

immunosuppressed by *L. braziliensis* infections. Our observation was consistent with another report showing downregulation of Jag2 in DCs activated by various TLR agonists[149], suggesting that Jag2 inversely correlates with DC activation. To further look into the role of Jag2, we induced Jag2 expression in DCs (J2-DC) by retroviral transduction; as a control we transduced DCs with MiT vector alone (MiT-DCs) (**Fig. 13**). However, we noticed that despite induced Jag2 expression, once stimulated with LPS, J2-DCs downregulated Jag2 expression on the cell surface (**Fig. 13A**). As opposed to infected MiT-DCs, the J2-DCs co-cultured with L. *braziliensis* did not show a further upregulation in Jag2 expression (**Fig. 13B**). The extent of Jag2 upregulation in infected cells was inconsistent with experiments, which might be due to the variability in the infectivity of the parasite. When compared with MiT-DCs, J2-DCs weakly responded to LPS or CpG stimulation and significantly less percentage of the cells became $CD86^{+}Notch2^{+}$ (**Fig. 13D**). These data implied that maintenance of low Jag2 levels is necessary for DC activation.

3.8 Jagged2 inhibits IL-12 production

Among cytokines, IL-12 is an important inducer of T_H1 polarization, however it is shown to be dispensable for efficient production of IFN-γ and clearance of respiratory viral infection in IL-$12^{-/-}$ mice[152]. Additionally, mice deficient for both Jag2 and IL-12 were shown to be as strong of a T_H2 inducer as IL-$12^{-/-}$ mice, suggesting that lack of Jag2 is not capable of skewing a T_H1 response[142]. However the observation made could also imply that the role of Jag2 is masked by the absence of IL-12. Since Jag2 overexpression

prevented Notch2 augmentation (**Fig. 13D**), required for enhanced IL-12 production (**Fig. 10**), we measured IL-12 production in J2-DCs. In accordance with $N2^{-/-}$ DCs, p40 synthesis was significantly reduced with Jag2 induction (**Fig. 14A**). Further analysis of p40 expression of each DC based on its Jag2 levels, showed that within the activated J2-DC population, those with the highest Jag2 proteins contributed the least to p40 production (**Fig. 14B**). These results indicate that such Jag2 modulation of IL-12 is not an indirect consequence of alteration in the DC's local microenvironment but is intrinsic to the cell itself. Taken together, these data confirm that Jag2 depresses DC activation and, subsequently, attenuates IL-12 production.

3.9 MSCV-based Notch reporter constructs require further development

Considering the limitation of existing tools used to study Notch signaling, we aimed to produce Notch reporter constructs that would allow us to not only quantify the nuclear activity of RBP-J but also distinguish between intrinsic and extrinsic Notch signaling. We used a MSCV retrovirus to genetically modify BMDCs for the expression of a vector containing RBP-J binding region upstream of the coding sequence of H2B histone protein fused to either Citrine or mCherry fluorescent proteins (**Fig 15A**). We aimed to study the role of Notch ligands in DCs, with respect to *trans*-activation or *cis*-inhibition mechanisms, by co-transducing BMDCs with our Notch reporter and Notch ligand overexpression vectors (**Fig. 15B**). The two Notch reporters allow us to measure the Notch activity within a heterogeneous population of transgenic DCs with a WT or an induced Notch profile. We then can quantify whether overexpression of Notch ligands in

one sub-population of DCs alters the Notch signaling only in neighboring cells (extrinsic signaling) or it affects Notch activity within the same cell (intrinsic signaling). However, we were not able to detect any significant Notch reporter activity in DCs by Flow Cytometry in response to any stimuli. On the other hand, our preliminary data shows noticeable Notch reporter activity among transduced mouse embryonic fibroblasts (MEFs), suggesting that the construct is functional (data not shown).

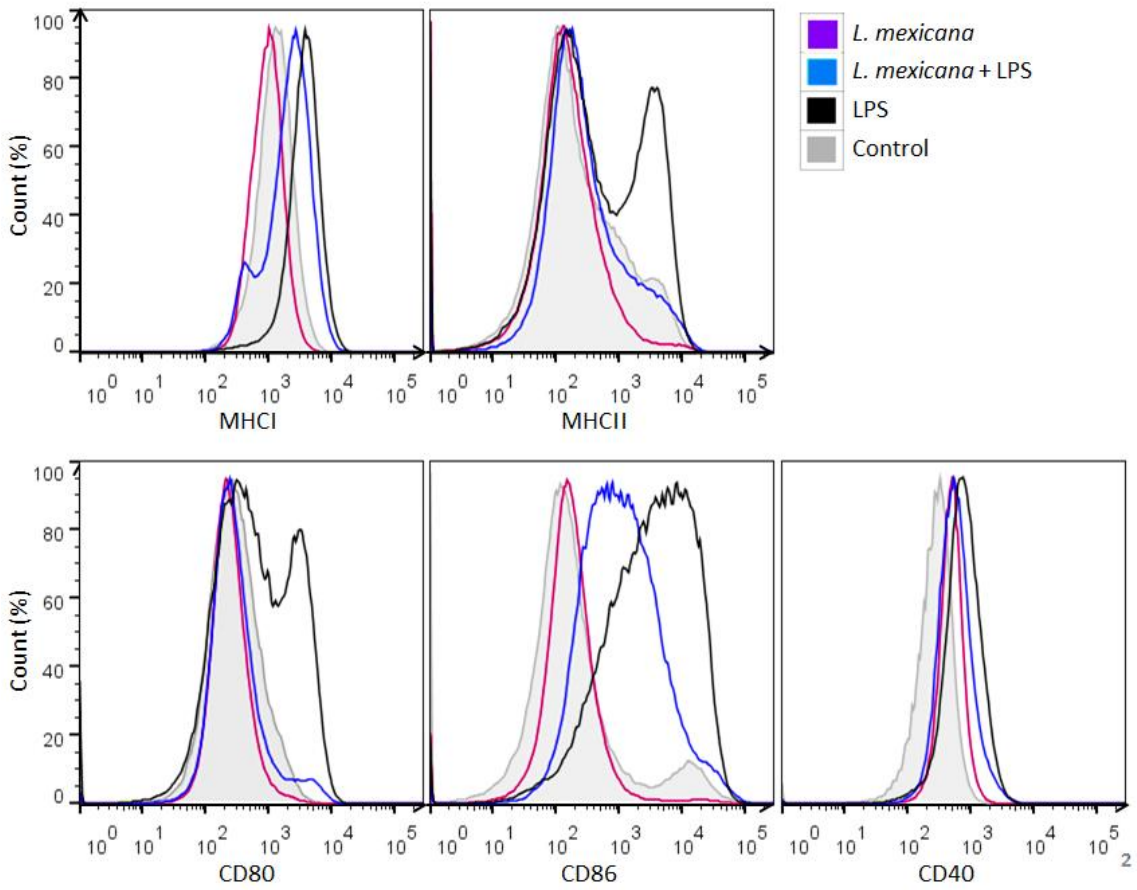

Fig. 4. Infected DCs retain immature characteristics after stimulation with LPS. DCs from C57BL/6 mice were either untreated, stimulated with 100ng/mL LPS, or infected with *L. mexicana* at DC to parasite ratio of 1:20. DCs co-cultured with the parasite were later either cultured in plane CDCM media or treated with LPS for an additional 16 hours. DC activation status (MHC I and II, CD80, CD86, and CD40) post-treatment was assessed by flow cytometry. Histograms are gated on $CD11c^{+}$ cells. Results are from one experiment and representative of three independent experiments.

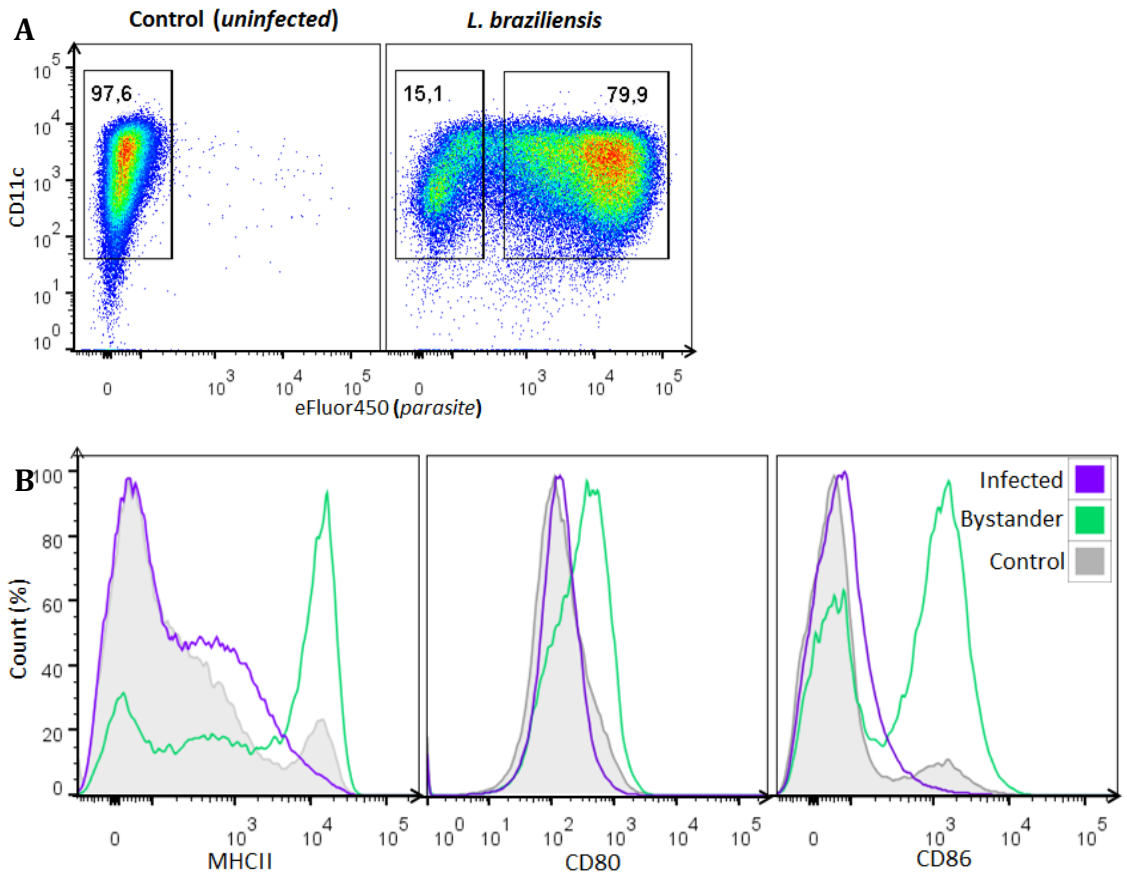

Fig. 5. Activation dichotomy of DCs following their interaction with *L. braziliensis*. BMDCs were either untreated or co-cultured with eFluor450-labeled *L. braziliensis* for 12 hours. **A)** In the control culture, 97.6% of the cells were $CD11c^+$. After 12 hours of infection at DC/parasite of 1:10, 79.9% of DCs were infected (eFluor450 bright) and the remaining 15.1% were uninfected/bystander (eFluor450 dim). **B)** Expression of surface MHCII, CD80, and CD86 in untreated/control DCs (*grey*), bystander DCs (*green*), and infected DCs (*purple*). Histograms are gated on $CD11c^+$ cells. Results are from one experiment and representative of three independent experiments.

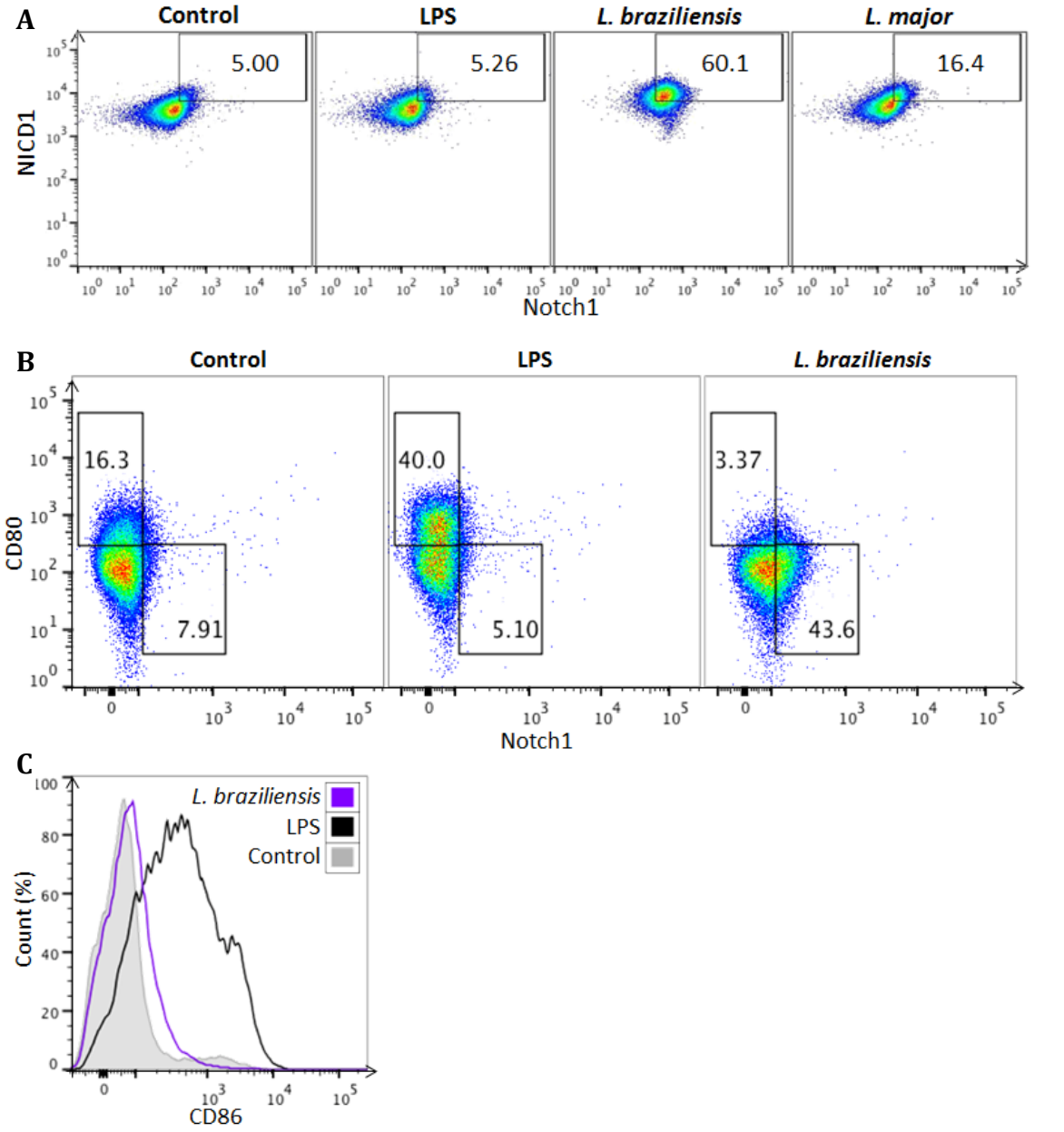

Fig. 6. Notch1 expression is inversely correlate with DC activation. BMDCs were either untreated, infected with stationary phase promastigote *Leishmania* parasites at DC/parasite of 1:20 or stimulated with 100ng/mL LPS for 16 hours. **A)** The density plots show the percentage of CD11c+ DCs expressing higher levels of surface (Notch) and intracellular (NICD) of Notch1. To account for the variability among subgenera of *Leishmania*, DCs were infected with either *L. major* or *L. braziliensis*. **B)** Percentage of CD11c+ cells expressing increased levels of activation marker CD80 and low levels of surface Notch1 *versus* cells expressing low levels of CD80 and higher levels of Notch1. **C)** Histogram overlay of activation marker CD86 among three cultures of control, LPS-treated, and *L. braziliensis*-infected CD11c+ DCs. Results are from one experiment and representative of three independent experiments.

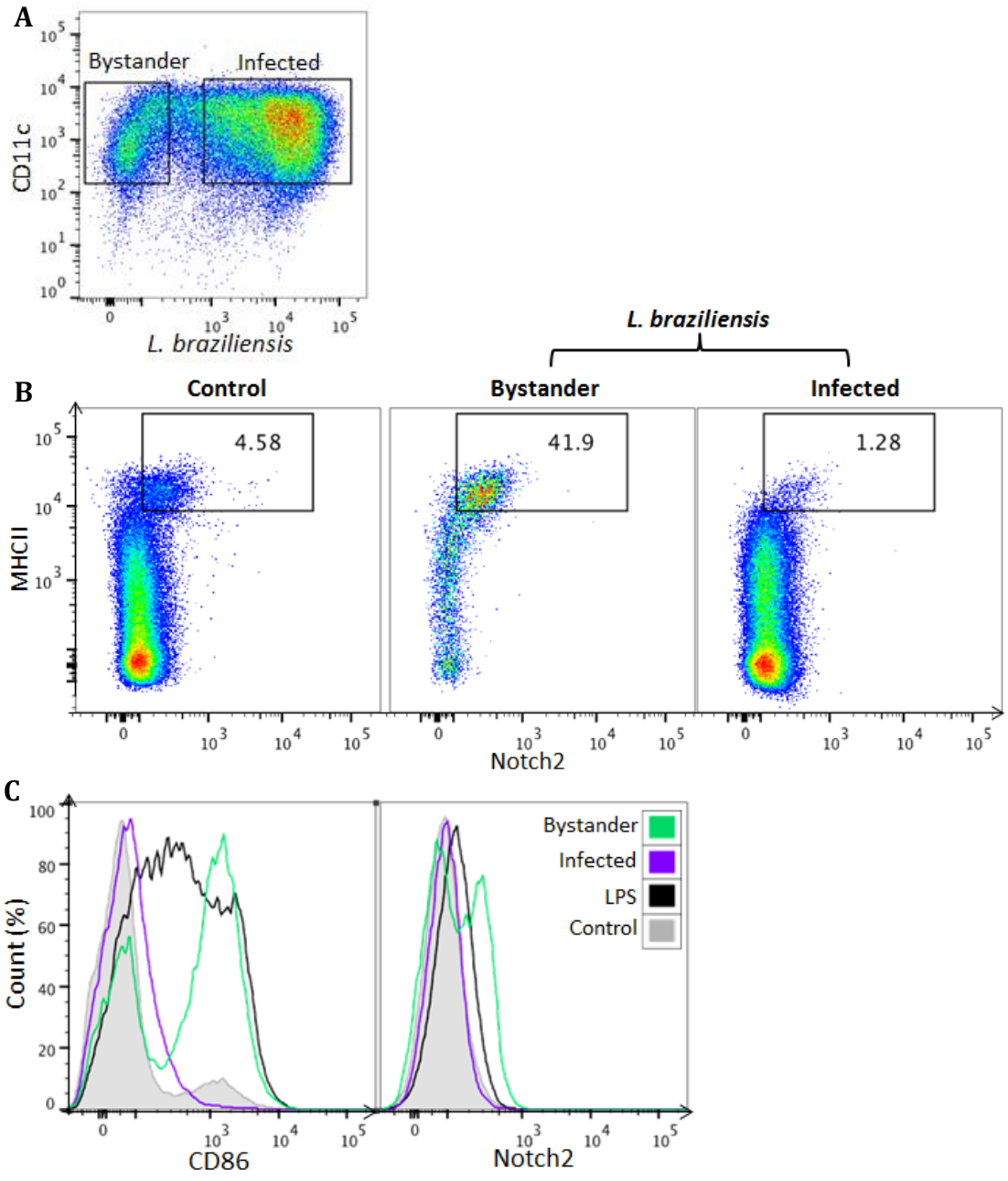

Fig. 7. Notch2 expression is positively correlated with DC activation. BMDCs were infected with eFluor450-labeld *L. braziliensis* at DC/parasite of 1:10 for 12 hours. **A)** The density plot of DCs co-cultured with *L. braziliensis*. CD11c+ DCs that were dim for eFluor450 are bystander cells while bright CD11c+ are infected. **B)** Among the control, bystander, and infected DC populations, 4.58%, 41.9%, and 1.28% were positive for surface Notch2 and high for activation marker MHCII, respectively. **C)** In comparison with control (*grey*) and infected (*purple*) $CD11c^{+}$ cells, 100ng/mL LPS-stimulated (*black*) and bystander (*green*) DCs had increased surface expression of CD86 (*left*) and Notch2 (*right*). Results are from one experiment and representative of three independent experiments.

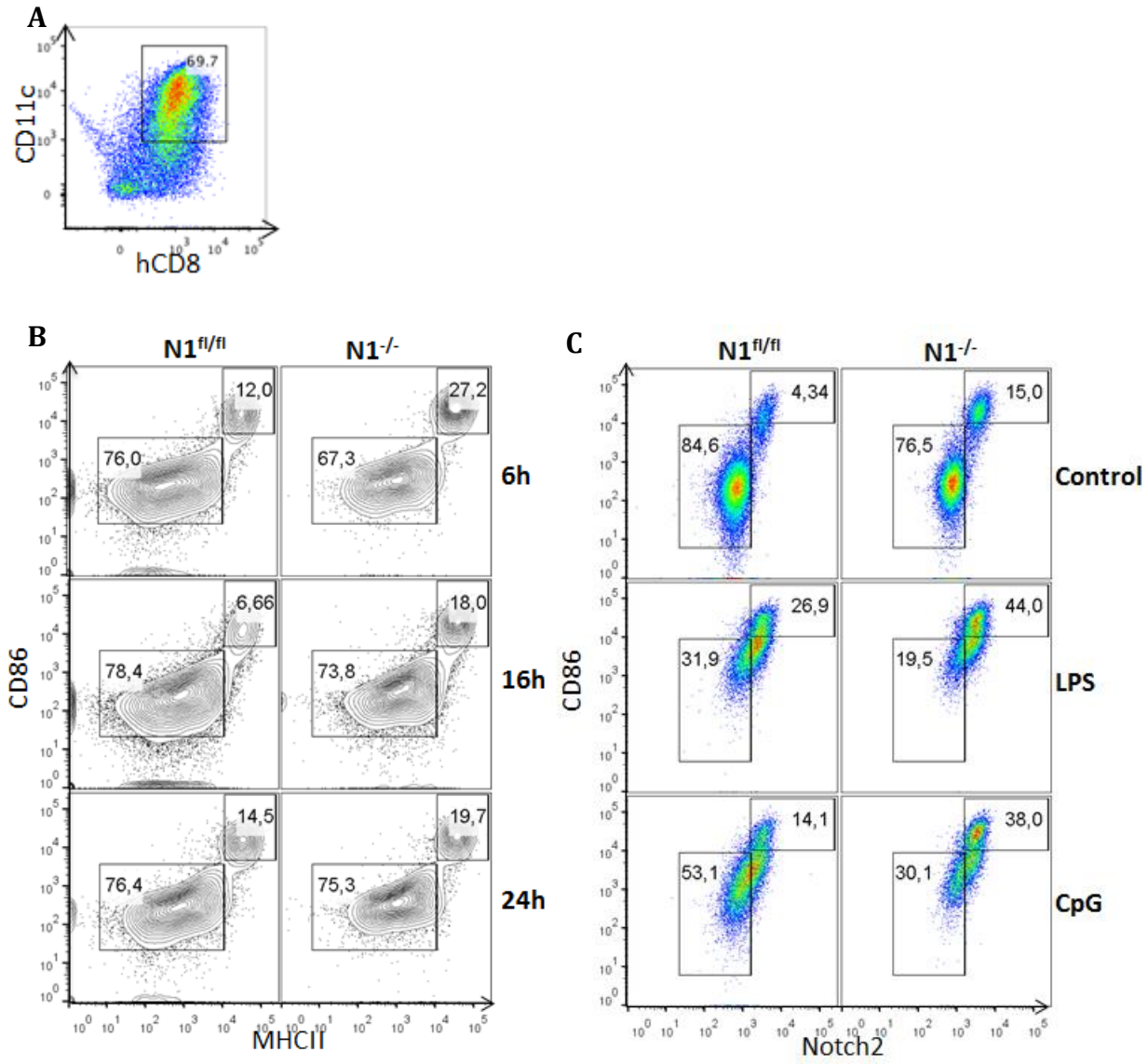

Fig. 8. Notch1 negatively regulates DC activation. The bone marrow cells of floxed Notch1 mice were transduced with either control retrovirus MSCV-IRES-hCD8 (Mi8) or Cre-recombinase virus (Mi8/Cre) at day 2 of the culture. **A)** Percentage of $CD11c^+$ DCs transduced with the retrovirus. The density plot is a representation of floxed Notch1 or Notch2 transduced with either Mi8 or Mi8/Cre. **B)** Contour plot of floxed Notch1 transduced with either Mi8 ($N1^{f/f}$) (*left panel*) or Mi8-Cre ($N1^{-/-}$) (*right panel*) show the percentage of $CD11c^+$ DCs expressing activation markers MHCII and CD86 overtime. **C)** Percentage of $CD11c^+$ $N1^{f/f}$ and $N1^{-/-}$ cells expressing surface Notch2 and CD86 when untreated (control) (*top row*), stimulated with 100ng/mL LPS (*middle row*), or stimulated with 10ug/mL CpG (*bottom row*) for 16 hours.

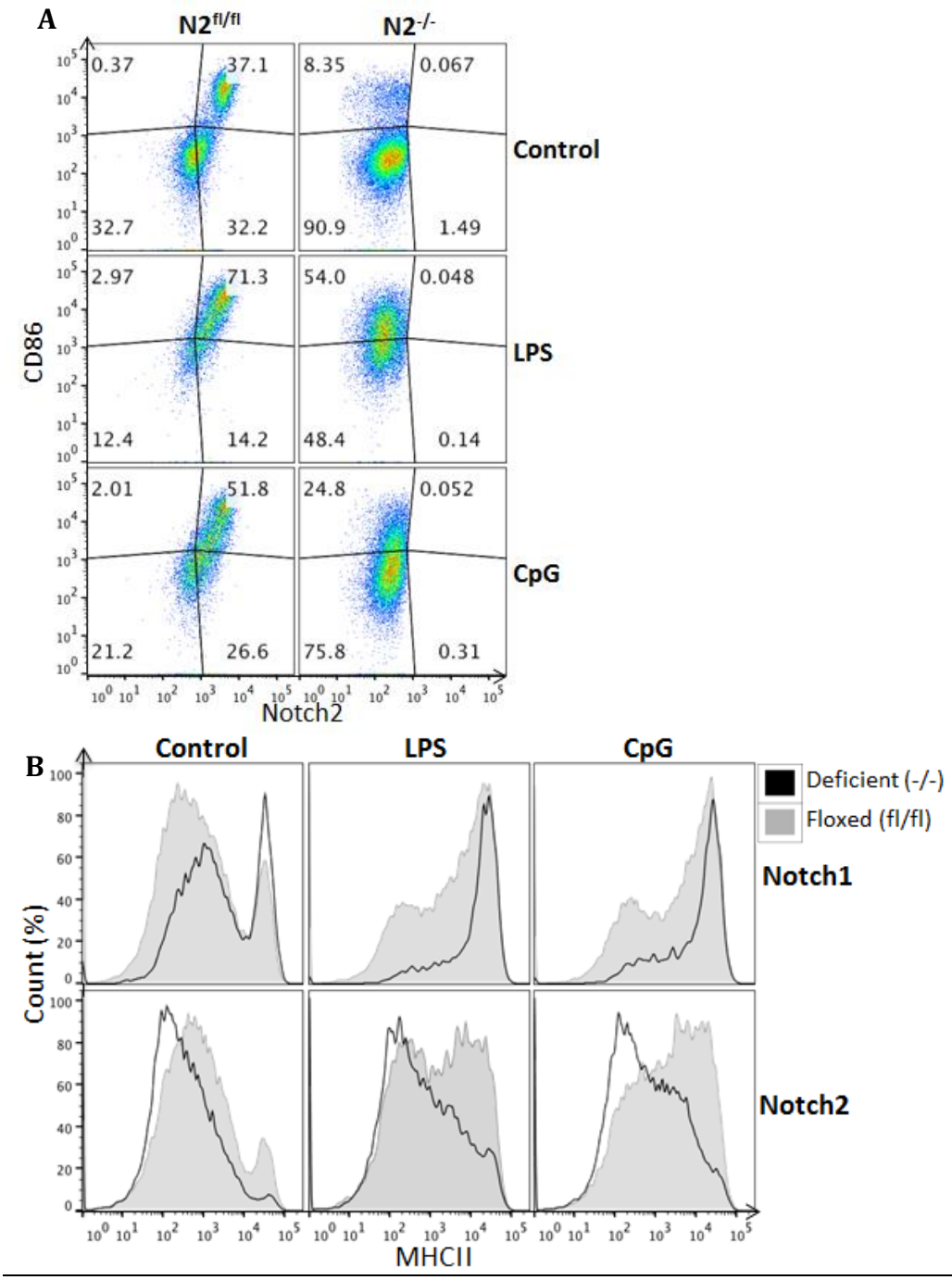

Fig. 9. Notch2 positively regulates DC activation. The bone marrow cells of floxed Notch2 mice were transduced with either control retrovirus MSCV-IRES-hCD8 (Mi8) or Cre-recombinase virus (Mi8/Cre) at day 2 of the culture. **A)** Percentage of $CD11c^{+}$ $N2^{f/f}$ and $N2^{-/-}$ cells expressing surface Notch2 and CD86 when untreated (control) (*top row*) or stimulated with LPS (*middle row*) or CpG (*bottom row*) for 16 hours. **B)** Overlaid histogram of MHCII expression among $N1^{f/f}$ and $N1^{-/-}$ (*top row*) or $N2^{f/f}$ and $N2^{-/-}$ (*bottom row*) when untreated (*left panel*) or stimulated with LPS (*middle panel*) or CpG (*right panel*) for 16 hours.

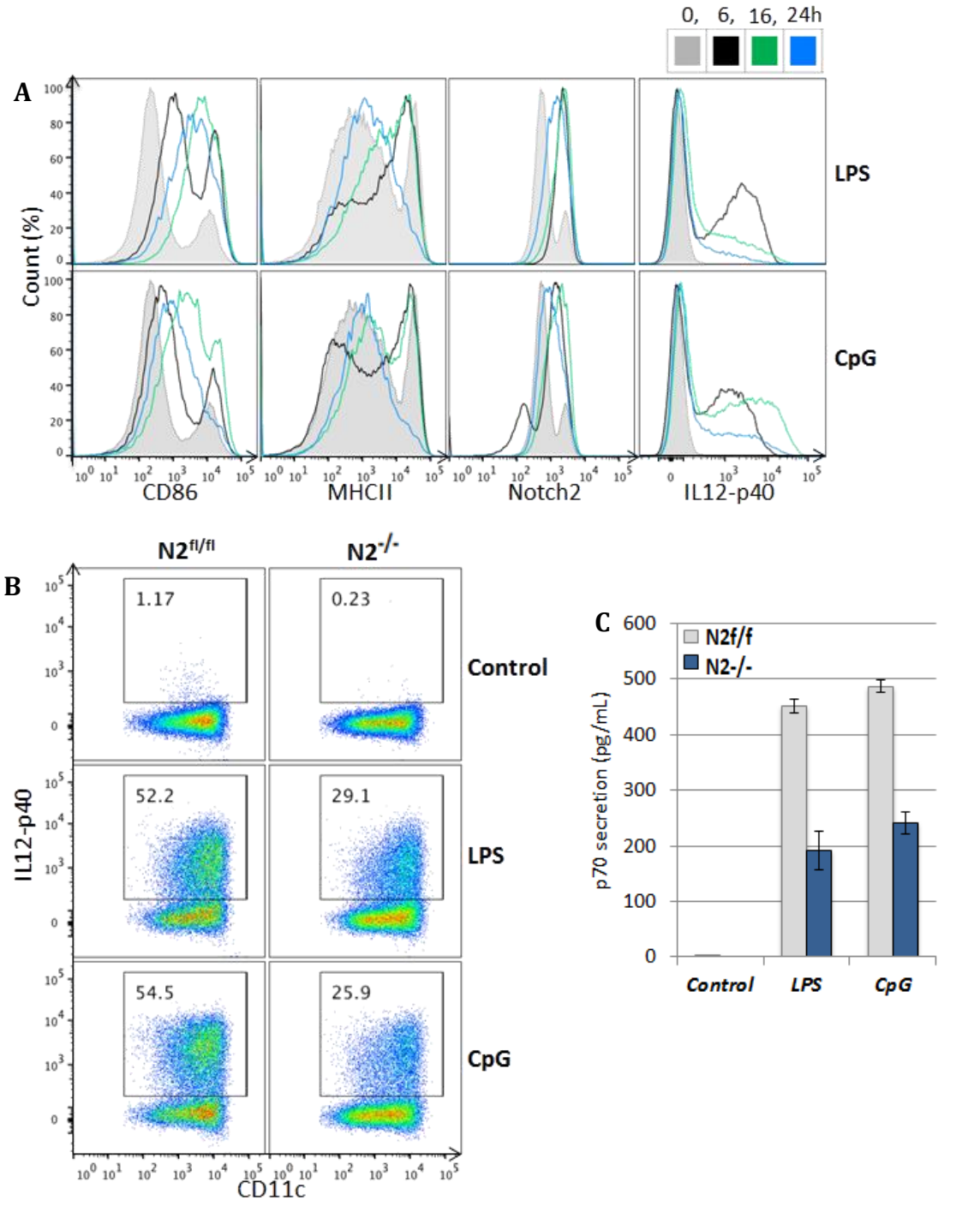

Fig. 10. Notch2 expression correlates with and positively regulates IL-12 production. A) Expression of activation surface markers CD86, MHCII, and Notch2 and proinflammatory cytokine IL-12-p40 overtime of CD11c+ DCs stimulated with 100ng/mL LPS or 10ug/mL CpG. **B)** Percentage of CD11c+ N2f/f (*left panel*) and N2-/- (*right panel*) cells producing IL-12-p40 cytokine (control) (*top row*), stimulated with LPS (*middle row*), or stimulated with CpG (*bottom row*) for 16 hours. **C)** IL-12-p70 secretion by N2f/f (*grey*) was higher than that of N2-/- (*red*) DCs, measured by ELISA after 6 hours of stimulation with either LPS or CpG. The control DCs were left untreated. Each data point represents the mean ± SD.

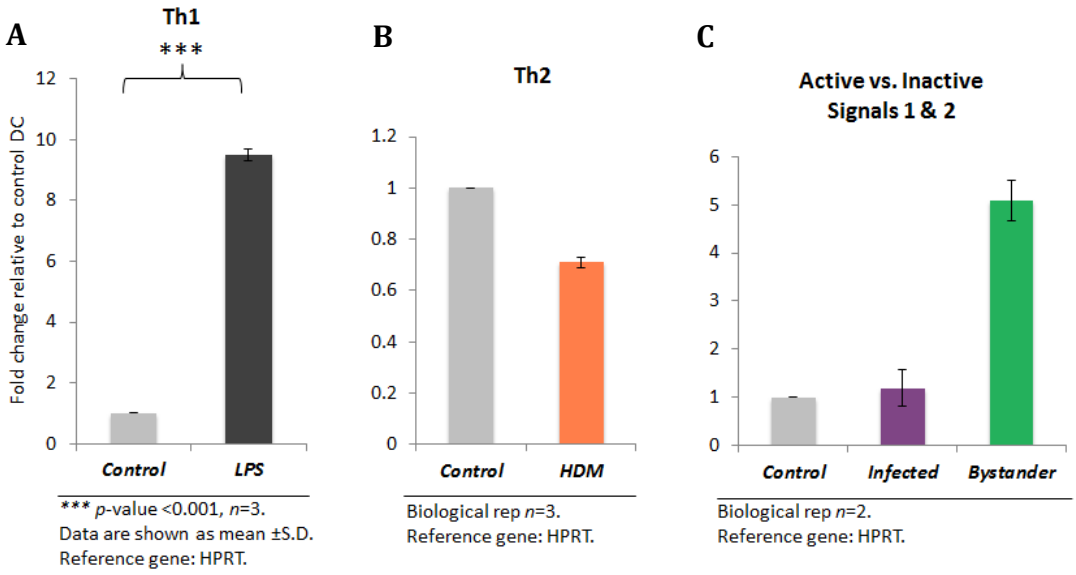

Fig. 11. Dll4 expression is induced when DCs are proinflammatory. The mRNA levels of *Dll4* gene was measured by quantitative Real-Time PCR (qRT-PCR). A) Dll4 expression between untreated DCs (*grey*) and 100ng/mL LPS-stimulated DCs (*black*) after 16 hours. B) Dll4 expression between untreated DCs (*grey*) and 50ug/mL HDM-stimulated DCs (*orange*) after 16 hours. C) DCs were either untreated or co-cultured with eFlour450-labeled *L. braziliensis* for 6 hours. Dll4 expression was measured among control DCs (*grey*) or DCs sorted based on eFlour450 brightness (infected=*purple*, bystander=*green*). The control DCs were left untreated. Each data point represents the mean ± SD.

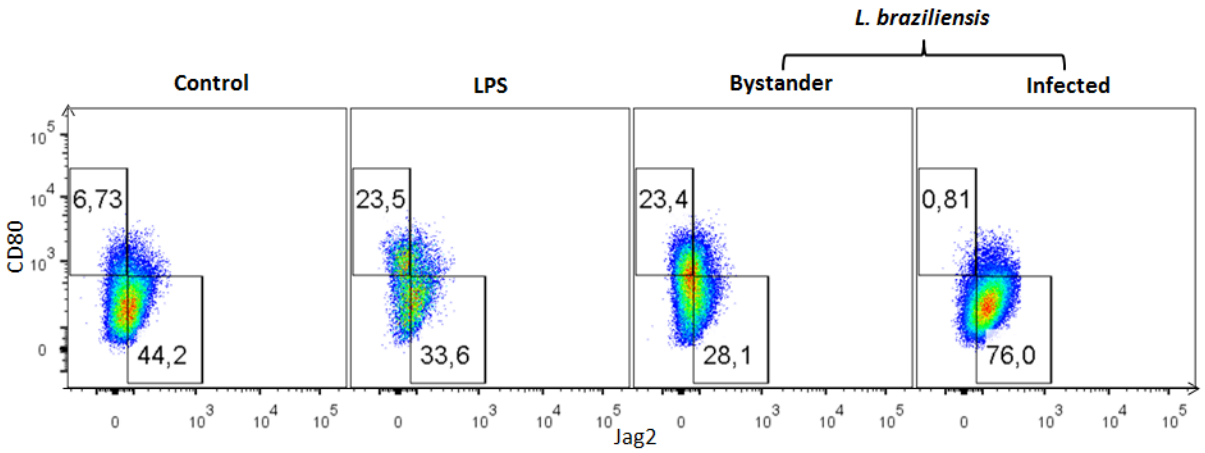

Fig. 12. Jag2 expression inversely correlates with DC activation. BMDCs were infected with eFluor450-labeld *L. braziliensis* at DC/parasite of 1:10 or stimulated with 100ng/mL LPS for 12 hours. The CD11c+ DCs were subsequently analyzed for surface Jag2 and activation marker CD80. The percentages of populations negative for Jag2 but positive for CD80 are shown.

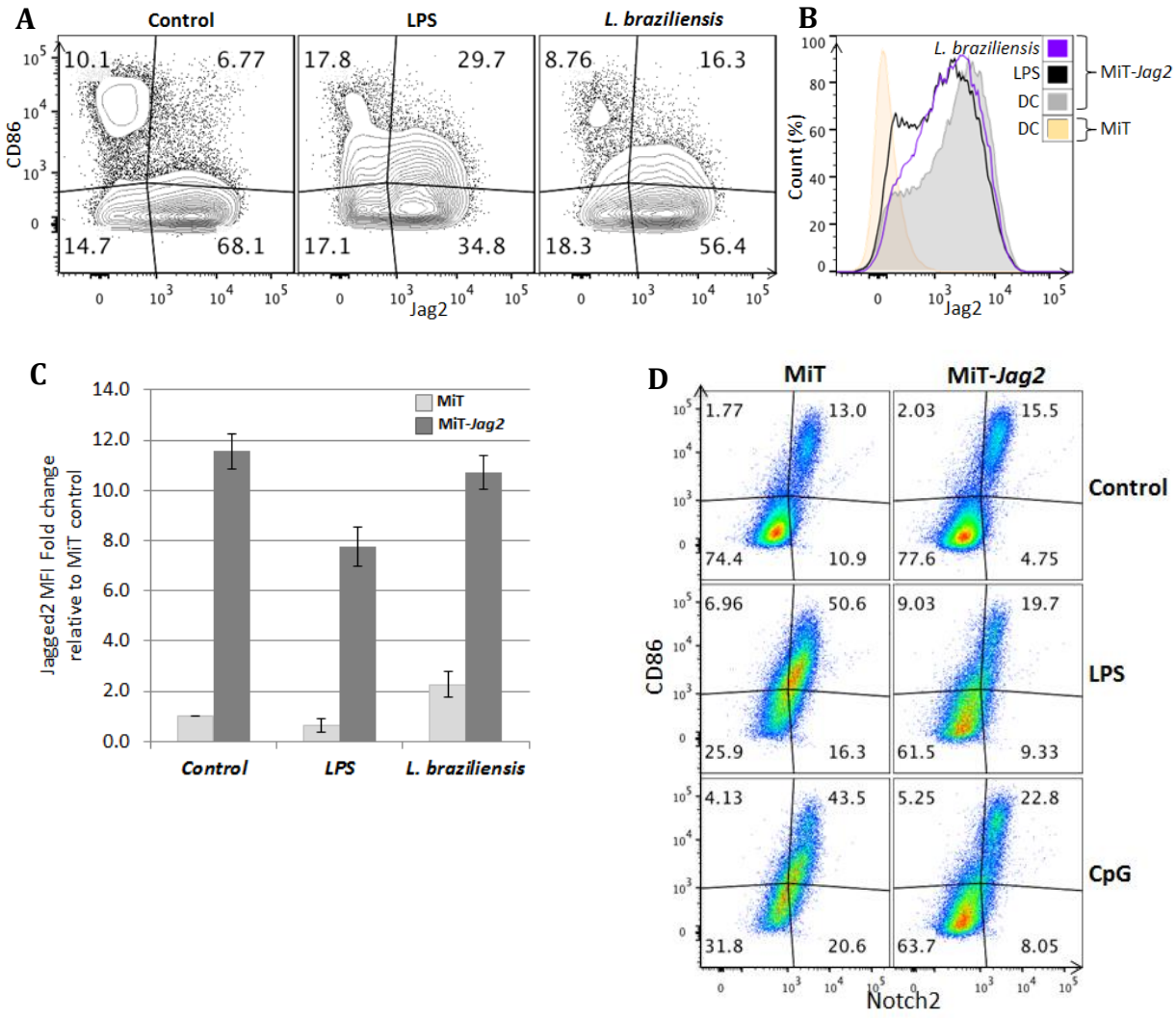

Fig. 13. Jag2 expression negatively regulates DC activation. Bone marrow cells of C57BL/6 mice were transduced with control retrovirus MSCV-IRES-Thy1.1 (MiT) or Jag2-expressing virus (MiT-*Jag2*) at day 2 of the culture. The Jag2 expression was then analyzed by flow cytometry. **A)** The contour plot of DCs overexpressing Jag2 (transduced with MiT-Jag2) showed 10.1%, 17.8%, and 8.76% of control, LPS-stimulated, and infected CD11c^{+} DCs, respectively, expressing low levels of Jag2 and high amounts of CD86 after 16 hours. **C)** The average mean fluorescence intensity of surface Jag2 ± SD among control, and LPS or CpG-stimulated DCs after 16 hours showed increased surface Jag2 expression when DCs were transduced with MiT-*Jag2*, compared to the control vector MiT. **D)** Percentage of CD11c^{+} MiT and MiT-*Jag2* cells expressing surface Notch2 and CD86 when untreated (control) (*top row*), stimulated with LPS (*middle row*), or stimulated with CpG (*bottom row*) for 16 hours. With Jag2 induction, the percentage of CD11c+ DCs expressing high levels of Notch2 and CD86 decreased from 50.6% to 19.7% and from 43.5% to 22.8% among LPS- and CpG-stimulated cells. Results are from one experiment and representative of three independent experiments.

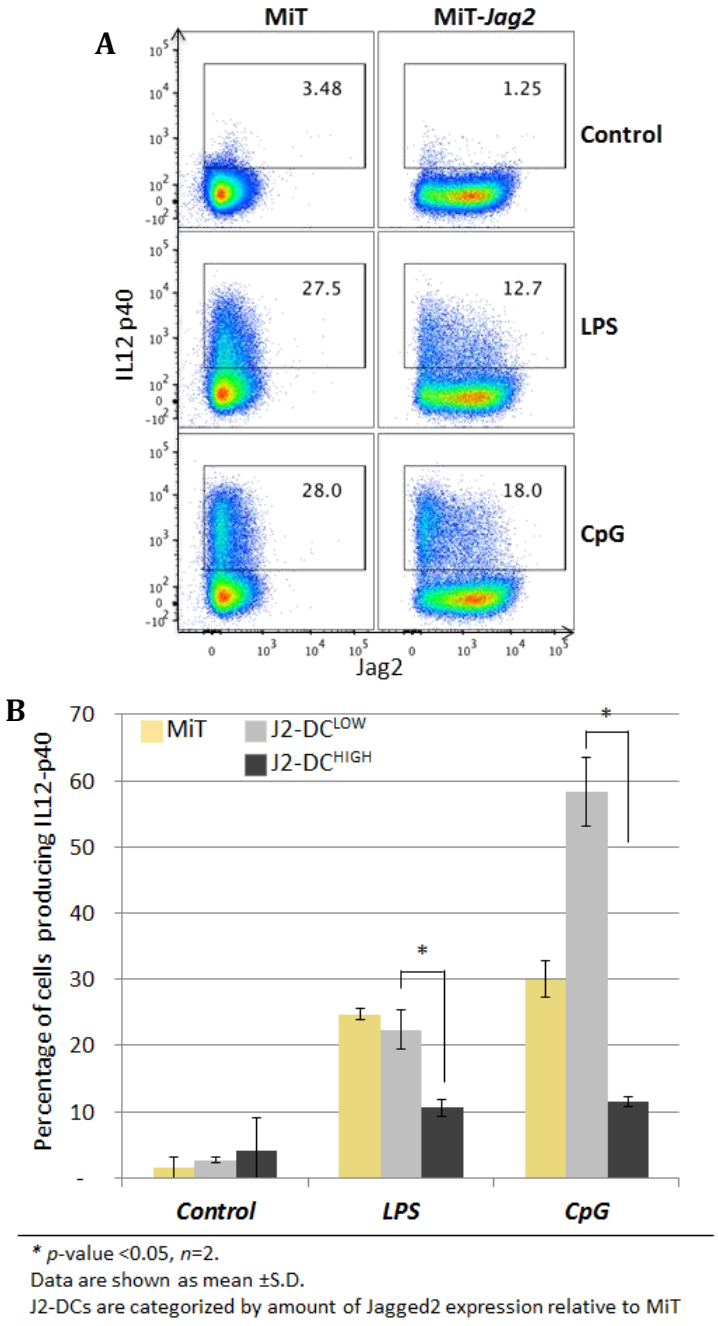

Fig. 14. Jag2 expression negatively regulates IL-12-p40 production. Bone marrow cells of C57BL/6 mice were transduced with control retrovirus MSCV-IRES-Thy1.1 (MiT) or Jag2-expressing virus (MiT/Jag2) at day 2 of the culture. **A)** Percentage of CD11c+ MiT (*left panel*) and MiT/Jag2 (*right panel*) cells producing IL-12-p40 cytokine (control) (*top row*), stimulated with 100ng/mL LPS (*middle row*), or stimulated with 10ug/mL CpG (*bottom row*) for 16 hours. Results are from one experiment and representative of three independent experiments. **B)** Percentage of IL-12-p40-producing cells among DC populations with different levels of Jag2 expression in response to LPS or CpG stimuli after 16 hours. Compared with MiT-transduced DCs, Jag2 expression was classified as low (J2-DC^{LOW}) or high (J2-DC^{HIGH}) among MiT/Jag2 DCs. Data are shown as mean ± SD.

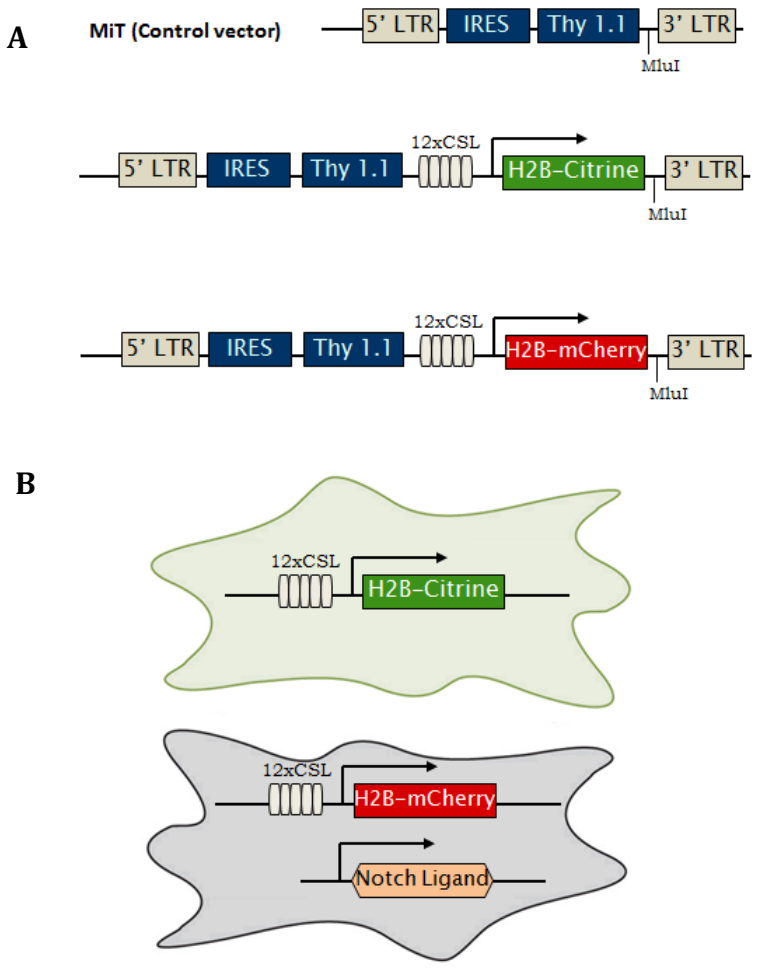

Fig. 15. The experimental model for Notch reporter assays. A) Notch reporter cDNA containing RBP-J response element (12×CSL), promoter, and coding sequence of H2B histone protein fused to Citrine or mCherry fluorescent protein was cloned downstream of Internal ribosome entry site (IRES) and Thy1.1 (marker for transduction) at the MluI restriction site of retrovirus MSCV-IRES-Thy1 (MiT). **B)** Co-culture assays of Notch reporter transgenic DCs, with parallel induction of Notch ligand expression allows quantification of Notch signaling at the single cell level by flow cytometry. This model could be used to study both the intrinsic and extrinsic effects of altered ligand expression on Notch signaling.

4. DISCUSSION/CONCLUSION

Despite the accumulating evidence for the role of Notch signaling in T cell differentiation, the molecular mechanisms underlying Notch orchestration of innate and adaptive immune system is poorly understood. In this thesis we were able to show that Notch1 and Notch2 play opposite roles in regulating DC activation phenotype. Further we illustrated that transcription of *Dll4* is induced when DCs are stimulated with a T_H1-inducing agent, which inversely correlates with Notch1 expression. We also showed that Jag2 expression negatively regulates the proinflammatory phenotype of DCs.

In order to quantify the type and extent of Notch signaling among DCs in the context of various microenvironment, we first examined the activation phenotype of DCs in response to microbial agents, including *Leishmania* and LPS. The infection of BMDCs with *L. mexicana* not only maintained DCs in their inactivated stage but also immunosuppressed DCs and held them from responding to the following LPS stimulation (**Fig. 4**). Activation of immature DCs by proinflammatory agents such LPS and CpG results in a T_H1 response that is important for clearance of intracellular pathogens[16,27]. Taking into account the ability of the parasite to replicate inside the host cell[28], these observations suggest that *Leishmania* can sustain DCs in an immunosuppressed state and may limit its ability to induce T_H polarization. Infected DCs showed equal or even slightly lower expression of activation markers compared with uninfected control DCs (**Fig. 5**), which could be explained by the preferential infection of more immature, thus more phagocytic, DCs[153]. Further investigation of the heterogeneous population of DCs

co-cultured with *Leishmania* revealed that, in contrast to the infected DCs, the bystander population (not infected) had an upregulated expression of activation markers on the cell membrane (**Fig. 5**). A recent study has proposed the microbial agents released by the parasite to be responsible for the activation of bystander cells, by a mechanism which is yet not understood[143]. Similarly, our findings support the evidence that the increased antigen presentation capacity of the bystander population and their ability induce the T_H1 response necessary for the clearance of the parasite.

Our investigation of Notch protein expression among proinflammatory or immunosuppressed DCs revealed that, in contrast to the published work on RAW264 macrophage cell lines[139], Notch1 levels were maintained upon TLR stimulation but augmented when DCs were infected with *Leishmania* (**Fig. 6**). These results suggested that DCs retain low levels of Notch1 with activation, which supports the evidence for the recent association of Notch1 signaling with enhanced transcription of *SOCS3* in *M. bovis* infection model[35] and, similarly, with the suppression of TLR-mediated cytokine production in macrophages[139]. The expression of Notch2, on the other hand, followed an opposite trend, in agreement with several reports[135,139,140]. DCs that were either activated by LPS or CpG stimulation or via the bystander effect had an elevated Notch2 expression, while infected cells and immature DCs did not, which suggests that Notch1 and 2 may challenge one another in regulating proinflammation of DCs.

Despite the discrepancy in literature, maintenance of low Notch1 expression struck us as beneficial to APCs in order to achieve a proinflammatory state. Thus, in order to investigate the functional significance of Notch1 expression, we depleted Notch in BMDCs via Cre-recombinase transduction. The loss of Notch1 expression resulted in a

pronounced response to TLR ligation, evident by increased expression of MHCII, CD86, and Notch2 (**Fig 8B** and **8C**). Our preliminary data also showed that in absence of Notch1, DCs produced more IL12, even after a 24-hour LPS stimulation, which supports the conjuncture that the Notch1/RBP-J complex, in association with NFκB, enhances expression of *SOCS* [35]. Considering Notch1-mediated SOCS3 expression[35], these results are consistent with the decreased activation in SOCS3-transduced DCs[154]. Likewise, NICD1 overexpression was shown to hinder proinflammation by inducing IL-10 and inhibiting TNF-α production[139]. Additionally, the same group reported that Notch1 expression significantly increases after a 24-hour stimulation of macrophages with LPS or CpG[139]. During such lengthy stimulations, DCs tend to quiesce and become immunosuppressive, a phenotype observed by reduced MHCII expression, and IL-12 production (**Fig 10A**). At such circumstances, human DCs were shown to utilize Notch1 to regulate a PI3K-dependent switch form IL-6 to immunosuppressive enzyme indoleamine-pyrrole 2,3-dioxygenase (IDO)[155] production. It is important to note that induction of NICD1 expression may not be a physiological representation of cellular mechanisms involved with Notch regulation, as it bypasses the regulatory action of ADAM protease at the extracellular domain of Notch. Additionally, susceptibility to ADAM protease was shown to alter capability of Notch ligands to participate in *cis*-inhibition. For example, a mutant version of non-canonical Dlk-1 ligand, resistant to ADAM proteolytic activity, was shown to be a stronger inhibitor of Notch receptors[156]. Furthermore, the increased expression of Notch2 in Notch1-deficient DCs not only supports the evidence for positive association of Notch2 with activation, but also suggests the possibility for non-classical compensatory or redundant regulatory interplays among

the Notch proteins. For example, the consequences of altering the expression of a Notch protein on other Notch receptors and ligands, possibly through *cis*-interaction at the cell surface, should be addressed. For example, due to the inverse correlation between Notch2 and Jag2 expression, it could be possible that Notch1 deletion or Notch2 overexpression result in Jag2 downregulation. Such mechanism may be mediated through *cis*-inhibition between Notch2 and Jag2.

Additionally, similar to the report of Notch2 deletion causing a significant reduction in the number of splenic $CD11c^{+}MHCII^{+}$ DCs[23], we showed that in the absence of Notch2, BMDCs were less responsive to TLR agonists and were severely impaired in CD86 and MHCII expression. These results, for the first time, show that Notch1 and Notch2 have opposing effects in modulating DC function. Despite the lack of functional segregation between Notch1 and 2 in several models, a few reports hint otherwise. For instance, in malignant mesothelioma (MM), which are mutated for the expression of constitutively active Notch1, it was shown that overexpression of NICD2 diminished MM survival[157]. In the proposed mechanism, increased tumor suppressor phosphatase and tensin homolog (PTEN) protein levels inhibited PI3K/Akt pathway[157]. From the developmental perspective, Notch1 was detected in multiple hematopoietic stem cell lineages while Notch2 was expressed in more differentiated cell lineages, mainly macrophages and activated B cells[158]. The next step to address the functional disparity between Notch1 and Notch2 is to analyze the activation profile of DCs deleted for both Notch1 and Notch2. Further co-culture studies can reveal the capacity of such cells to promote or suppress various T_H differentiations. One could argue that DCs abrogated for both Notch1 and Notch2 signaling mimic the behavior of WT DCs.

Interestingly, DC expression of Notch2 in response to CpG, and more so to LPS, was transient and decreased as IL-12 production decayed overtime (**Fig. 10A**). Moreover, IL-12 production was markedly reduced when Notch2 was deleted (**Fig. 10B** and **10C**). In contrast to our results, siRNA knockdown of Notch2 in BM macrophages did not diminish transcription of RBP-J-dependent genes such as *il12b*, however mice heterozygous for Notch1 deletion showed significant reduction in *il12a* and *b* transcription[159]. Further, our preliminary findings showed reduced capability of Notch2 depleted DCs in inducing $CD4^+$ T cells to become $CD25^+CD44^+CD69^+$ (data not shown), which signifies their impaired functionality. However the basis for such phenotype is yet not clear. A recent study showed the deletion of Notch2 results in depletion of $CD11b^+$ DC subsets in the spleen and intestine[25]. The lack of such DC population resulted in impaired IL-17 production by $CD4^+$ T cells[25], thereby diminishing protection against extracellular pathogens. It is important to address whether such impaired DC activation and, consequently, T cell priming stems from Notch2-dependent developmental anomalies in DCs. Alternatively, intrinsic or extrinsic Notch regulatory mechanisms, which are not fully understood to date, may shape the microenvironment T cells interact with. It is noteworthy to state that despite the use of similar methods, such as induced expression of constitutively active Notch proteins or treatment with γ-secretase inhibitors, there is yet a consensus to be reached on the role of Notch proteins in APCs. Some studies show that Notch1 and Notch2 negatively regulate TLR-mediate inflammatory cytokine production[35,139,140] while others relate Notch signaling to enhancement of proinflammatory cytokine production[96,134,135,137].

In APCs, in addition to the receptors, Notch ligands are also expressed, and even though their role has been tackled more frequently than Notch receptors, they are still a source of significant controversy in the field. The first comprehensive analysis of Notch ligand profile in APCs revealed that Delta ligands were upregulated with LPS stimulation while Jag2 was induced upon exposure of APCs to cholera toxin (CT) of *Vibrio* bacterium or prostaglandin E2 (PGE2)[52]. In accordance with these findings, we were able to show upregulation of *Dll4* transcription in LPS stimulated DCs and activated bystander cells to *L. braziliensis*, as opposed to DCs activated by T_H2-inducing allergen house dust mite (HDM) (**Fig. 11**). Consistent with our observation, *in vivo* administration of polyclonal Abs against Dll4 significantly induced T_H2-type cytokine production by isolated lung $CD4^+$ T cells during RSV infection[147]. Furthermore, Dll4 and Jag1 regulation was mediated through MyD88-dependent TLR activation[42,54]. Later, it was shown that soluble Dll1, which shares sequence identity with Dll4, enhanced T_H1 polarization shown by increased IFN-γ and reduced IL-4 production[118]. Such mechanism, nonetheless, might have been facilitated via an IL-12-independent T_H2-inhibitory mechanism[54,119]. Similarly, inhibition of Dll4 was shown to reduce T_H1/T_H17 differentiation and prevented development of multiple sclerosis in mice[160]. However, one study showed Dll4-treated DCs were more immunosuppressive and induced $CD4^+$ T cell proliferation to a lesser extent[161].

The co-expression of Notch and their ligands brings up the question of how, then, Notch pathway coordinates its directionality and what role Notch receptors in APCs play during such circumstances. One emerging mechanism is the *cis*-interaction of Notch proteins, which is not restricted to classical Notch ligands only. Several other membrane-

bound or soluble non-canonical Notch ligands such as Dlk-1 and MAPG-1 were discovered to participate in *cis*-inhibition[162] or *cis*-activation[163], respectively. As opposed to *trans*-activation, upon *cis*-inhibition, independent of NICD, the resultant receptor/ligand heterodimer was shown to be endocytosed and proteolyzed, thus bypassing the necessity of cleavage by γ-secretase complex. The Dll4 expression patterns support the evidence for *cis*-inhibition, as Notch1 upregulation was seen only in the infected DCs (**Fig. 6B**) with maintained Dll4 levels (**Fig. 11**); the opposite of which holds true for LPS stimulated DCs. Such observations suggest that when Dll4 levels are increased at the cell surface, Notch1 is outcompeted and subsequently eliminated from the surface through *cis*-inhibition. Likewise, co-transfection experiments showed that induced Dll4 expression contains Notch1 levels, and when Dll4 expression is limited, Notch1 levels increase[73]. Further, *cis*-inhibitory mechanisms can be studied by FRET Flow which allows for analysis of speculated physical interactions between surface ligands and receptors. Most commercially available antibodies against Notch proteins are conjugated with either PE or APC, making ideal donor/receptor pairs for the FRET assay. If a Notch receptor and ligand are in physical proximity of one another, the emission spectrum of PE-conjugated antibody will excite the APC-conjugated antibody. It would be interesting to see whether *Leishmania* employs such *cis*-inhibitory mechanism to evade the inflammatory response of DCs.

In contrast to Dll4, Jag2 expression was suppressed in response to T_H1-inducing stimuli and increased with *L. braziliensis* infection (**Fig. 12**). Similarly, Jag2 expression was shown to be elevated in response to CT and SEA[149]. However, despite Jag2 upregulation in response to such T_H2-inducing microbial agents and the failure of Jag2-

deficient DCs to mediate such $CD4^+$ T_H2 response *in vitro*[150], splenic $CD4^+$ T cells harvested from mice immunized with SEA- or CT-pulsed Jag2-deficient DCs did not show impaired IL4 production[149]. Moreover, in an alternative scenario, LPS-pulsed Jag2-overexpressing DCs were significantly impaired in IL-12 production (**Fig. 14A**); however, there was no report of enhanced T_H2 responses *in vivo*[149]. Such findings supported the evidence for the splenic $CD8^-$ DC guidance of IL-12-independent T_H1 polarization, which presumably was mediated through MyD88-independent Dll4 induction[119,164]. In contrast to these findings, Jag2 mAb administration in a murine cardiac transplantation model, showed increased graft rejection and enhanced T_H2 response, indicated by increased levels of IL-4, IL-6, and GATA3[151]. Nonetheless, we were able to show that despite ubiquitous expression of Jag2 in transgenic DCs, TLR stimulation retained its ability to diminish Jag2 expression on the cell surface (**Fig. 13B**). Existence of such mechanism could explain the observed T_H2 phenotype *in vivo* by administration of soluble Jag2 that is invulnerable to transmembrane-dependent modulation. The artificial dosing of Notch ligand may override any self-regulatory mechanisms that exist among Notch proteins. These data suggested that notwithstanding the imposed Notch profile, the local microenvironment could provoke readjustment of DC's proinflammatory response through induction of intrinsic regulatory mechanisms (**Fig. 13B** and **14B**). Hence, an alternative explanation to the preserved T_H1 polarization seen in mice immunized with Jag2-overexpressing DCs could be Notch-mediated *cis*-inhibition of membane Jag2 expression, which in turn could restore IL-12 production. In concordance with such hypothesis, we showed that despite the induction of Jag2 expression, IL-12 producing DCs came from the $Jag2^{low}Notch2^{high}$ population (**Fig. 14**),

which might be sufficient to induce T_H1 differentiation. Moreover, Jag1 expression increased in LPS-pulsed T_H1-promoting wild type or T_H2-promoting MyD88-deficient BM-DCs[27,165]. Additionally, our preliminary results implicated that manipulation of Jag2 was not inert to Jag1 levels (data not shown), which might branch from redundancy in Jagged function. Thus, it would be beneficial to assess $CD4^+$ T cell responses adapted to $Notch2^{-/-}Jag2^{high}$ or $Jag1^{-/-}Jag2^{-/-}$ DCs in order to address *cis*-inhibition or functional redundancy of Jag2, respectively. Moreover, by deleting Notch2 and inducing Jag2 expression in $Notch2^{-/-}Jag2^{high}$ we may abrogate IL-12 production sufficiently, which then could diminish T_H1 polarization. Since Jag2 expression alone was shown to be dispensable for T_H2 polarization, it would be interesting to see whether co-deletion of Jag1 expression in $Jag1^{-/-}Jag2^{-/-}$ DCs will overcome functional redundancy and sway T_H polarization.

Bearing in mind the significance of noncanonical Notch pathways and their extensive interplay with other pathways in the immune cells, it becomes clear that the conventional methods of studying Notch signaling are no longer capable of accurately addressing our questions, and hence require novel modifications. In the pursuit of developing such tools, we constructed retroviral based Notch reporter vectors that contained the promoter and coding sequence for H2B-Citrine or H2B-mCherry fusion proteins downstream of the RBP-J response element (**Fig. 15A**). The advantages of these constructs include the ability to measure the Notch activity via all the receptors combined by Flow Cytometry at a single cell level. Co-culture assays further allow us to quantify ligand-induced Notch activity both intrinsically and extrinsically (**Fig. 15B**). But this model could only detect the activity of the classical RBP-J-dependent Notch pathway.

Nonetheless, we were not able to detect any Notch reporter activity in transgenic BMDCs, which may be due to fusion of florescent proteins to H2B. Nuclear localization of Citrine or mCherry through the action of H2B histone protein allows for increased longevity and amplification of the fluorescent signal[166]. However, primary BMDCs do not proliferate, and, thereby, their DNA predominately exists in fine and loose structures[166]. In accordance with this speculation, our prelimneray data showed detectable Notch reporter activity in proliferating MEFs (data not shown). The linearity of Notch signaling (no amplification step) and the absence of nucleosome-based supercoils could, together, lead to the dissipation of an already weak signal. In order to address this issue, the vector, with or without H2B sequence, should be tested in APC primary cells or cell lines. By removing the H2B coding sequence we can predict to detect Notch activity in BMDCs.

In conclusion, our findings illustrate that there is functional differences among Notch proteins and support the evidence for presence of a *cis*-inhibitory mechanism in DCs. However, the indirect role of Notch signaling in regulating the adaptive immune system requires further investigation by performing co-culture assays of T cells and transgenic DCs, with an induced Notch profile. In addition, the direct role of Notch signaling in DCs through *cis*-inhibition can be studied by development of tools such as FRET Flow. A better understanding of Notch-mediated T cell modulation can further expand our targets in developing therapies that favor certain immune response against viral cancer or other human tumors and immunological disorders.

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