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Notch Signaling and Immune Regulation in Alloimmunity

Naoka Murakami^a, Ivan Maillard^{b,c}, and Leonardo V. Riella^a

^aTransplantation Research Center, Renal Division, Brigham & Women's Hospital, Harvard Medical School, Boston, MA

^bLife Sciences Institute and Division of Hematology-Oncology, Department of Internal Medicine, University of Michigan, Ann Arbor, MI

^cDepartment of Cell and Developmental Biology, University of Michigan, Ann Arbor, MI

Abstract

Notch signaling plays a pivotal role in the differentiation and fate determination of T cells, B cells, dendritic cells (DCs) and innate lymphoid cells (ILCs). Recent gene-targeting and antibody approaches advanced our knowledge of the importance of Notch signaling in fine-tuning the peripheral immune response. Here we review current knowledge of the Notch pathway, focusing on solid organ transplant and graft-versus-host disease preclinical models, and discuss the potential of targeting Notch to suppress the immune response and improve transplant outcomes.

Keywords

Notch; alloimmunity; transplant; graft-versus-host disease

Introduction

Organ transplant serves as an excellent therapeutic measure for patients with end-stage organ dysfunction and hematopoietic malignancies. Though long sought, tolerance induction protocols have not been widely applicable and long-term global immune suppression is currently necessary for essentially all post-transplant patients, which put them at substantial risks of undesired infections and malignancies. Targeted immune modifying agents therefore are under extensive investigation, such as CTLA4-Ig, anti-CD20 or anti-CD40 monoclonal antibodies, for which detailed understanding of alloimmune responses is necessary.

The alloimmune response is mediated by both cellular and humoral immunity. The process initiates with alloantigen presentation by antigen presenting cells (APCs) to T cells, via the direct or indirect pathway. In either case, alloantigens are presented to cognate T cell receptors (TCR) in the context of MHC molecules. In addition to this peptide-MHC-TCR interaction (Signal 1), full T cell activation requires additional co-stimulatory signals (Signal 2), mediated by co-stimulatory molecules (e.g. CD28/B7 and CD40/CD40L). There is an additional third signal that may further modulate T cell activation both in a stimulatory or

*Address for Correspondence: Leonardo V. Riella, MD, PhD, lriella@bwh.harvard.edu, 221 Longwood Ave, Boston MA 02115, USA. Tel: 617-732-5252; Fax: 617-732-5254.

inhibitory manner. Signal 3 is conveyed by means of cytokine signaling pathways such as IL-2/CD25, as well as via cell-cell interaction molecules such as Notch receptors-ligands. Therefore, Notch signaling participates in the fine-tuning of the immune response. In this review, we introduce how Notch signaling plays an important role in immune cell differentiation and function and how it is involved in the mechanism of allograft rejection and tolerance in the context of solid organ transplant and graft-versus-host disease (GVHD), exploring the potential of using Notch as a therapeutic target in alloimmunity.

Notch Signaling: a complex and orchestrated pathway

The Notch family is an evolutionally conserved group of cell surface signaling molecules originally identified in *Drosophila*. The mammalian Notch family consists of 4 Notch receptors (Notch1-Notch4) and five ligands (delta-like 1 (DLL1), DLL3, DLL4, Jagged1 and Jagged2). Upon interaction with their ligands, Notch receptors transduce intracellular signals via the Notch intracellular domain (NICD) after sequential cleavages by ADAM family metalloproteinase (ADAM10) and by gamma-secretase. NICD then translocates into the nucleus, where it binds to the DNA-binding factor RBPJ and recruits co-activators such as MAML1-3, modulating targeted gene expression (Figure 1). In addition to this canonical pathway, Notch has also been reported to use non-canonical signaling pathways, which are independent of RBPJ. Non-canonical signaling includes interaction with other signaling pathways, such as nuclear factor- κ B (NF- κ B), transforming growth factor- β (TGF- β), hypoxia-induced signaling and Akt-PI3K pathways via mechanistic target of rapamycin complex 2 (mTORC2) activation (1). Overall, evidence indicates that the predominant effects of Notch signaling in the immune system are mediated via the canonical pathway.

With these different signaling molecules and diverse pathways involved in Notch signaling, how is Notch tightly regulated in a highly cell type-specific and context-dependent manner? One determinant is the specific expression pattern of Notch receptor-ligand pairs. For example, DLL4 is specifically expressed on thymic epithelial cells, providing a specific niche for T cell progenitors to mature via interaction with Notch1 (2). Another mechanism is related to the chromatin landscape downstream of Notch. It was elucidated that Notch target gene transcription was not only regulated by direct binding of CSL/RBPJ complex to the promoter region of target genes, but also by other epigenetic modification factors (3) interacting with distant superenhancer regions in a context-dependent fashion (4, 5). Lastly, Notch receptors undergo post-translational changes by Fringe family glycosyltransferases, which alter the relative binding avidity of ligand-receptor pairs, translating into different efficiencies or signaling strength of Notch receptors (6, 7). With this extremely orchestrated system, Notch signaling plays a critical role in cell-fate determination throughout embryonic development and in almost all adult tissues, and deregulation of Notch pathway is associated with diverse human diseases including genetic disorders and cancers, especially T cell acute lymphoblastic leukemia (T-ALL) (8). Hereafter, we discuss the roles of Notch signaling pathway in the peripheral immune system especially in the context of transplantation.

Notch signaling in immune cell differentiation and function

The fundamental roles of Notch signaling in early lymphocyte development, especially in T lineage commitment and maturation were well described in early 2000s and reviewed in detail elsewhere (9, 10). Herein, we review the roles of Notch signaling in the differentiation and function of peripheral T helper and effector cells, dendritic cells and innate lymphoid cells, which are relevant to organ transplantation (Figure 2).

Mature T cell differentiation and function

During the past decade, knowledge advanced extensively on how T helper subsets differentiate, especially through understanding of the fate-determining transcription factors and the key-polarization cytokines. Early studies showed naïve CD4 T cells express Notch1 and 2 (11) and T cell receptor (TCR) stimulation considerably increases Notch receptors' expression (12, 13), suggesting a role for Notch signaling in activation- induced T cell differentiation and functions. Th1 cells differentiation is dependent on transcription factor T-bet and cytokine signaling pathways IL-12/Stat4 and IFN- γ /Stat1. Early studies by Flavell's group demonstrated that overexpression of DLL1 or NICD led to Th1 differentiation and blocking of Notch signaling by DLL1-Fc or T cell-specific knockout of Notch1 and Notch2 inhibited Th1 differentiation (11, 14). However, later studies reported seemingly conflicting results. Dominant-negative Mastermind-like (DNMAML), which shuts off Notch-mediated transcriptional activation in a dominant-negative fashion, did not affect Th1 responses in the *Leishmania major* infection model (15), though it inhibited the magnitude of the Th1 response in a transplant model (16). These different results highlight the context-dependent nature of Notch signaling, requiring further studies to reconcile these findings (17).

Th2 cell differentiation is driven by Gata3 together with IL-4/Stat6 and IL-2/Stat5 pathways. The ability of Notch signaling to promote Th2 differentiation has been attributed to its direct transcriptional regulation of *Gata3* (18, 19) and *Il4* (20). In addition, overexpression of NICD skewed Th2 differentiation and T cell specific DNMAML expression inhibited Th2 cytokine production *in vitro* (15), providing other evidence of Notch requirement in Th2 differentiation. Th17 differentiation is dependent on the master transcription factor retinoid-related orphan receptor- γ t (ROR γ t) and IL-6, IL-23/Stat3 pathway and the transcriptional networks controlling Th17 differentiation was recently further identified using large scale transcriptome analyses (21, 22), which included Notch signaling and RBPJ. More recently, Kuchroo's group demonstrated that RBPJ determines development of pathogenic and non-pathogenic Th17 by reciprocally controlling IL-23R and IL-10 (23). These findings, together with others demonstrating that Notch directly regulates expression of ROR γ t (24) and IL-17 (25, 26), indicate the critical role of Notch in promoting Th17 differentiation.

Regulatory T cells (Treg) are dependent on Foxp3, IL-2/Stat5 and TGF- β /SMADs for their differentiation and exhibit suppressive activity to effector T cells via multiple mechanisms. Studies suggested that the Notch-Hes1 axis promotes Treg function via regulation of Foxp3 expression cooperatively with membrane bound TGF- β (27, 28) and direct binding of RBPJ to Foxp3 promoter (29). On the other hand, later studies showed different results in GVHD models: Maillard's group showed that DNMAML recipients had increased Treg populations (16) and Chatila's group showed that Treg-specific deletion of Notch1 or RBPJ promoted

the differentiation of peripheral Treg, as discussed below (30), again, highlighting the context-dependent role of Notch signaling in Treg differentiation and function.

Follicular helper T cells (T_{fh}) are a recently described subset of helper T cells that support cognate B cells to enhance germinal center (GC) formation, the generation of high-affinity antibody and further differentiation to memory B cell and long-lived plasma cells. Their differentiation is regulated by Bcl-6 and IL-21. Genetic deletion of both Notch1 and Notch2 in T cells resulted in decreased T_{fh} differentiation, leading to abrogated production of high-affinity antibodies (especially IgG1 and IgE) against T-dependent antigen (31). Further, utilizing a series of conditional Notch ligand knockout mice, Fasnacht *et al.* meticulously showed that DLL4-expressing stromal cells, but not hematopoietic cells, provide a critical niche for T_{fh} differentiation in lymph nodes. By using *Cc119*-Cre driver mice to target fibroblastic reticular cells (FRCs), they showed that the deletion of DLL4 in FRCs resulted in severe reduction in T_{fh} and GC B cells, with concomitant reduction in NP-specific high affinity antibody generation after immunization, which was not compensated by the presence of DLL1 (32).

In addition to T helper subsets, genetic studies support a role of Notch2 activation in cytotoxic CD8⁺ T effector cells via direct regulation of Eomesodermin (Eomes), granzyme B and Perforin expression (14, 33). Finally, activated T cells may become memory or terminally differentiated effectors T cells (TEC), which are mostly short-lived. Notch signaling regulates, in part, key transcription genes for the commitment of CD8⁺ T cells to TEC while suppressing the memory fate. In models of infection such as influenza and *Listeria*, combined deficiency of Notch1 and 2 or RBPJ in CD8 T cells led to reduced TEC differentiation, compromising infection clearance in those settings (34, 35). Overall, Notch signaling is capable of fine-tuning and modulating the fate of T lymphocytes.

Innate immune cells—More recently, multiple lines of evidence showed that Notch is involved in dendritic cell (DC) development and activation. Splenic DCs consists of plasmacytoid DCs and other subsets of conventional DCs defined by expression of CD8 and CD11b. In mice lacking Notch2 specifically in DCs, CD8⁺CD11b⁺ endothelial cell-selective adhesion molecule (ESAM)⁺ DCs were missing in the spleen, without changes in other conventional or plasmacytoid DC subsets (36–38). This DC subset resides in the marginal zone in close contact to stromal cells expressing DLL1. Fasnacht *et al.* thoroughly showed that DLL1⁺, but not DLL4⁺, expressing stromal cells are indispensable for ESAM⁺DC development, using *Cc119*-specific DLL1 knockout as well as reverse chimera animals reconstituted with DLL1-deficient cells (32). In intestines, Notch2 deficiency in DCs leads to lack of CD103⁺CD11b⁺ migratory DCs, thereby decreasing the priming especially of Th17 cells (37). In summary, Notch2 plays a tissue-specific role in DC homeostasis.

Innate lymphoid cells (ILCs) are a group of hematopoietic effector cells identified relatively recently and involved in innate immune responses to clear infections or tissue injury. They have emerged as modulators of conditioning-induced tissue damage and of the development of GVHD in the context of hematopoietic stem cell transplantation as discussed below. There is little data in regards to ILCs and solid organ transplantation. They are subdivided into three groups (ILC1, ILC2 and ILC3) that parallel the T helper cell subsets of Th1, Th2

and Th17, respectively (39). Recently, more data highlighted the importance of Notch signaling in ILCs development and differentiation (10). Studies showed that Notch signaling are necessary in early development of ILC3s (40), including lymphoid tissue-inducer (LTi) cells (41) and IL-22-producing ILC3 cells (42) through the regulation of ROR γ t expression. Furthermore, DLL1 was shown to support plasticity of NCR⁻ILC3 and NCR⁺ILC3 in conjunction with TGF- β signaling (43, 44). In another study, ROR γ t⁺ ILCs near the marginal zone were shown to stimulate marginal zone B cells via production of B cell-activation factor (BAFF), co-stimulatory receptor ligand CD40L and DLL1 (45), providing an evidence of crosstalk between innate and adaptive immune system. Lastly, T-cell specific Notch1/2 deletion as well as FRC-specific (*Cc119*-Cre) DLL1 or DLL4 deletion led to severe reduction, both in number and in function, of hepatic invariant NKT (iNKT) cells, CD8 $\alpha\alpha$ TCR $\alpha\beta$ small intestinal intraepithelial lymphocytes and innate memory phenotype CD8 T cells, providing another line of evidence that Notch signaling is indispensable for innate-like T cells differentiation and function (46).

In summary, Notch receptor-ligand interactions modulate T cell, DC and ILC differentiation and function via multiple downstream signaling pathways in a context-dependent manner.

Notch and immune regulation of the alloimmune response

The Notch pathway not only plays a critical role in the physiological differentiation of immune cells but also is highly involved in pathologic processes. Here we review recent advances that were made by gene targeting and antibody-mediated approaches in understanding how Notch signaling plays a significant role in alloimmunity. In addition, we also discuss the contribution of Notch signaling to graft-versus-host disease (GVHD), which carries significant similarity in mechanisms of allorecognition and allograft injury to solid organ transplants.

Notch and solid organ transplantation

A series of early studies using Notch ligand overexpression models initially showed that the Notch signaling pathway could induce immune suppression or antigen-specific tolerance. For example, exogenously overexpressed DLL1 together with MHC class I or II in a fibroblast L cell line, used as adoptively transferred “artificial” antigen presenting cells (APCs), attenuated APC-T cell interactions and blunted alloimmune responses via decreased IFN- γ production and enhanced IL-10 production by splenic CD8⁺ T cells in a MHC-mismatched cardiac transplant model (47). More recently, adoptive transfer of an antigen-pulsed Jagged1-overexpressing dendritic cell line DC2.4 led to antigen-specific hyporesponsiveness and achieved long-term tolerance in a murine cardiac transplant model when combined with CD40L administration (48). However, these overexpression models raised the concern as to whether supra-physiological levels of Notch ligands cause uncontrolled activation of Notch signaling that may not provide accurate information about the role of endogenous Notch signals in alloreactivity.

Instead of the gain-of-function approaches described above, the role of Notch signaling in solid organ transplant was further explored with the use of blocking antibodies to specific Notch receptors or ligands. Our group first demonstrated that blockade of DLL1 by

monoclonal antibody delayed allograft rejection in a fully MHC-mismatched cardiac transplant model (49). DLL1-blocking antibody treatment in CD28-deficient animals or in combination with CTLA4-Ig, resulted in decreased proinflammatory cytokine production, such as IL-2, IFN- γ , IL-6 and IL-17, while increased Th2 cytokines (IL-4 and IL-5) in a Stat6-dependent manner. Similarly, an activating monoclonal antibody against Jagged2 precipitated rejection both in a MHC-fully mismatched cardiac transplant model in CD28-deficient recipients and in a MHC-class II mismatched cardiac transplant model. This accelerated rejection was associated with upregulated IL-6 production and Th2-skewing (50). Although these studies elucidated significant roles of Notch signaling in allograft rejection, systemic treatment with monoclonal antibodies did not provide a clear answer on which specific cells were involved in the process. To answer the question more specifically, Wood *et al.* took advantage of a cell-specific pan-Notch signaling inhibition model of dominant negative Mastermind-like 1 (DNMAML) (51). By utilizing pan-T cell DNMAML mice (15), they showed that pan-Notch inhibition led to prolonged cardiac allograft survival. DNMAML recipient mice revealed less graft-infiltrating cells and their effector T cells produced less IFN- γ *ex vivo*. Furthermore, systemic treatment with anti-DLL1 or anti-DLL4 antibodies alone or in combination significantly improved allograft survival and was associated with decreased IFN- γ , perforin and granzyme B expression in effector T cells as well as with greater infiltration of Foxp3⁺ regulatory T cells in the allograft. These results indicated that Notch blockade in T cells could be a potential effective therapy to prevent cell-mediated rejection. Interestingly, combination treatment with anti-DLL1 and anti-DLL4 antibodies in the setting of CD8 depletion drastically blunted allospecific antibody production long term with lower plasma cells and germinal center reactions in the spleen. Although it is still unclear which cells are responsible for DLL1/4 blockade-mediated alloantibody inhibition (i.e. subsets of B cells, plasma cells, or Tfh cells), further investigations might elucidate interference with Notch receptor-ligand interactions as another intriguing strategy to prevent alloantibody-mediated rejection.

Notch and Graft-versus-host disease (GVHD)

Graft-versus-host disease (GVHD) is a potentially fatal complication of hematopoietic stem cell transplantation (HSCT) caused by allogeneic donor T cells attacking host tissues, GVHD remains a major barrier to the success of HSCT. The pathophysiology of GVHD has been studied extensively, and the involvement of multiple T cell subsets as well as the association of disease severity with the degree of tissue damage due to the pre-transplant conditioning have been a major focus of investigation (52).

Seminal studies by Maillard's group uncovered the importance of Notch signaling in GVHD. First, Zhang *et al.* identified a critical role of Notch in allogeneic CD4⁺ cells in GVHD using the DNMAML pan-T cell Notch inhibition model (16). DNMAML alloreactive CD4⁺ T cells produced less IFN- γ and IL-17 and showed enhanced expansion of Tregs, resulting in less severe GVHD. Importantly, DNMAML T cells retained cytotoxic and proliferative functions, as well as graft-versus-leukemia (GVL) activity, which is imperative for the success of clinical applications of this strategy. Furthermore, using the same model, Sandy *et al.* demonstrated that DNMAML CD8⁺ cells also induced less GVHD while still maintaining their cytotoxic potential against tumors. Mechanistically, both CD4⁺

and CD8⁺ DNAM1 T cells revealed blunted Ras/MAPK signaling, leading to hyporesponsiveness to alloantigen (53). Using antibody-mediated approaches, Tran *et al.* demonstrated that DLL1/4-mediated signaling through Notch1 was the dominant pathway for Notch signaling activation in alloreactive T cells (54). Interestingly, in contrast to the severe intestinal toxicity with treatment of combination of Notch1/Notch2 inhibition by antibody administration, combination blockade of DLL1/4 did not reveal such toxicity and still achieved reduced Notch-mediated IL-2 and IFN- γ production with concomitant enhanced Treg expansion, similar to the effect observed in T-cell pan-Notch inhibition by DNAM1. And again, DLL1/4 double blockade retained GVL effects and preserved hematopoietic recovery after allogeneic HSCT.

More recently, Chatila's group demonstrated that Notch negatively regulates Treg numbers and function *in vivo* using Treg cell lineage-specific Notch1-or RBPJ-knockout mice. The adoptive transfer of Notch1-deficient Tregs was able to ameliorate GVHD, suggesting specific inhibition of Notch1 in Treg may have immune regulatory benefits (30). However, the relative importance of Notch signaling in conventional and regulatory T cells during GVHD remains to be fully investigated.

In summary, Notch signaling plays a critical role in promoting GVHD, and blocking Notch signaling result in hyporesponsiveness of effector T cells and enhanced regulatory T cells both quantitatively and qualitatively, while preserving GVL effects.

In addition to T cells, dendritic cells (DCs) recently emerged as relevant cell populations for the GVH-related response. Zhang's group identified host-type DLL4-high inflammatory DCs (iDCs) as a key player to elicit alloreactive T cell response in the very early phase after allo-HSCT (55). These iDCs carry a phenotype of CD11c⁺B220⁺PDCA1⁺, and express higher levels of costimulatory molecules (CD80, CD86 and CD40). Functionally, they are more capable of inducing IFN- γ and IL-17 in alloreactive T cells when co-cultured. Further, the same group utilized this population to manipulate alloreactive T cell functions. First, murine bone marrow was cultured with Flt3 ligand followed by stimulation with lipopolysaccharide (LPS) and R848 (TLR 7/8 agonist) to induce DLL4-hi iDCs. Donor naïve T cells were then primed with DLL4-hi iDCs *in vitro*, resulting in IFN- γ and IL-17 producing effector T cells. When these DLL4-hi iDC-primed T cells were transferred at the time of allo-HSCT, mice that received these cells developed minimal GVHD, while mice receiving naïve donor T cells developed lethal GVHD (56). Although these findings are interesting, this study did not assess if Notch signaling specifically was the responsible pathway for the outcome rather than other signaling pathways.

More recently, critical roles of ILCs in GVHD have started to be recognized (57). Especially ILC3s (58) and their production of IL-22 (59) play significant roles in maintaining intestinal epithelial stem cells after condition-induced tissue damage, reducing the severity of GVHDs in acute myeloid leukemia patients (60) and in a murine GVHD model (61). In addition, more data suggests that ILCs modulate adaptive immunity (62). Direct evidence indicating involvement of Notch signaling in maintenance of ILC3 in GVHD has not been described yet, but this might be an intriguing target for intervention.

Altogether, Notch signaling modulates alloimmune response in multiple cell types, including effector and regulatory T cells, DCs and presumably ILCs, suggesting Notch blockade as a potential clinical therapeutic target to prevent allograft rejection or GVHD (Figure 2).

Therapeutic approach via Notch to modulate the immune response

Notch receptor-ligand interactions play a key role in immune cell differentiation and in the pathophysiology of allograft injury and GVHD, creating the possibility of targeting Notch for therapeutic approaches (63).

There are several potential intervention strategies to target the Notch signaling pathway, including: (a) blockade of ligand-receptor interaction, (b) blockade of receptor cleavage such as with gamma secretase inhibitors, and (c) inhibition of NICD-transcriptional complex formation. Several trials with different strategies above are ongoing especially in the oncology field (63). Gamma secretase inhibition (b) has been tried in animal models, for example dibenzazepine, leading to profound toxic adverse effect in gastrointestinal system (54). Studies using blocking antibodies against Notch receptors (64) or ligands (65) have led to promising results in diverse preclinical models, including transplant models (predominant driven by Th1 responses), asthma models (a Th2-mediated pathologic process) (66, 67), and experimental autoimmune encephalomyelitis (EAE), a disease model of multiple sclerosis with both Th1 and Th17 response (68, 69). However, given the nature of highly cell-type specific and context-dependent Notch signaling with distinctive combinations of receptor/ligands, targeting certain ligand(s) or receptor(s) might be challenging since it must take into account the immune setting and the various potential cell targets. A next therapeutic approach would be a combination of tissue-specific delivery of drugs and bi-specific targeting of Notch receptor/ligand pair in a cell specific- and context-dependent manner. Further investigation of this approach in disease models is awaited.

Concluding remarks

Notch receptor-ligand interactions play a critical role in immune cell development and differentiation, but they also significantly contribute to pathological processes in allograft injury and GVHD. Further understanding of Notch's immune modulatory mechanisms may lead to the development of promising targeted therapies in transplantation and other immune-related diseases.

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Figure 1.

Notch signaling on T cells. Antigen-presenting cells provide multiple signals required for full T cell activation, including MHC-peptide complex (signal 1), costimulatory/coinhibitory signals (signal 2) and signal 3 that includes cytokines and ligand-receptor interactions such as Notch signaling. Notch signaling is initiated by ligand binding to the receptor, which subsequently undergoes two successive proteolytic cleavages, leading to the intracellular release of Notch intracellular domain (NICD). Once NICD is released, it may either combine with RBPJ and other coactivators such as MAML1-3 (A) and induce transcriptional expression of downstream target genes (canonical pathway), or it may bind to NF- κ B (B) in a non-RBPJ dependent manner (non-canonical pathways). The ultimate fate of the T cell will depend on the combination of these signals with Notch playing a critical role on this process. For simplification, different Notch ligands and receptors as well as additional cofactors and binding partners of NICD were not depicted. MHC, major histocompatibility complex; TCR, T cell receptor; RBPJ, recombination signal binding protein for immunoglobulin kappa j region, MAML, mastermind-like; NF- κ B, nuclear factor kappa B.

Figure 2.

Notch signaling in T cells, dendritic cells and innate lymphoid cells differentiation in the context of transplantation. Notch receptor-ligand interaction promotes effector T cell differentiation, together with the fate-determining transcription factors and cytokines network. For example, Th1 differentiation is regulated by the master transcription factor T-bet, cytokine signaling via IL-12 and Notch ligand DLL1. In addition, Notch signaling enhances dendritic cells (DC) differentiation in tissue-dependent manner, such as CD103⁺CD11b⁺ DC in intestine lamina propria and CD8-ESAMhi DC in spleen. Overall, activated Notch signaling drives toward rejection/organ injury (bottom right), while inhibition leads to tolerance. Th1, T helper 1; Th2, T helper 2; Th17, T helper 17; Treg, regulatory T; Tfh, follicular helper T; DC, dendritic cells; ESAM, endothelial cell-selective adhesion molecule; ILC, innate lymphoid cells.