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DLL4+ Dendritic Cells: Key Regulators of Notch Signaling in Effector T Cell Responses

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Abstract

Dendritic cells (DCs) are critical regulators of adaptive immune responses. DCs can elicit primary T cell responses at low DC:T cell ratios through their expression of high levels of antigen-presenting molecules and costimulatory molecules. DCs are important for induction of functionally diverse T cell subsets such as $CD4^+$ T helper (Th)1 and Th17 cells and effector $CD8^+$ T cells able to reside in epithelial tissues. Recent studies begin illuminating the underlying mechanism by which DCs regulate specialized T cell subsets. DCs are composed of subsets that differ in their phenotype, localization and function. DCs expressing high levels of DLL4 ($DLL4^+$ DCs), which is a member of Notch ligand family, are newly discovered cells that have greater ability than $DLL4^-$ DCs to promote the generation of Th1 and Th17 $CD4^+$ T cells. DLL4 derived from $DLL4^+$ DCs is also important for promoting the differentiation and expansion of effector $CD8^+$ T cells. Experimental studies have demonstrated that selective deletion of DLL4 in DCs causes impaired antitumor immunity. In contrast, blocking DLL4 leads to dramatic reduction of inflammatory T cell responses and their-mediated tissue damage. We will discuss emerging functional specialization within the $DLL4^+$ DC compartment, $DLL4^+$ DC biology and the impact of pharmacological modulation of DLL4 to control inflammatory disorders.

Graphical abstract

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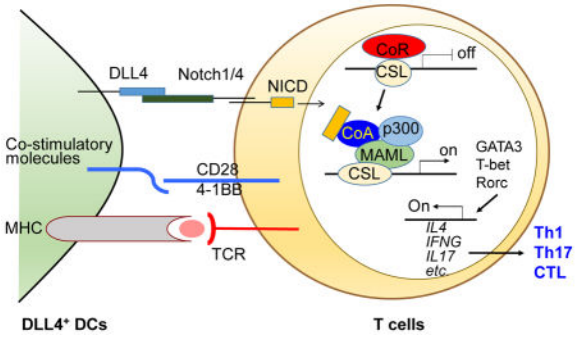

Keywords

DLL4; DLL4$^+$ DCs; Notch signaling; tumor immunity; alloimmunity and autoimmunity

A. INTRODUCTION

Dendritic cells (DCs) are essential for eliciting primary T cell responses.[1–3] DCs express high levels of antigen presenting molecules, which present antigen peptides to prime naïve T cells by triggering T cell receptor (TCR) signaling, and costimulatory molecules, which are able to amplify TCR signaling to promote proliferation and survival of activated T cells.[1–3] DCs also produce cytokines to direct effector differentiation,[4–8] however, DCs can polarize the generation of distinct lineages of effector cells independent of cytokines.[9,10] We now appreciate that Notch ligands expressed on the surface of DCs are crucial for promoting effector differentiation.[11–14] In mammalians, there are five Notch ligands (i.e., Delta-like 1 (DLL1), DLL3, DLL4, Jagged1 and Jagged2).[15–17] Notch ligands interact with Notch receptors (Notch 1, 2, 3, and 4),[15,17,18] triggering the release of intracellular Notch and the subsequent transcription of Notch target genes.[15,17,18] Among these Notch ligands, DLL4 shows greater capacity than others to promote the generation of T helper (Th)1 and Th17 CD4$^+$ T cells, which are characterized by producing high levels of IFN-γ and IL-17, respectively.[14,19–21] This leads to our discovery of DLL4-positive (DLL4$^+$) DCs that have greater capacity than DLL4-negative (DLL4$^-$) DCs to induce Th1 and Th17 cells.[14,19,20] A recent study reports that DC-derived DLL4 was able to regulate CD4$^+$ T cell metabolism and proliferation through potentiating T cell receptor (TCR)/CD28 signaling.[22] Thus, DLL4 activation of Notch may represent a ‘fourth’ signal that is crucial for instructing effector development.[11,12,14,19,20,23,24] This review will focus on discussing our current understanding of biology of DLL4$^+$ DCs and their-derived DLL4 in the generation of specialized effector T cells and their-mediated inflammatory disorders.

B. INDUCTION OF DLL4$^+$ DCS AND LINEAGE-SPECIFIC EFFECTOR T CELLS

Early studies revealed that DLL4 was highly and selectively expressed within vascular endothelium and crucial for the control of endothelial cell development.[25] In 2004, studies by Amsen and colleagues suggested that DLL4 was induced in lipopolysaccharides (LPS)-stimulated bone marrow (BM) cells that contained antigen-presenting cells (APCs). Ectopic

expression of DLL4 in IE^k-expressing L cell lines enhanced the production IFN-γ by naïve $CD4^+$ T cells of AND TCR transgenic T cells.[12] In 2007, Skokos and colleagues reported that $CD8^-$ DCs induced IL-12-independent Th1 differentiation through DLL4 activation of Notch.[10] Subsequent studies showed that DLL4 regulated pathogenesis of inflammatory diseases in experimental mice, including respiratory viral (RSV) infection,[26,27] experimental allergic conjunctivitis,[28] experimental autoimmune encephalomyelitis[29] and mycobacteria-elicited pulmonary granulomatous.[30] These studies open the perspective to explore the importance of DLL4 and $DLL4^+$ DCs in inflammatory T cell responses. However, under steady state conditions, DLL4 was barely detected on the surface of DCs and only a small numbers of $DLL4^+$ DCs could be recovered from normal mice,[10,26–28,30,31] which limited our capacity to investigate the biological properties of these $DLL4^+$ DCs. By 2013, we discovered that DLL4 was dramatically upregulated on the surface of approximately 40% of host DCs from the spleen of mice undergoing preparative conditioning for allogeneic hematopoietic stem cell transplantation (HSCT). This finding allowed us to characterize these $DLL4^+$ DCs.[14] Most recently, we have established a novel culture system that can produce large amount of $DLL4^+$ DCs from murine BM, providing a unique opportunity to investigate their biology and clinical implications.[19]

B.1. DC expression of DLL4 under inflammatory conditions

DCs are heterogeneous cell populations.[32] Based on their surface phenotype, anatomical location and function, murine DCs at the steady state condition are broadly categorized into conventional DCs (cDCs, $CD11c^+PDCA\text{-}1^-B220^-$) and plasmacytoid DCs (pDCs, $CD11c^+PDCA\text{-}1^+B220^+$).[32,33] Under inflammatory conditions, immature DCs profoundly change their phenotype, acquire enhanced antigen-presenting capacity and alter migration capability.[33–37] To define how inflammatory DCs induce specialized effector T cells, we used murine models of allogeneic HSCT that cause graft-versus-host disease (GVHD). In the GVHD model, recipient mice were pre-conditioned using lethal irradiation, followed by transfer of BM with or without T cells from allogeneic donors. Lethal irradiation would cause tissue injury and gastrointestinal (GI) dysfunction, leading to the release of danger signals and entry of LPS. Within three days after transplantation, DCs were all of host origin and expressed high levels of MHC class II molecule Ia and costimulatory molecules CD80 and CD86.[14] Thus, DCs generated in hosts undergoing preparative conditioning resemble the phenotype of inflammatory DCs.[33,34,36,37]

The expression of DLL4 is induced in immature DCs upon inflammatory stimulation. Data from our studies and others indicate that under steady state condition in mice, only a small fraction of pDCs expressed low levels of DLL4, whereas cDCs did not produce DLL4.[14,38] In contrast, DLL4 was dramatically upregulated on the surface of approximately 40% of murine whole DC population from the spleen of allogeneic HSCT recipients. Notably, while approximately 10% cDCs derived from mice undergoing HSCT also upregulated the expression of DLL4, as many as 80% of murine $DLL4^+$ DCs derived from mice undergoing HSCT were $PDCA\text{-}1^+B220^+$ cells, resembling to pDCs under steady state conditions. However, these $DLL4^+$ pDC-like cells expressed CD11b, which is normally not seen in steady state pDCs.[32] Upon inflammatory stimulation, immature DCs may alter their phenotype and functionality.[33–35,39] Indeed, our subsequent studies showed that while

immature pDCs derived from BM cells cultured in the presence of Flt3 ligand (referred to as FL) did not express high level of DLL4, they rapidly upregulated DLL4 transcript and protein upon activation of TLR signaling, accompanied with their increase of CD11b and other costimulatory molecules (e.g., CD40, CD80 and CD86).[19] These observations suggest that DLL4$^+$ pDC-like cells may be derived from immature pDCs upon inflammatory stimulation. Thus, both murine DLL4$^+$ pDCs and DLL4$^+$ cDCs occurred in mice under the allogeneic HSCT condition, with the former being a major subset. Notably, these DLL4$^+$cDCs did not express CD8.[14,19]

Our understanding of human DCs derived predominantly from studies of cells isolated from peripheral blood.[40] Under steady state conditions, human peripheral blood (PB) DCs lack lineage (Lin) markers (i.e., CD3, CD15, CD19, CD14, CD20 and CD56) and constitutively express HLA-DR (DR$^+$), and can be broadly categorized into two major subsets: cDCs and pDCs.[6] Human cDCs are characterized as Lin$^-$HLA-DR$^+$CD11c$^+$ cells, whereas pDCs are Lin$^-$HLA-DR$^+$CD11c$^-$CD123$^+$cells.[6,41] In PB, cDCs can be further classified into at least two subsets: CD1c$^+$ DCs and CD141$^+$ DCs.[42] The former comprises the predominant cDC subsets, whereas the latter is a relatively small population. We found that only a small fraction of CD1c$^+$ DCs (2.2%) and pDCs (0.8% ± 0.2%) from PB of healthy donors expressed low levels of DLL4 on their surface.[20] In contrast, allogeneic HSCT recipients had a 16-fold more DLL4$^+$CD1c$^+$ DCs (32.4%) than healthy donors. Human pDCs (3.2%) from HSCT patients also upregulated DLL4, but to a much less extent than CD1c$^+$ DCs.[20] These studies clearly identify the existence of human counterpart of mouse DLL4$^+$ DCs. Furthermore, the expression of DLL4 represents a unique marker of activating DCs under inflammatory conditions.

B.2. Induction of specialized T cell subsets by DLL4$^+$ DCs

Induction of specialized T cell subsets is important for both protective and inflammatory immune responses. DLL4 derived from CD1c$^+$ DCs is critical for the induction of Th1 and Th17 CD4$^+$ T cells. In mixed lymphocyte reaction (MLR) cultures, activated DLL4$^+$ DCs were better able to promote Th1 and Th17 differentiation than unstimulated PB DCs. Blocking DLL4 using a neutralizing antibody (Ab) decreased Notch signaling in T cells stimulated with DLL4$^+$ DCs, and reduced the generation of Th1 and Th17 CD4$^+$ T cells.[20] Like their human counterparts, murine DLL4$^+$ DCs had greater ability than DLL4$^-$ DCs to promote the development of effector CD4$^+$ T cells producing IFN-γ and IL-17.[14] Furthermore, in vivo administration of neutralizing anti-DLL4 Ab led to decreased generation of inflammatory Th1 and Th17 cells, and the reduction of GVHD in mice receiving allogeneic HSCT. Notably, blocking DLL4 had no significantly impact on regulatory T cells (Treg).[14,19,24] Thus, DLL4$^+$ DCs and their derived DLL4 play critical roles in regulating functionally specialized CD4$^+$ T cell subsets.

Recent studies suggested that both human CD1c$^+$ DCs and CD141$^+$ DCs acquired influenza antigens in vivo and expanded specific cytotoxic CD8$^+$ T cells in vitro using humanized mice infected by live-attenuated influenza virus (LAIV).[43] Interestingly, lung-tissue-resident CD1c$^+$ DCs but not CD141$^+$ DCs, were able to drive CD103 expression on CD8$^+$ T cells and promoted CD8$^+$ T cell accumulation in the lung epithelia in vitro and in vivo.[43] Given

the importance of DLL4 on human CD1c$^+$ DC-mediated IFN-γ, it will be intriguing to determine whether DLL4 derived from CD1c$^+$ DCs may play important roles in the generation of specialized effector CD8$^+$ T cells, such as the capacity to produce different profile of inflammatory cytokines and chemokines and the ability to express chemokine receptors and adhesion molecules. Data from our studies and others suggest that tissue resident DCs play important roles in regulating T cell function in the local tissues.[43–48] In mice undergoing allogeneic HSCT, DLL4$^+$ DCs were identified in the intestine where the occurrence of GVHD is associated with increased risk of mortality in recipients.[14] Other studies suggested that tissue resident DCs expressing CD103 were important for expanding tumor-reactive T cells to sufficient numbers for controlling tumor growth. Better understanding of how tissue resident DLL4$^+$ DCs influence T cell immunity in the local tissue may lead to new strategies of modulating alloimmunity and tumor immunity.

C. DLL4$^+$ DC ACTIVATION OF NOTCH SIGNALING IN T CELLS

C.1. Notch signaling in effector T cell subsets

Notch is a conserved signaling pathway that plays important roles in multiple cell processes, including cell growth, proliferation, differentiation and fate determination[15–17]. Notch receptor is composed of an extracellular ligand-binding domain, a single-pass transmembrane domain and an intracellular domain (Figure.1). Binding of Notch ligand to Notch receptors leads to its cleavage by γ-secretase complex, and release of active intracellular domain of Notch (NICD). NICD is translocated from cytoplasm into nucleus, where it binds to the DNA-binding protein complex CSL/RBP-J, recruits transcriptional co-activators including MAML and p300, and subsequently activates gene transcription. In peripheral immune responses, Notch signaling activates major transcription factors including T-bet, GATA3 and Rorc to instruct the differentiation of activated CD4 T cells into Th1, Th2 and Th17 cells, respectively. Recent studies suggested that DLL4 promoted CD4$^+$ T cells to secrete IL-17 via a mechanism of upregulating Rorc and Il17 transcription.[49–51]

DLL4$^+$ DCs promote Th1 and Th17 polarization at least partially dependent on canonical Notch signaling. Data from our recent studies indicate that DLL4$^+$ DCs promote effector differentiation via a Notch-dependent mechanism. Dominant-negative (DN)-MAML is a specific pan-Notch inhibitor.[52] Upon allogeneic DLL4$^+$ DC activation, DN-MAML-expressing CD4$^+$ T cells produced five-times fewer Th1 cells in cultures compared to wild-type (WT) CD4$^+$ T cells.[53] Addition of anti- DLL4 Ab did not further decrease Th1 cells in cultures of DN-MAML CD4$^+$ T cells. Thus, Notch signaling is essential for DLL4$^+$ DC-mediated Th1 differentiation. Anti-DLL4 Ab treatment reduced activation of Notch 1 signaling and downstream targets of Notch signaling, including Hes1 and Dtx1, in CD4$^+$ T cells cocultured with DLL4$^+$ DCs. This effect of anti-DLL4 Ab was associated with significantly decreased expression of Tbx21 and Rorc transcripts.[14,19] Interestingly, an elegant study by Bailis and colleagues showed that Notch signaling was able to simultaneously orchestrate multiple helper T cell programs via a mechanism independently of cytokine signals. They found that Notch signaling directly bound to the *Ifng* locus to activate *Ifng* transcription independently of T-bet.[9]

Emerging data from recent studies indicate that DLL4 activation of Notch1 signaling is also important for proliferation of antigen-activated $CD8^+$ T cells.[19] These findings are in agreement with previous observation showing that Notch signaling is crucial for activating T-bet to promote the differentiation of $CD8^+$ T cells into effector cells.[54,55] Notch 1/2 deficiency also reduced effector cell differentiation through impairing AKT and mTOR activation.[9,54] Notch 1/2 deficiency led to increased expression of transcription factors (Bcl6, Foxo3, Foxo1, Tcf7, Id3), promoting memory precursor cell generation.[55] It appears that Notch signaling has a broad impact on $CD8^+$ T cell responses.

C.2. High binding affinity between DLL4 and Notch1/4

Better understanding of the molecular structure of DLL4 will be important for understanding why DLL4 has greater capacity than other Notch ligands to activate Notch signaling in T cells in the context of instructing their Th1 and Th17 differentiation. The human *DLL4* gene was located on 15q21.1, and the mouse *Dll4* gene was mapped to chromosome 2E3, a region that shows conservation of synteny with human chromosome 15q.[25] The open reading frame (ORF) of human *DLL4* is ~86% identical at the nucleotide level and 87% identical at the amino acid level to murine *Dll4*.[25] Like Notch receptors and other Notch ligands, DLL4 is composed of a module of N-terminal domain of Notch ligand (MNNL), followed by NSL domain, EGF-like repeats, transmembrane domain and intracellular domain (Figure 2).[15,17,56] Unlike Jagged family Notch ligands, DLL4 has different numbers of EGF-like repeats and lacks an extracellular cysteine-rich domain and insertions that interrupt some EGF-like repeats. These structural differences categorize a Notch ligand as a Delta or Serrate family member.[15,17,56] Besides, DLL4 is ~50% identical to DLL1 at the amino acid level.[57] These structure differences and the dramatic difference of amino acid sequence among Notch ligands may explain their functional differences.

DLL4 activates downstream Notch signaling mainly through Notch1 and Notch4 receptors.[25,58] Coexpression of DLL4 and Notch1 or Notch4 induces the activation of target genes of Notch signaling in neural ectoderm in *Xenopus* embryos. In vitro binding affinity assay showed that DLL4 had an at least 10 fold higher binding affinity to Notch1 than DLL1.[25] The molecular basis for DLL4 binding with Notch1 has been demonstrated with the analysis of crystal structure, further validating DLL4-Notch1 signaling pathway.[58] Upregulation of Notch1 and Notch2 was seen in both $CD4^+$ and $CD8^+$ T cells after TCR activation, with clear increase of activated Notch1 NICD being detected.[11,17,18,59] However, expression of Notch3 and Notch4 in activated T cells remains elusive.[21] Our studies have shown that in vivo anti-DLL1 neutralizing antibody treatment did not affect IFN-γ- and TNF-α-producing T cells, indicating that DLL4-Notch signaling may play more important roles in vivo in T cell responses.[14,19] Whether and how DLL4-Notch4 signaling regulates T cell immune responses remains to be explored. Report from other groups also found that blocking DLL4 in vivo had more dramatic effect in ameliorating GVHD and improving overall survival, further supporting this hypothesis.[24]

D. MECHANISMS THAT REGULATE DLL4+ DC DIFFERENTIATION

D.1. The role of Toll-like receptor (TLR) signaling in DC expression of DLL4

The capacity of different DC subsets to produce DLL4 under inflammatory conditions suggests that immature DCs may respond differentially to inflammatory stimuli in the context of upregulating DLL4. DCs become mature after encountering pathogens through activation of pattern recognition receptors including TLRs, Nod-like receptors (NLRs), C-type lectin receptors, mannose receptors and etc.[60] $CD1c^+$ DCs express TLR4 and TLR7, whereas pDCs express TLR7 and TLR9 but lack TLR4.[6,7,61–63] Data from our published studies indicate that while human immature $CD1c^+$ DCs and pDCs expressed low levels of DLL4, they rapidly upregulated the expression of DLL4 upon activation with TLR7/8 agonist R848 (Resiquimod) and / or TLR4 agonist LPS. Interestingly, monocyte-derived DCs (MoDCs) were unable to produce high levels of DLL4. MoDCs represent a subset of DCs of particular importance under inflammatory conditions, and have been widely used as vaccine adjuvants.[6,64] We found that both monocytes and their-derived MoDCs failed to produce high levels of DLL4 when they were stimulated with R848 and LPS. Real-time RT-PCR analysis further revealed that activated monocytes expressed 2 to 5-fold less *DLL4* transcripts compared to pDCs and $CD1c^+$ DCs. In contrast, MoDCs upregulated the expression of costimulatory molecules (e.g., CD40, CD80, CD83, and CD86), suggesting that the capacity of immature DCs to become $DLL4^+$ DCs has been programmed during DC precursor development stages.[20] These observations indicate that the capacity of immature DCs to upregulate DLL4 has been established during the period of time when they are generated from their progenitor cells.

This hypothesis is further confirmed by our findings showing that human pDCs are composed of two distinct subsets in terms of their ability to produce DLL4 upon triggering TLR signaling. When being activated with high concentration of R848, Lin^-DR^+ pan-DCs gave rise to approximately 90% of $DLL4^+CD1c^+$ DCs and 35% of $DLL4^+$ pDCs.[20] This allowed us to isolate pDCs into two subpopulations: $DLL4^+$ pDCs and $DLL4^-$ pDCs. When cocultured with allogeneic $CD4^+$ T cells, we found that $DLL4^+$ pDCs induced 2-fold more Th1 cells than $DLL4^-$ pDCs. Interestingly, as compared to $DLL4^-$ pDCs, $DLL4^+$ pDCs had higher levels of *IL6*, but were lower in *IL1B* expression, two inflammatory cytokines important for Th17 cell differentiation.[65] In addition, $DLL4^+$ pDCs expressed 2-fold higher *IRF8*, a transcription factor important for pDC development, compared to $DLL4^-$ pDCs.[66] This difference was unlikely the result of pDC activation, because both subsets showed similar levels of surface markers related to mature DCs (e.g., CD40, CD80, CD86 and CD83). [66]

Collectively, all these observations raise some important questions. For example, can DLL4 be used as a DC lineage marker for defining DC differentiation from hematopoietic progenitor cells (HPCs)? What is the intrinsic molecular mechanism that specifies the fate of $DLL4^+$ DCs during hematopoietic progenitor cell differentiation and subsequently activate DLL4 transcription in activated immature DCs? Under what kind of environmental signals are HPCs driven to immature $DLL4^+$ DCs?

D.2. Cytokine-mediated fate determination of immature DLL4$^+$ DCs

To answer these questions, we have recently established a culture system to generate murine DLL4$^+$ DCs from mouse bone marrow.[19] Being cultured in the presence of FL, murine bone marrow (BM) cells differentiated into immature pDCs and cDCs,[19] which were characterized by the surface phenotype of CD11b$^-$B220$^+$CD11c$^+$ and CD11b$^+$B220$^-$ CD11c$^+$ cells, respectively.[7,67] Both subsets were DLL4 negative, but rapidly upregulated the expression of DLL4 upon overnight incubation with LPS or R848. Concurrent stimulation with LPS and R848 induced much higher frequency of DLL4$^+$ DCs. However, culture of BM cells in the presence GM-CSF failed to induce DLL4$^+$ DCs despite stimulation with LPS and R848.[19] These data suggest that the capacity of immature DCs to increase DLL4 upon inflammatory stimulation has been established during their differentiation from hematopoietic progenitor cells. These results are also in agreement with those aforementioned findings that upon GM-CSF culture human MoDCs were also unable to upregulate DLL4. Thus, FL promotes the generation of immature DLL4$^+$ DCs from HPCs, whereas GM-CSF diverts the differentiation of HPCs into DLL4$^-$ DC subsets.

Recent studies have demonstrated that inflammatory environment has significant impact on the generation of immature DLL4$^+$ DCs and their differentiation into distinct DC subsets.[12,68] Preparative conditioning for allogeneic HSCT may lead to increased release of LPS and other danger signals.[69,70] The time kinetic analysis revealed that host-type DLL4$^+$ DCs markedly increased 3 days after transplantation and declined by day 7. This is in agreement with previous observations that host DCs may finally diminish during the GVHD process.[45,71–73] In mice undergoing HSCT, DCs can be de novo generated from infused donor BM cells. Interestingly, despite their exposure to inflammatory stimuli, newly generated donor origin DCs did not express DLL4 seven days after transplantation. It has been shown that seven days after HSCT in mice, alloreactive T cells differentiated into effector T cells that are able to produce inflammatory cytokines, including IFN-γ, TNF-α and GM-CSF.[70] It is speculated that upon simulation with GM-CSF in vivo, donor HPCs may reduce their capacity to differentiate into DLL4$^+$ DCs. We therefore propose that an impaired reconstitution of donor-type DLL4$^+$ DCs in hosts undergoing allogeneic HSCT might reflect the presence of virulent GVH reactions that produce high levels of inflammatory cytokines such as TNF-α and GM-CSF.

E. REGULATION OF DLL4 BY NFκB and STAT3 IN IMMATURE DCS

Studies of mechanisms that induce DLL4 in DCs will be important for better defining the ontogeny of DLL4$^+$ DCs and targeting these cells for immunotherapy. Our recently studies have demonstrated that Pam3 (TLR1/2 stimulus), Poly I:C (TLR3 stimulus), LPS and R848 induced high levels of DLL4 expression on the surface of 50% to 80% of CD1c$^+$ DCs, whereas IFN-α (pro-inflammatory cytokine) and CD40L (signal from activated T cells) did not.[20] CpG oligodeoxynucleotides (TLR9 agonists) did not increase DLL4 in CD1c$^+$ DCs,[20] consistent with the absence of TLR9.[74,75] pDCs increased DLL4 expression when activated by R848 (16.0% ± 2.7%) and to a lesser extent by CpG oligodeoxynucleotides (8.6% ± 0.8%).[20] These results demonstrate that activation of TLR signaling induces high levels of DLL4 in CD1c$^+$DCs and pDCs, with R848 being the most potent stimulus.

NFκB is a critical pathway downstream of TLR signaling[76]. We found that the NFκB inhibitor PDTC completely blocked DLL4 induction in both DC subsets, suggesting the important role of NFκB in inducing DC expression of DLL4.[20] However, stimulation of monocytes with R848 + LPS, which is known to activate NFκB signal in these cells,[77] activated NFκB as evidenced by increased expression of phosphorylated P65 (p-P65), but induced low levels of DLL4 on the surface of monocytes.[20] Furthermore, MoDCs derived from cultures in GM-CSF and IL-4 had elevated p-P65 following stimulation by R848 + LPS and but were DLL4 negative.[20] These data suggest that activation of NFκB is important but not sufficient for inducing DLL4 in human DCs.

Many studies have demonstrated that STAT3 plays multiple roles in both innate and adaptive immunity.[78–81] For example, STAT3 is essential for production of indoleamine-2,3-dioxygenase (IDO) by murine antigen-presenting cells,[80] thereby repressing T cell response. Conversely, data from clinical studies indicate that about half of these patients treated with a STAT3 inhibitor experienced pathogen-mediated diarrhea[81], suggesting that STAT3 is required for protective mucosal immunity. In mice, loss of STAT3 results in deficiency of common DC precursors and their DC progenies, but has no effect on monocyte/macrophage differentiation of hematopoietic precursor cells.[82] We found that activation of TLR7/8 signaling increased STAT3 and p-STAT3 in human PB $CD1c^+$ DCs and pDCs.[20] Inhibiting STAT3 led to dramatic decrease in expression of DLL4 transcripts and proteins in circulating DCs. Promoter reporter assay revealed that STAT3 activated DLL4 transcription. In contrast, monocytes or MoDCs expressed low levels of STAT3 mRNA and active p-STAT3 protein in response to R848 and LPS.[20] This may explain the inability of monocytes and MoDCs to produce high level of DLL4. Thus, DC expression of DLL4 may have been developmentally programmed through a mechanism of permitting or repressing STAT3 transcription.

It has been shown that the interaction between NFκB and STAT3 plays an important role in regulating inflammatory immune cells.[78] This appears to be true in regulating the expression of DLL4 in human DCs.[20] Freshly isolated $CD1c^+$ DCs and pDCs expressed higher levels of STAT3 than MoDCs. Activation of TLR4/7 led to increased amount of both STAT3 and phosphorylated STAT3 (p-STAT3) in $CD1c^+$ DCs and pDCs, but not in MoDCs. The increased level of STAT3 and p-STAT3 in these $CD1c^+$ DCs and pDCs was accompanied by induction of high levels of DLL4 protein. Notably, inhibiting either STAT3 or NFκB caused a decrease in DLL4 expression in circulating $CD1c^+$ DCs and pDCs.[20] These finding argue the notion that both NFκB and STAT3 are required for the induction of $DLL4^+$ DCs. It is likely that NFκB and STAT3 cooperate to activate DLL4 transcription in human DCs.

F. DLL4 AND $DLL4^+$ DCS IN T CELL IMMUNITY

Autoimmunity

Several lines of evidence indicate that DLL4 and $DLL4^+$ DCs may play important roles in regulating T cell immunity. DLL4 has important roles in the pathogenesis of autoimmune diseases. In a mouse model of respiratory syncytial virus (RSV)-exacerbated allergic airway disease, neutralizing DLL4 led to increased production of IL-4, enhanced Th2 immunity the subsequent development of airway hyper-responsiveness.[83] In contrast, in mice with multiple sclerosis (MS) induced by TMEV,[84] treatment with anti-DLL4 Ab resulted in

significantly decreased expression of cytokines transcripts derived from Th1 and Th17 cells, reduced number of infiltrating mononuclear inflammatory cells in the spinal cords and suppression of the disease development.[84] A recent study further revealed that DLL4 influenced inflammatory T-cell response via modulating chemokine receptor expression in activated T cells during experimental autoimmune encephalomyelitis, thereby regulating migration of effector T cells.[29] These observations indicate that DLL4 dual functions in T cell autoimmunity. It has a regulatory role in initiation on Th2 cytokine production and allergic responses.[83] Furthermore, DLL4 activation of Notch signaling promotes Th1- and Th17 cell-mediated demyelinating disease. However, whether these effects result from DLL4 expressed by DCs or non-DCs are not defined in these studies.

It will be intriguing to determine whether DLL4 and DLL4$^+$ DCs may be associated with autoimmune diseases in humans. Given the importance of DLL4$^+$ DCs in regulating inflammatory T cell response in HSCT patients and a variety of inflammatory disorders in mice, it is envisioned that DLL4$^+$ DCs may have significant implications in the pathogenesis of autoimmune diseases in humans, such as lupus.

Tumor immunity

T cell immune responses play a central role in cancer immune surveillance, and the efficient induction of effector T cells against tumor antigens is required for successful immunotherapy for cancer patients.[85,86] Data from a recent study has demonstrated that DLL4 on DCs is essential for an effective anti-tumor response.[22] They observed that Notch signals enhanced CD4$^+$ T cell priming, which could critically alter the course of immune response. In a model in which eradication of a tumor is dependent on cross-presentation of tumor Ag to CD4$^+$ T cells by host MCH-II$^+$ APCs, they found that adoptively transferred Marilyn T cells responded to antigen derived from the tumor cells and constrained tumor growth in wild-type mice.[53] In contrast, the antitumor effect of Marilyn T cells was impaired in conditional DC-specific DLL4 deficient mice (DLL4$^{-/-}$ mice).[53] The inability of T cells to control tumor growth in the absence of DLL4$^+$ DCs were associated with less efficient stimulation of CD4$^+$ T cell activation, metabolism, proliferation and cytokine secretion.[53] Other studies by Sugimoto et al. have demonstrated the importance of Notch signaling in mediating tumor immunity.[87] Mice with Notch2-deficient-CD8$^+$ T cells die earlier than control mice after inoculation with OVA-expressing EG7 thymoma cells.[87] Furthermore, administration of anti-Notch2 agonist Ab augments tumor immunity.[87] These findings are interesting as they imply that enhancing the presence of functional DLL4$^+$ DCs in the lymphoid tissues and tumor sites might augment antitumor immunity.

Alloimmunity

GVHD is caused by donor T cells that recognize and react to alloantigens of the recipient.[70] Upon APC activation, donor T cells become alloreactive effector T cells producing high levels of inflammatory cytokines (e.g., TNF-α, IFN-γ, IL-4 and IL-17) and cytotoxic molecules, leading to host tissue injury and the subsequent life-threatening complication.[47,69,88–93] However, blockade of individual effector molecules has limited efficacy in controlling GVHD.[69,93] We provide evidence for a function of DLL4 and DLL4$^+$ DCs in eliciting allogeneic T-cell responses early during GVHD.[14,19,20] As compared to

DLL4$^-$ DCs, DLL4$^+$ DCs had greater ability to stimulate the generation of alloreactive effector T cells producing IFN-γ and IL-17. Blockade of DLL4 could abrogate this effect of DLL4$^+$ DCs. Furthermore, in vivo administration of anti-DLL4 Ab caused a marked reduction of alloreactive effector T cells in GVHD target organs, leading to reduction of GVHD and significantly improved survival of mice after allogeneic HSCT.[14] Our findings indicate that DLL4$^+$ DCs and DLL4 are important for the generation of alloreactive effector T cells capable of mediating host tissue injury and could be beneficial targets for improving the efficacy of allogeneic HSCT. Elegant studies by Tran and colleagues have also demonstrated that inhibiting DLL4 and DLL1 blocked GVHD while preserving substantial anticancer activity in mice undergoing allogeneic HSCT.[24] These findings establish that DLL4 and DLL4$^+$ DCs are important for induction of GVHD.

Tissue resident APCs play critical roles in inducing organ-specific GVHD.[44–47,94] Depletion of APCs in the spleen and liver reduced the GVHD in the liver but had no effect on the development of GVHD in the skin.[45] Inhibiting Langerhan's cells in the skin resulted in inhibition of cutaneous GVHD.[44] In our experimental studies, we found that DLL4$^+$ DCs predominantly localized in the intestine of mice three days after allogeneic HSCT. In vivo administration of anti-DLL4 Ab dramatically reduced GVH reactions in the gastrointestinal (GI) tract, leading to significantly reduced GVHD in mice after allogeneic HSCT.[14] This is in agreement with our recent observations that donor T cells lacking Notch signaling have drastically reduced ability to mediate intestinal GVHD.[23] We proposed that DLL4 and intestinal DLL4$^+$ DCs could play important roles in mediating GVH reaction in these tissues. However, many studies have shown that DLL4 was expressed in inflammatory macrophages and endothelial cells in other models.[13,17,29,95,96] Furthermore, GVHD still occurred after profound depletion of CD11c$^+$ DCs in mice after allogeneic HSCT.[71] More importantly, a recent study reports that non-hematopoietic APCs in the GI tract were important for production of GVHD in the local tissue.[94] Thus, the impact of DLL4 blockade on T-cell responses in vivo should consider the potential contribution of DLL4-expressing non-hematopoietic APCs (such as endothelial cells and epithelial cells, etc.). Nevertheless, it will be intriguing to determine whether DLL4$^+$ DCs may be sufficient to cause GVHD, and if non-hematopoietic APCs expressing DLL4 might have similar effect to DLL4$^+$ DCs on promoting alloreactive effector T-cell responses.

Most recently, we have demonstrated that DLL4$^+$ DCs can be used to program donor T cells to reduce their GVHD toxicity while retaining antitumor activity.[19] Using an in vitro culture system, we activated donor T cells with allogeneic DLL4$^+$ DCs and transferred them into allogeneic recipient mice. Mice receiving allogeneic DLL4$^+$ DC-induced CD4$^+$ T cells developed only minimal GVHD and complete survival. Importantly, these DLL4$^+$DC-induced alloreactive effector T cells had acquired the capability of killing leukemic cells.[19] This may potentially improve the anti-leukemic response early after HSCT, and overcome some barriers to the GVL response such as high disease burden and pharmacologic immunosuppression. Given the fact that DC activation of naïve T cells allows priming them with antigens, we propose that DLL4$^+$ DCs loaded with leukemia-associated antigens may facilitate the selection and expansion of leukemic cell-reactive T cells that mediate anti-leukemia activity. Notably, we have recently identified human DLL4$^+$ DCs that possess great ability to promote allogeneic CD4$^+$ naïve T cells to become effector cells producing high

levels of IFN-γ.[20] If sufficient numbers of human DLL4$^+$ DCs can be produced, a novel strategy can be potentially devised for human patients.

G. FUTURE PERSPECTIVE

The recent years have seen major advances in our understanding of the impact of distinct DC subsets on inducing specialized T cell subsets both in mice and humans.[14,19,20,22,43,48,54,63,97] CD1c$^+$ DCs and CD141$^+$ DCs have differential roles in the generation of CD8$^+$ T cells with unique functional properties and tissue localization.[43] Although both DC subsets from blood can induce efficient CTL immune responses, CD1c$^+$ DCs from human lung have significant higher capacity than lung CD141$^+$ DCs to induce the differentiation and expansion of local CD103$^+$CD8$^+$ effector T cells. In addition, while CD1c$^+$ DCs from lung and blood have been shown to promote Th17 generation, CD141$^+$ DCs are more potent than CD1c$^+$ DCs to induce IL-4- and IL-13-producing Th2 differentiation after LAIV challenge.[98,99] Moreover, human skin dermis CD141$^+$ DCs are able to induce regulatory CD4$^+$ T cells, which inhibit inflammation during allogneneic skin transplantation.[100] It remains to be determined if the expression of DLL4 on the surface of CD1c$^+$ DCs and CD141$^+$ DCs may be important for them to regulate and maintain effector T cells in these local tissues where these DCs reside. Given that DLL4 expression is induced on the surface of DCs upon inflammatory stimulation, future studies will investigate whether DLL4$^+$ DCs may be responsible for the induction of different subsets of specialized effector T cells in local tissues and whether DLL4$^+$ DCs are associated with the development and progression of organ-specific inflammatory disorders, such as autoimmune disease and GVHD. Better defining the tissue distribution of DLL4$^+$ DCs and their function in local tissues will be important to answer these questions.

Available data indicate that whether the immature DC can produce DLL4 upon inflammatory stimulation may have been established during differentiation of HPCs into immature DCs.[20] DCs develop along a defined differentiation pathway from HSCs/HPCs, common myeloid progenitors (CMP), myeloid and DC progenitors (MDP), DC precursor cells and immature DCs.[33,67,68] In response to inflammatory stimuli, immature DCs undergo maturation by increasing the expression of antigen-presenting molecules, costimulatory molecules and some cytokines.[1,6] However, at which differentiation stages, including CMP, MDP, DC precursors and immature DCs, the fate of HSCs/HPCs to become DLL4$^+$ DC precursor cells is established and why monocytes cannot become DLL4$^+$ DCs, have yet to be determined. It is likely that DLL4$^+$ DCs may be epigenetically programmed by environmental factors. Understanding of the epigenetic mechanism that regulates the generation of DLL4$^+$ DCs may lead to new strategies to target DCs for improving the efficacy of cancer immunotherapies and controlling inflammatory disorders such as GVHD and autoimmune diseases.

Finally, since DLL4$^+$ DCs play important roles in promoting the generation Th1 and Th17 cells, absence of DLL4$^+$ DCs may lead to inadequate immune responses. After allogeneic HSCT, the reconstitution of DC network appeared to be attenuated both in patients and experimental mice.[14,45,72,101–105] For example, in experimental mice, we found that while host DCs upregulated DLL4 early after transplantation, donor DCs reconstituted later in

mice with GVHD did not express DLL4 despite the presence of potent inflammatory factors in vivo. In human patients, a substantial proportion of patients in clinic early after HSCT did not have or have very low level of circulating $DLL4^+$ DCs.[20] Determining whether impaired generation of $DLL4^+$ DCs may reflect the presence of severe GVHD and/or other co-morbidity and / or correlate with infection or relapse in patients may have significant implications in the improvement of immune reconstitution after allogeneic HSCT, chronic infection and cancer immunotherapies in a broad context.

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Abbreviation

APC	antigen presenting cell
CTL	cytotoxic T lymphocyte
DC	dendritic cell
cDC	conventional dendritic cell
pDC	plasmacytoid dendritic cell
CMP	common myeloid progenitor
HPC	hematopoietic progenitor cell
MDP	macrophage and DC progenitor
HSCT	hematopoietic stem cell transplantation
DLL	delta-like ligand
GVHD	graft-versus-host disease
NICD	Notch intracellular domain
moDC	monocyte-derived dendritic cell
Th	T helper
TLR	toll-like receptor
TCR	T cell receptor

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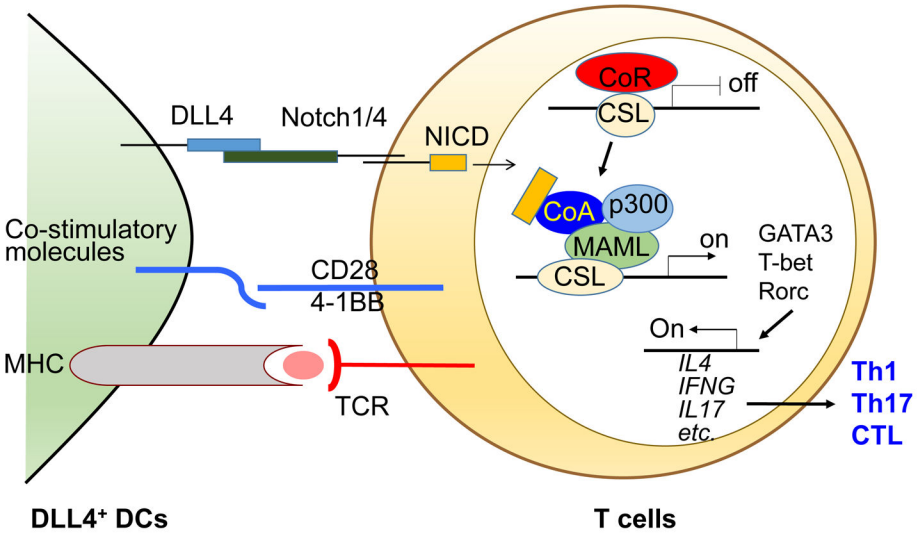

Figure 1. DLL4 activation of Notch signaling in T cells

DLL4 derived from DCs activates Notch receptor expressed on the surface of T cells, and triggers Notch cleavage by the ADAM metalloproteases and γ-secretase complex, generating active intracellular domain of notch (NICD). This leads to release of NICD from cytoplasm into nucleus, after which NICD binds to the DNA-binding protein complex CSL/RBPj. Interaction of NICD with CSL leads to the release of co-repressor which are inhibitory interaction proteins with CSL when there is no NICD in the nucleus, followed by recruitment of transcriptional co-activator including MAML and others. NICD, CSL and MALM and other co-activator form a complex and induce the active transcription of downstream Notch targets (e.g., GATA3, T-bet, Rorc, ec.). Following TCR and costimulatory signaling induced T cell activation, DLL4 activation of Notch signaling is crucial for instructing the development of Th1, Th17 and cytotoxic T lymphocytes (CTL).

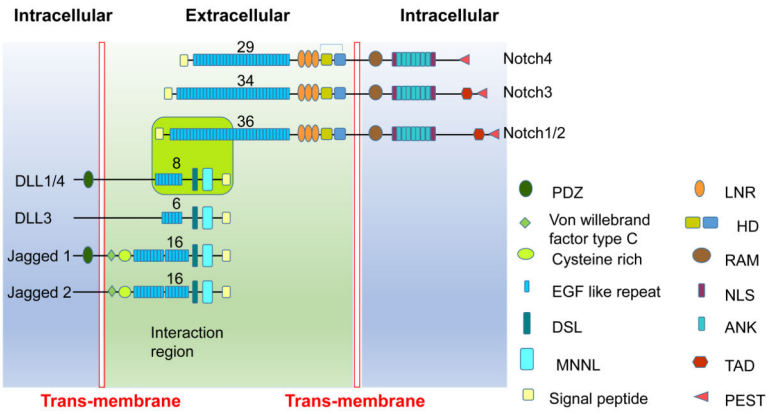

Figure 2. Biochemistry of Notch ligands and Notch receptors

There are five Notch ligands (Jagged 1, Jagged2, DLL1, DLL3 and DLL4) and four Notch receptors (Notch 1, 2, 3 and 4). Both Notch ligands and Notch receptors are transmembrane proteins, containing extracellular domain, transmembrane domain and intracellular domain. EGF-like repeats constitutes the majority of extracellular domain for both ligands and receptors, with each receptor and ligand having different number of repeats specified as indicated. Noteworthy, although DLL1 and DLL4 have almost same structure, they are only ~60% identical in protein sequence. The situation for Notch1 and Notch2 is the same. The interaction region for DLL4 ligand and Notch1 include most of the extracellular domain of DLL4 and some of the EFG-like repeats of Notch1 extracellular domain as indicated in this figure in bright green shade. Each domain is indicated in the right corner.