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Notch signaling in hematopoietic cell transplantation and T cell alloimmunity

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Abstract

Notch signaling can regulate both hematopoietic progenitors and alloimmune T cells in the setting of allogeneic bone marrow or hematopoietic cell transplantation (allo-HCT). Ex vivo culture of multipotent blood progenitors with immobilized Delta-like ligands induces supraphysiological Notch signals and can markedly enhance progenitor expansion. Infusion of Notch-expanded progenitors shortened myelosuppression in preclinical and early clinical studies, while accelerating T cell reconstitution in preclinical models. Notch also plays an essential role in vivo to regulate pathogenic alloimmune T cells that mediate graft-versus-host disease (GVHD), the most severe complication of allo-HCT. In mouse allo-HCT models, Notch inhibition in donor-derived T cells or transient blockade of Delta-like ligands after transplantation profoundly decreased GVHD incidence and severity, without causing global immunosuppression. These findings identify Notch in T cells as an attractive therapeutic target to control GVHD. In this review, we discuss these contrasting functions of Notch signaling with high translational significance in allo-HCT patients.

Keywords

Notch; Notch ligands; bone marrow transplantation; hematopoietic stem cells; T cells; graft-versus-host disease

Introduction

Allogeneic bone marrow or hematopoietic cell transplantation (allo-HCT) is a critically important and potentially curative therapy for many patients with hematological diseases.[1, 2] In the absence of underlying cancer, allo-HCT provides a source of healthy progenitors to

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replace failing or diseased cells (e.g. in bone marrow failure syndromes, congenital immunodeficiencies and hemoglobinopathies). However, the majority of allo-HCT procedures are performed for patients with leukemias, lymphomas and other clonal hematological disorders. In these cases, the allogeneic graft provides T cells and other immune cells that play major therapeutic roles through recognition and elimination of cancer cells in the host (graft-versus-tumor, or GVT, effect).[3–5] Unfortunately, donor-derived T cells also lead to immune-mediated damage in normal host tissues, a life-threatening complication referred to as graft-versus-host disease (GVHD).[6–8]

Multiple shortcomings limit the success and broader applicability of allo-HCT: absence or insufficient numbers of adequately matched progenitors in some patients; prolonged myelosuppression and lymphopenia after transplantation; high morbidity and mortality associated with GVHD; and insufficient graft-versus-tumor effects leading to post-transplant relapse.[2] Progress in the field requires creative new solutions to these problems. Interestingly, the Notch signaling pathway was recently identified as a target for intervention to mitigate several complications of allo-HCT. Both ex vivo and in vivo observations have been reported, reflecting diverse effects of Notch signaling, different target cells (hematopoietic progenitors vs. T cells) and contrasting interventions (induction vs. blockade of Notch signaling). In this paper, we review emerging work describing important effects of Notch signaling in allo-HCT with a focus on potential translational impact in patients.

Use and limitations of allogeneic hematopoietic cell transplantation

The devastating health effects of radiation exposure from nuclear warfare in World War II prompted pioneering studies and ultimately the first bone marrow transplantations, which were of limited benefit.[1] Intense subsequent clinical and laboratory research improved success rates and made allo-HCT available to an expanding number of patients. Recent estimates indicate that ca. 25’000 allo-HCT procedures are being performed annually worldwide. Multiple advances contributed to this success, including progress in HLA matching and donor selection, improved donor registries, access to alternative sources of hematopoietic progenitors such as cord blood, better conditioning regimen and supportive care, as well as systematic use of prophylactic immunosuppression to control GVHD.

Despite advances in transplant care, several major problems limit the safety and effectiveness of allo-HCT. First, a sizable subset of patients lacks a related or unrelated donor with a sufficiently high degree of HLA matching.[9, 10] This problem affects ethnic minorities to a disproportionate extent. In these cases, cord blood transplantation (CBT) can be considered as an alternative approach, as a higher degree of HLA mismatch can be tolerated with this source of hematopoietic progenitors and T cells.[11] However, a significant limitation of CBT especially for adult recipients is the low progenitor content of cord blood grafts. Low progenitor numbers typically lead to delayed engraftment with prolonged myelosuppression and an increased risk of serious infections. Slow lymphoid reconstitution is also particularly prevalent and severe after CBT. The first historic use of Notch signaling in allo-HCT addresses these significant issues via enhanced ex vivo expansion of cord blood progenitors.[12]

As a second major problem, acute and chronic GVHD remains a source of high morbidity and mortality after allo-HCT.[6, 7] Current strategies to prevent GVHD rely either on T cell depletion from the donor inoculum, or on global immunosuppression (typically with calcineurin inhibitors such as cyclosporin A or tacrolimus, plus other agents).[13] However, severe acute GVHD still occurs in a high proportion of patients (up to 50% or even more depending on donor/recipient characteristics, conditioning and GVHD prophylaxis). Patients with severe acute GVHD are treated with steroids, but only about half demonstrate a

sustained response. Allo-HCT recipients with steroid-refractory acute GVHD have unacceptably high mortality (>70%).[14] Furthermore, T cell depletion and global immunosuppression increase the risk of opportunistic infections and also decrease the potency of graft-versus-tumor activity.[4, 7] This problem is best illustrated by studies of T cell depletion as a preventative approach for GVHD: improved GVHD control was counterbalanced by a markedly increased risk of tumor relapse, so that overall patient outcome was not improved.[15–17] Finally, chronic GVHD represents a major unmet clinical need, as all current treatment strategies perform poorly in this condition.[18] Altogether, the field would benefit from novel interventions that control GVHD without causing global immunosuppression and without eliminating potent GVT activity. Notch inhibition in T cells is emerging as an attractive new strategy to achieve these goals.[19–22]

Overview of Notch signaling

Notch is a highly conserved intercellular communication pathway with important functions in health and disease.[23, 24] In mammalian organisms, four Notch receptor genes have been identified (*Notch1-4*) (Fig. 1). Notch1-4 receptors are expressed as transmembrane proteins after constitutive cleavage at the S1 site during transport through the Golgi complex. Notch receptors interact with ligands of the Delta-like (Dll1, 3, 4) or Jagged family (Jag1, 2) on adjacent cells. Ligand-receptor interaction generates a physical force that displaces a negative regulatory region of the Notch receptor and opens access to proteolysis at the S2 site by an ADAM-family metalloprotease.[25–27] S2 cleavage generates an unstable intermediate that becomes a substrate for intramembrane proteolysis by the γ-secretase complex (S3 cleavage), releasing intracellular Notch (ICN).[28] ICN migrates into the nucleus where it interacts with the CSL (CBF-1, Su(H), Lag-1) transcription factor.[29] In turn, ICN and CSL recruit a key transcriptional coactivator of the Mastermind-like (MAML) family that nucleates assembly of a large transcriptional activation complex to mediate target gene activation.[30–32] The *Hairy/enhancer-of-split* (*Hes*) gene family encodes recurrent direct transcriptional targets of Notch signaling, although many other targets have been reported or remain to be identified.[23]

The biochemical features of Notch activation have been reviewed in detail elsewhere.[23] Our increasing understanding of the pathway has set the stage for multiple interventions to activate or inhibit Notch signaling, both experimentally and in clinical studies (Fig. 1). Unlike soluble ligands, plate-bound or cell-bound Notch ligands can induce high levels of Notch activation in cultured cells (e.g. hematopoietic progenitors).[33, 34] Neutralizing monoclonal antibodies were developed to target Delta-like Notch ligands and prevent their productive interaction with Notch receptors.[20, 21, 35] Other antibodies block Notch activation by preventing S2 cleavage after ligand binding.[20, 36] Originally developed for their activity in Alzheimer's disease, γ-secretase inhibitors block the rate-limiting step of intramembrane proteolysis during Notch activation, leading to pan-Notch inhibition.[37] Finally, genetic approaches have been instrumental to capture the effects of Notch signaling mediated by the ICN-CSL-MAML complex downstream of all Notch receptors and ligands. This can be achieved either by genetic inactivation of *Rbpj* (encoding CSL) or by expression of a dominant negative form of Mastermind-like1 (DNMAML) in specific cell types.[38–42]

Notch signaling is involved in multiple aspects of organ development, with additional functions during tissue homeostasis in adults. We will focus here on the effects of Notch in hematopoiesis and the immune system that are relevant to allo-HCT. Regarding the important role of Notch as an oncogene or tumor suppressor in an expanding range of malignancies, we refer the reader to several recent comprehensive reviews.[43–45]

Notch signaling in hematopoiesis and immunity

In the hematopoietic system, an essential role for Notch signaling was first recognized at early stages of T cell development in the thymus.[46] Rare bone marrow-derived progenitors seeding the thymus experience a high intensity of Notch signaling after exposure to Dll4 Notch ligands expressed by the thymic epithelium.[47, 48] In the absence of Notch signaling, T cell development is arrested at a very early stage, while cells differentiating along alternative lineages accumulate in the thymus.[49–51] Notch is required continuously until T cell progenitors successfully clear the pre-T cell receptor or β selection checkpoint.[42, 52–54] Multiple mechanisms then actively inhibit Notch signaling, so that $CD4^+CD8^+$ double positive (DP) thymocytes experience little, if any Notch signals during positive and negative selection. Due to this careful regulation of signaling intensity, Notch blockade in DP thymocytes does not interfere with T cell development.[41,55, 56] In contrast to DP thymocytes, mature $CD4^+$ and $CD8^+$ T cells regain the ability to respond to Notch signaling during antigen-mediated immune responses in secondary lymphoid tissues. Emerging data highlight multiple context-dependent Notch functions in peripheral T cell immunity.[57–59] These effects will be discussed in detail below in the regulation of T cell alloimmunity after allo-HCT.

Besides the role of Notch1 in T cell development, Notch2-mediated signals control the homeostasis of splenic marginal zone B cells and $ESAM^{hi}$ myeloid dendritic cells.[60, 61] Other developmental functions of Notch signaling continue to be reported, such as the requirement for Notch to generate subsets of innate lymphoid cells (ILCs).[59] Given this multiplicity of functions, cell-specific Notch inhibition strategies have been essential to dissect the effects of Notch in the hematopoietic system.

In addition to lineage-specific effects of the pathway, much attention has been devoted to the putative role of Notch signaling in hematopoietic stem cells and multipotent progenitors. Work from several groups showed that in vitro exposure of mouse or human hematopoietic stem and progenitor cells to a high density of Notch ligands can vastly expand progenitor numbers, especially when Notch signaling intensity and concomitant cytokine use are optimized.[12, 34, 62–70] Progenitor expansion was also reported upon coculture with immortalized endothelial cells expressing endogenous Notch ligands.[69] These findings have been exploited to achieve expansion of cord blood progenitors in human patients and will be discussed in detail below for their high relevance to allo-HCT (Table 1).

In contrast to these gain-of-function studies, the overall physiological impact of Notch signaling at the apex of the hematopoietic hierarchy in vivo remains controversial. During fetal life, Notch1 is essential for the emergence of definitive hematopoietic stem cells from specialized hemogenic endothelium.[71] However, most studies using well-validated methods of Notch inhibition failed to identify a role for canonical Notch signaling in the maintenance of adult hematopoietic stem cells in vivo.[70, 72–74] This was true in steady-state conditions and upon transplantation, although recent data from the Bernstein group revealed an effect of Notch2 on the initial speed of reconstitution after transplantation.[70] Other laboratories have reported an inhibitory function for Notch signaling on myeloid cell fate in multipotent progenitors, downstream of hematopoietic stem cells.[75] The nature of the Notch ligands mediating these effects is not known. However, as compared to Notch activity in early T cell progenitors, the overall intensity of Notch signaling remains low in multipotent bone marrow progenitors.[73, 76] Expression of the Dll4 ligand is actively suppressed in the bone marrow environment, suggesting that other ligands might be involved.[77] Open questions include how findings in the mouse apply to human progenitors and whether non-canonical Notch signals that do not require CSL and MAML could be important.

Notch ligand-mediated expansion of multipotent hematopoietic progenitors

Pioneering studies from Bernstein's group first described Notch1/2 receptor expression in human and mouse hematopoietic progenitors.[62, 78] These observations triggered a string of studies exploring the functional effects of engaging Notch receptors in ex vivo cultures, followed by in vitro and in vivo readouts of progenitor function (Fig. 2, Table 1). Co-culture of murine Lin^{-} $Sca\text{-}1^{hi}c\text{-}Kit^{hi}$ (LSK) hematopoietic stem and progenitor cells with cytokines and 3T3 cells expressing the Notch ligand Jagged1 led to a 4–8-fold expansion in clonogenic progenitors over cells cultured with cytokines and parental 3T3 cells.[62] Although the degree of expansion remained modest in these conditions, this was the first demonstration that inducing Notch signals could increase the numbers of primitive progenitors. Bhatia's group exposed human cord blood $CD34^{+}$ cells to a soluble recombinant Jagged1-IgG1 fusion protein. Modest in vitro $CD34^{+}$ cells expansion was observed, but importantly leading to enhanced engraftment in NOD/SCID mice.[63] Similar findings were reported with Dll1 but not Dll4 fusion proteins.[64]

Notch-mediated progenitor expansion was perfected by the demonstration that immobilization of Dll1 ligands was necessary to efficiently induce Notch signaling.[33, 34] When combined with optimized cytokine cocktails, Notch induction led to multi-log expansion of progenitors capable of long-term multilineage reconstitution in irradiated recipients.[12, 34, 65] Genetic studies showed that *Notch2* but not *Notch1* was essential to mediate the effects of Notch ligands in multipotent hematopoietic progenitors.[70] Another interesting lesson learned was the dose-dependent effects of Notch signals: intermediate doses enhanced expansion of multipotent progenitors, while high Notch signaling intensity promoted T lineage development.[66–68] These considerations are important to target the desired clinical outcome, i.e. accelerated myeloid and overall hematopoietic reconstitution vs. enhanced T cell recovery. Rafii's group described an interesting system using primary human endothelial cells transduced with adenoviral E4ORF1.[69] Coculture of mouse LSK progenitors with these cells induced potent Notch-dependent expansion of progenitors capable of long-term multilineage engraftment. Thus, provision of cell-bound Notch ligands by endothelial cells in this system achieved comparable expansion of primitive hematopoietic progenitors as plate-bound ligands.

Based on these preclinical observations, Delaney and colleagues initiated human clinical trials using Notch-expanded progenitors in allo-HCT recipients.[12] Cord blood transplantation involve administration of two cord blood units to mitigate the impact of low progenitor numbers.[11] Building on this practice, investigators subjected one unit to Dll1-based ex vivo expansion, before reinfusing these cells in tandem with an unmanipulated second unit.[12] Clinical-grade $CD34^{+}$ cells expanded on average 140-fold in culture. In comparison to historical controls receiving two unmanipulated units, the duration of profound neutropenia was reduced from 26 to 16 days in study subjects. The expanded cord generated mature myeloid cells within weeks after transplantation, before being replaced in most patients by cells derived from the unmanipulated cord. These results met the endpoint of abbreviating the period of myelosuppression, likely from short-term progenitors. It remains more difficult to ascertain if the Notch-expanded product also contained cells capable of long-term reconstitution in patients, as seen in preclinical mouse models. Indeed, other work suggests that the dominance of one cord blood unit over the other is immunologically mediated (cord-versus-cord reactivity).[79] As mature lymphocytes do not survive ex vivo culture, Notch-expanded progenitors could have ultimately been rejected by T cells or NK cells from the unmanipulated cord in this study.[12] In any case, the findings provided a landmark observation that the procedure was safe and effective to improve early recovery of allo-HCT patients. Additional work is currently being performed to assess the utility of this approach in an expanding range of clinical allo-HCT situations.

Notch ligand-mediated expansion of T cell progenitors

Notch-based ex vivo culture systems were explored in preclinical models to mitigate the slow recovery of de novo T cell production after transplantation, a major problem following HCT in human patients (Fig. 2, Table 2).[80] In these studies, a high intensity of Delta-like-mediated signals and provision of lymphoid cytokines (e.g. IL-7) led to preferential development and expansion of T lineage cells as opposed to multipotent progenitors. Either plate-bound Notch ligands or cocultures with stromal cells expressing Delta-like ligands were used successfully.[34, 66–68, 81–84] As an overall approach, hematopoietic stem and progenitor cells were allowed to differentiate into the T cell lineage in culture, before being administered to transplant recipients. The bulk of mouse progenitors were infused after reaching the DN2-DN3 stage of T. cell development.[68, 81] These in vitro specified T cell progenitors could complete their T cell development program in vivo, generating functional $CD4^+$ and $CD8^+$ T cells early after transplantation. Interestingly, expanded T lineage progenitors enhanced thymic reconstitution, but also led to extrathymic T cell development, in particular within mesenteric lymph nodes.[81, 84] In aging recipients with compromised thymic function, the contribution of extrathymic development tended to be higher. The existence of extrathymic sites supporting T cell development from primitive progenitors after transplantation had previously been reported.[85–87] Genetic studies showed that extrathymic T cell development was Notch-dependent, although the nature of the Notch ligand(s) involved is unknown.[87] For ex vivo expanded T cell progenitors, it remains to be determined whether and how long they require Notch signaling to complete T cell development in vivo after reinfusion. Moreover, it is currently unknown if extrathymic T cell development happens in humans. This pathway could be relevant to older HCT recipients in whom integrity and function of the thymic epithelium are compromised.[80]

Besides work with mouse cells, human Notch-expanded T cell progenitors have been studied for their capacity to enhance T cell reconstitution in vivo (Table 2).[34, 83] Subsets of human pro-T cells with a $CD34^+CD7^{++}$ phenotype were most efficient at thymic reconstitution in immunodeficient mice.[83] The full clinical potential of this strategy remains to be investigated. Mouse studies have highlighted possible future applications. Infusion of T cell progenitors showed additive effects on T cell reconstitution over administration of keratinocyte growth factor as a trophic factor for the thymic epithelium.[81] Thus, combined interventions could be considered. As compared to administration of mature T cells, pre-T cell infusion had the advantage of ensuring enhanced tolerance to host alloantigens, as final stages of T cell development including negative selection happened in vivo. This was associated with the absence of severe GVHD in allo-HCT models. Nevertheless, anti-tumor activity was still observed for reasons that remain to be fully clarified, perhaps because negative selection to host alloantigens was not fully enforced in transferred T cell progenitors.[81, 82] Alloantigen-based anti-cancer effects remained relatively weak, but could be potentiated by genetic engineering of the T cell progenitors to express a chimeric antigen receptor against CD19. This could represent an interesting platform for treatment with engineered T cells, as off-the-shelf allogeneic progenitors could in principle be used with limited risks of inducing GVHD. It remains to be established whether using allogeneic pre-T cells will have advantages over engineering autologous mature T cells.

Notch and T cell alloimmunity

In addition to its involvement in T cell development, the Notch pathway is increasingly recognized for its context-dependent effects in the regulation of mature T cell function.[57, 58, 88] A new major role for Notch signaling was recently reported in alloreactive T cells mediating GVHD (Table 3).[19–22] To study the effects of Notch in T cell alloimmunity during GVHD, we took genetic and biochemical approaches to block all

Notch signals specifically in donor-derived alloreactive T cells after allo-HCT in mice.[19, 20] We observed efficient protection from acute GVHD in allo-HCT recipients of Notch-deprived T cells, as these mice survived as well as mice receiving only T cell-depleted bone marrow. Protection was observed in several models of major and minor histocompatibility antigen mismatched transplantation. Mechanistically, Notch inhibition blocked the production of multiple inflammatory cytokines by alloreactive T cells, including IFNγ, TNFα, IL-17 and IL-2, while enhancing the accumulation of regulatory T cells. Despite these potent effects on cytokine production, Notch blockade did not cause global immunosuppression, as the expansion of alloreactive T cells was preserved or even enhanced in vivo. Moreover, Notch-deprived T cells retained potent cytotoxic effects against allogeneic targets and were able to eliminate host-type leukemic cells, leading to long-term survival free of leukemia and severe GVHD. Thus, Notch inhibition in T cells had different effects on their individual effector functions and induced a unique pattern of beneficial immunomodulation in mouse allo-HCT models (Fig. 3).

A remarkable consequence of Notch inhibition in alloreactive T cells was its broad impact on multiple T helper $CD4^+$ subsets, suggesting that Notch did not merely control lineage-specific differentiation decisions during GVHD.[19, 22] In addition, Notch blockade induced parallel effects in $CD8^+$ alloreactive T cells, including markedly decreased production of the cytokine IFN γ.[22] Decreased IFNγ production occurred despite preserved expression of the master transcription factors T-bet and Eomesodermin, which regulate Th1 $CD4^+$ and $CD8^+$ effector T cell differentiation. In contrast, Notch-deprived alloreactive T cells acquired a decreased capacity to activate Ras/MAPK and NF-kB signaling after stimulation through the T cell receptor, as well as increased expression of multiple negative regulators of T cell activation. Altogether, Notch blockade induced a hyporesponsive phenotype with features reminiscent of T cell anergy. Interestingly, downstream effector pathways were influenced to a very variable extent by these changes, with profoundly decreased cytokine production but preserved in vivo expansion and cellular cytotoxicity. More work is necessary to identify the direct transcriptional targets of Notch signaling that are ultimately responsible for these effects. Finally, Notch was also reported to play a pathogenic role in the dendritic cell compartment of Ikaros-deficient mice during GVHD.[89] Thus, effects of Notch signaling beyond the T cell compartment must be considered.

Based on its importance in alloimmunity, it is tempting to speculate that Notch might also play an important role in other T cell-mediated disorders, including autoimmunity. Minter's group recently provided an interesting observation that Notch signaling regulates T cell function in immune-mediated aplastic anemia (Table 3).[90] Induction of aplastic anemia in this report was based on a major alloantigen mismatch using parent to F1 bone marrow transplantation. Pharmacological or genetic Notch blockade blunted immune-mediated damage to host hematopoietic progenitors and slowed progression to bone marrow failure. Given the use of alloantigens, these findings share characteristics with findings in GVHD models, with the main target organ being the bone marrow instead of epithelial organs. However, T cells isolated from human patients with aplastic anemia also showed evidence of Notch activation driving expression of *Tbx21*, *Ifng* and *GzmB*.[90] These findings suggest that Notch inhibition could ameliorate bone marrow damage by dampening autoreactive T cells in immune-mediated aplastic anemia, in the presence of autoantigens rather than alloantigens as drivers of the immune response.

Blockade of individual Notch ligands and receptors controls graft-versus-host disease

Initial observations about the role of Notch in T cell alloimmunity were based on genetic strategies, but pharmacological approaches are needed to harness the therapeutic potential of

Notch inhibition in GVHD. To this end, we first studied the effects of γ-secretase inhibitors (GSIs) after mouse allo-HCT. GSIs were effective at targeting Notch signaling in T cells, however systemic Notch blockade was poorly tolerated immediately after total body irradiation and allo-HCT due to major intestinal side effects.[20] Notch was previously reported to regulate the differentiation of intestinal progenitors into absorptive vs. secretory lineages.[91, 92] In addition to these effects, pan-Notch blockade after allo-HCT revealed a role of Notch in intestinal regeneration.[20] GSIs might be useful in other contexts, as shown for example by Minter's group in their model of aplastic anemia.[90] However, more selective targeting of Notch signaling appears a better option immediately after HCT.

To overcome the limitations of systemic Notch blockade, we investigated the role of individual Notch receptors and ligands in T cell alloimmunity. Among the four mammalian Notch receptors and five ligands, Notch1/2 and Delta-like1/4 mediated all the effects of Notch signaling in alloreactive T cells, with a predominant role for Notch1 and Delta-like4.[20] Notch1/Notch2 loss had a similar effect as blockade of CSL/MAML-dependent signaling in the nucleus, indicating that in this context at least the effects of the Notch pathway do not involve "non-canonical" pathway mediated by the Notch receptors independently of CSL/RBP-Jk and MAML. Regarding the role of Notch ligands, Mochizuki et al. also reported a dominant role for Dll4 using a different set of monoclonal antibodies, as well as a potential cellular source of Dll4 in host inflammatory dendritic cells.[21] More work is required to establish how Notch ligand expression is regulated after allo-HCT and to identify all the cellular partners involved in the delivery of Notch signals to incoming alloreactive T cells.

From a therapeutic perspective, the winning strategy to control GVHD turned out to be blockade of Delta-like1/4 ligands in vivo at early time points after allo-BMT (Fig. 3).[20] In contrast to GSIs and systemic Notch1/2 blockade, inhibition of Delta-like ligands preserved intestinal regeneration after allo-HCT, thus opening a therapeutic window in vivo. Interestingly, transient Dll1/4 blockade provided similar protection from GVHD as long-term or even permanent inhibition of Notch signaling in T cells. These observations suggest that incoming alloreactive T cells are rapidly exposed to a pulse of Notch signaling after transplantation, and that blockade of Notch signaling during this critical period can reprogram alloreactive T cells to a less pathogenic phenotype. At least in part, this could be related to the acquisition of permanent or long-lasting epigenetic changes. The effectiveness of short-term inhibition in GVHD has fundamental importance to understand the immunobiological effects of Notch signaling and its interaction with other signaling pathways that regulate alloreactive T cell function (e.g. costimulatory receptors). In addition, it has translational importance, as the use of short-term Notch blockade would mitigate safety concerns related to prolonged Notch inhibition in patients.[43, 75, 93] Additional work is needed to define optimal schedules and intervention strategies to prevent and/or to treat GVHD via Notch-based therapeutics.

Conclusions and perspectives

When reflecting on the initial description of Notch activity in flies by Thomas Hunt Morgan nearly a century ago, it is fascinating to consider how far we have traveled while continuing to discover major functions of Notch in physiology and disease, and while now considering Notch as a target for therapeutic intervention in humans.[94] Our increasingly sophisticated understanding of Notch genetics and biochemistry was derived from basic science investigations, but it is now providing potent experimental tools and potential therapeutic agents in patients.

In allo-HCT, Notch exerts very different effects on hematopoietic progenitors ex vivo and on alloreactive T cells in vivo. Notch-based expansion of multipotent hematopoietic progenitors primarily relies on ex vivo activation of Notch signaling above physiological levels to achieve the desired outcome. In contrast, GVHD prevention would involve transient inhibition of Notch signaling mediated by Delta-like ligands in vivo shortly after transplantation. Thus, these interventions are distinct and not mutually exclusive. Notch ligand-based expansion of hematopoietic progenitors has already progressed into clinical testing with promising early results. So far, pharmacological Notch inhibitors including neutralizing monoclonal antibodies have mostly been considered for therapeutic interventions targeting cancer cells or the tumor microenvironment, and some have been tested in early human clinical trials. Recent results highlight the potential of Notch-based therapeutics in immune diseases and also identify targeting of individual Notch receptors or ligands as a strategy to increase the therapeutic index of Notch inhibition. Although more preclinical studies are warranted, we hope that these findings will lead to carefully designed clinical trials that test the potential of Notch inhibitors in GVHD and in other T cell-mediated disorders.

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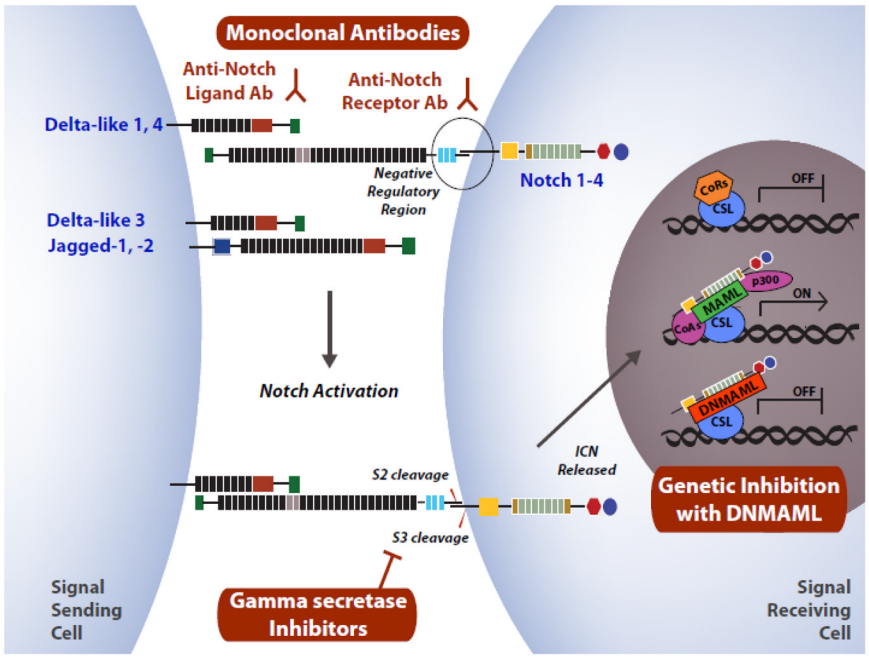

Figure 1. Overview of Notch signaling

Activation of Notch signaling is triggered by the interaction between one of five Notch ligands (Delta-like1, 3, 4; Jagged-1, 2) with one of four mammalian Notch receptors (Notch1-4). Ligand-receptor binding induces a mechanical change in the Notch receptor, displacing the Negative Regulatory Region to allow proteolytic cleavage at the S2 site by an ADAM family metalloprotease. S2 is rapidly followed by S3 cleavage mediated by the γ-secretase complex, releasing the intracellular portion of the Notch receptor (ICN) into the cytoplasm. ICN migrates into the nucleus to assemble a transcriptional activation complex together with the transcription factor CSL (CBF-1/Suppressor of Hairless/LAG-1) and a co-activator of the Mastermind-like family (MAML). During Notch activation, co-repressors (CoRs) are displaced and co-activators (CoAs) recruited, stimulating target gene transcription. Selected strategies of Notch inhibition are highlighted in red: neutralizing monoclonal antibodies against Notch ligands or receptors; pharmacologic inhibition of γ-secretase; and genetic blockade of the Notch transcription activation complex with dominant negative Mastermind-like (DNMAML). Other methods include gene inactivation of *Notch1-4* or *Rbpj* (encoding CSL).

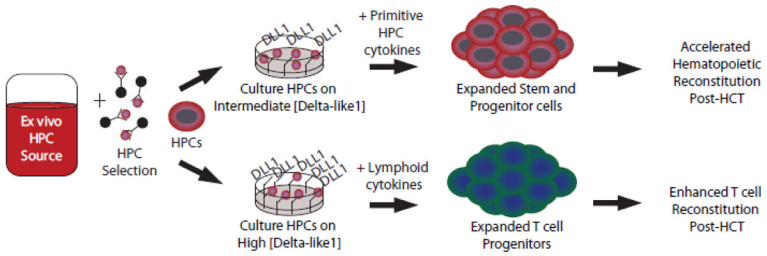

Figure 2. Notch-mediated ex vivo progenitor expansion promotes hematopoietic recovery or T cell reconstitution after hematopoietic cell transplantation

Culture of isolated hematopoietic progenitor cells (HPC) with cytokines and the Notch ligand Delta-like1 (DLL1) results in a multi-log expansion of hematopoietic progenitor cells (intermediate concentration of DLL1, primitive HPC cytokines) or differentiated T cell progenitors (high concentration of DLL1, lymphoid cytokines). Expanded progenitors can then be infused to enhance hematopoietic or lymphoid reconstitution after hematopoietic cell transplantation. To be effective, DLL1 must be provided as an immobilized plate-bound or cell-bound ligand. See Table 1 and 2 for applications in mouse models, xenograft systems and early clinical trials.

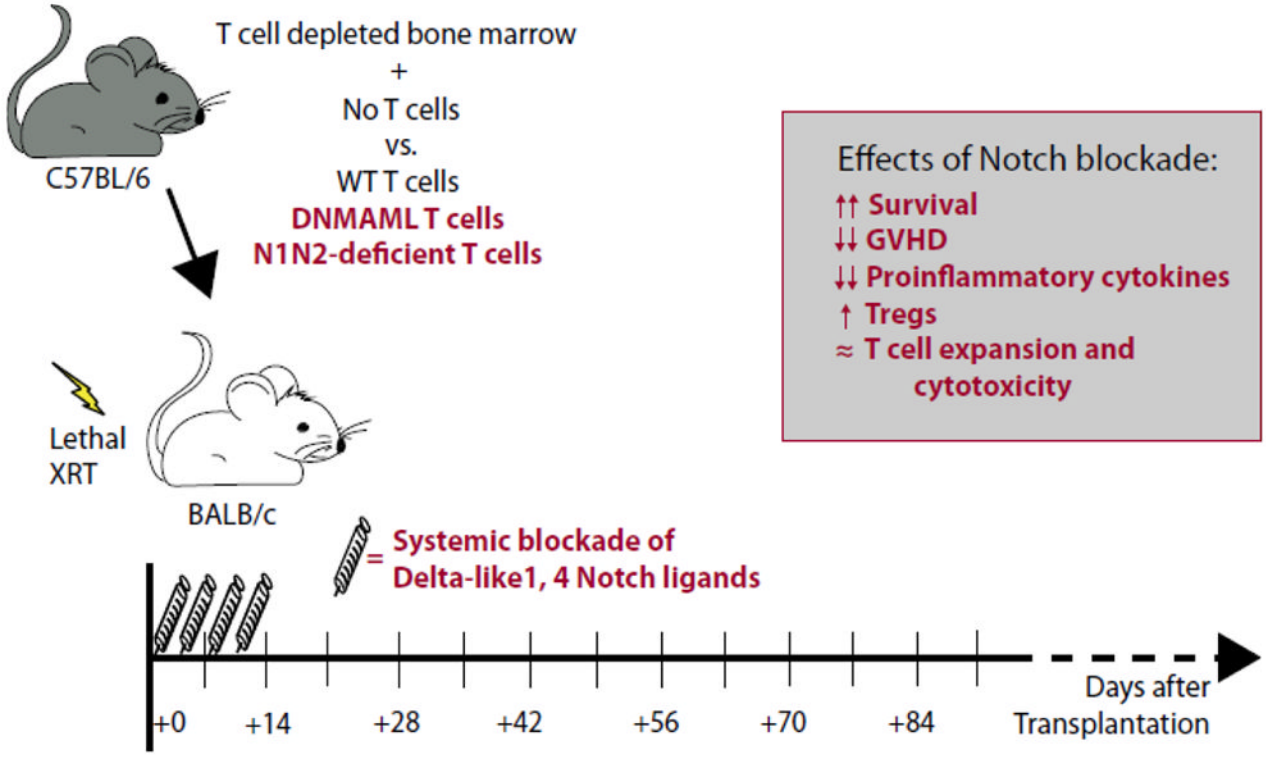

Figure 3. Notch inhibition in donor-derived T cells provides long-lasting protection from graft-versus-host disease

Schematic representation of a MHC-mismatched mouse model of allo-HCT used to identify a major function for Notch signaling in alloreactive T cells during GVHD. Lethally irradiated (8.5–10 Gy) BALB/c mice (H-2^d) are transplanted with C57BL/6 (H-2^b) T cell-depleted bone marrow, with or without C57BL/6 wild-type (WT) T cells, Notch-deprived DNMAML T cells or *Notch1/Notch2* (N1/N2) double-deficient T cells. Alternatively, neutralizing monoclonal antibodies against Notch ligands Delta-like1 and Delta-like4 were administered for a short course (day 0–10 post-transplantation). Genetic inhibition of Notch signaling in T cells or transient Dll1/4 blockade had similar effects, increasing survival and preventing GVHD (box). Protective effects of Notch inhibition were also observed in other GVHD models (see Table 3).

Table 1

Preclinical and early clinical interventions based on ex vivo Notch ligand-mediated expansion of hematopoietic progenitors

After the founding observation by Varnum-Finney et al. (1998), this table lists studies in which multipotent hematopoietic progenitors expanded in the presence of Notch ligands were evaluated functionally in vivo using transplantation assays. Additional studies not meeting these criteria are discussed in the text.

Progenitor source	Notch ligand source	Key Observation(s)	Reference
Mouse BM LSKs	Jag-1 transfected NIH-3T3 fibroblasts hJag-1^{ext} coated beads	Mouse BM LSKs express Notch2>Notch1 BM and fetal liver stromal cells express Jag-1 Jag-1 expands BM HPCs	Varnum-Finney et al., 1998 [62]
Human UCB: $CD34^+CD38^-$ cells	Soluble rhJag-1-IgG_1	Soluble Jag-1 expands HPCs	Karanu et al., 2000 [63]
Human UCB: $CD34^+CD38^-$ cells	Soluble rhDelta-1-IgG_1, Soluble rhDelta-4-IgG_1	Soluble Delta-like1, but not soluble Delta-like4, expands HPCs	Karanu et al., 2001 [64]
Human UCB: $CD34^+CD38^-$ cells	Immobilized Delta-1$^{ext-myc}$	Immobilization of Delta-like1 significantly improves HPC expansion	Ohishi et al., 2002 [34]
Mouse BM LSKs	Immobilized Delta-1$^{ext-IgG}$ Soluble Delta-1$^{ext-IgG}$	Immobilized Delta-like1 and cytokines cooperate to expand HPCs and inhibit differentiation	Varnum-Finney et al., 2003 [65]
Human UCB: $CD34^+CD38^-$ cells	Immobilized Delta-1$^{ext-IgG}$	Lower densities of Delta-like1 promote HPC expansion, higher densities promote T lineage development	Delaney, et al, 2005[66]
Human UCB: $CD34^+$ or $CD133^+$ cells	Immobilized Delta-1-Fc	Expanded $CD133^+$ fraction has better engraftment potential than expanded $CD34^+$ fraction	Suzuki et al., 2006 [95]
Human UCB: $CD34^+$ cells	Immobilized Delta-1$^{ext-IgG}$ OP9-Delta-like1 cells	Synergistic effect of HoxB4 and Delta-like1 on $CD34^+$ cell expansion and engraftment	Watts et al., 2010 [96]
Mouse BM LSKs	Adenoviral E4ORF1- transduced HUVECs	Endothelial cells express Notch ligands and support HPC expansion	Butler et al., 2010 [69]
Human UCB: $CD34^+CD38^-$ cells	Immobilized Delta-1$^{ext-IgG}$	Expanded HPCs are safe and shorten neutropenia after human double UCB allo-HCT	Delaney et al., 2010 [12]

BM, bone marrow; LSK, $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ hematopoietic stem and progenitor cells; Jag-1, Jagged-1; UCB, umbilical cord blood; rh, recombinant human; HPC, hematopoietic progenitor cells; Allo-HCT, allogeneic hematopoietic cell transplantation; HUVECs, Human umbilical vein endothelial cells.

Table 2

Preclinical observations based on ex vivo Notch ligand-mediated expansion of T cell progenitors

This table highlights studies in which Notch-expanded T cell progenitors were infused in vivo to improve immune reconstitution after transplantation. Additional studies not meeting these criteria are discussed in the text.

Cell source	Notch ligand source	Key Observation(s)	Reference
Mouse BM LSKs	OP9-Delta-like1 cells	Delta-like1 expands DN2 and DN3 pre-T cells Expanded pre-T cells increase thymic engraftment, bacterial clearance, and have NK-independent GVT effects	Zakrzewski et al., 2006 [81]
Mouse BM LSKs	Immobilized Delta-1$^{ext-IgG}$	Expansion of DN2 pre-T cells correlates with Delta-like1 density Expanded pre-T cells engraft the thymus and produce mature T cells	Dallas et al., 2007 [68]
Mouse BM LSKs	OP9-Delta-like1 cells	Expanded pre-T cells induce GVT, but not GVHD, and have potential for anti-tumor engineering	Zakrzewski et al., 2008 [82]
Human UCB: CD34^{+} cells	OP9-Delta-like1 cells	Expanded pre-T cells engraft the thymus and produce mature T cells	Awong et al., 2009 [83]
Mouse BM LSKs	OP9-Delta-like1 cells	Expanded pre-T cells engraft extra-thymically, producing mature T cells	Holland et al., 2012 [84]

BM, bone marrow; LSK, Lin^{-}Sca-1^{+}c-Kit^{+} hematopoietic stem and progenitor cells; GVT, graft-versus-tumor; GVHD, graft-versus-host disease; Ag, antigen; UCB, umbilical cord blood.

Table 3

In vivo Notch inhibition in alloreactive T cells controls GVHD and immune-mediated bone marrow failure

This table lists studies that investigated the effects of Notch inhibition in mature donor-derived T cells after allo-HCT, using GVHD or immune-mediated bone marrow failure as readouts of target organ damage.

Mouse model	Method of Notch blockade	Proposed cellular mechanisms	Reference
B6→BALB/c B6→B6 x DBA/2 F1 B6→BALB/b	*ROSA26*^{DNMAML1f} x *Cd4-Cre* *Rbpj*$^{f/f}$ x *Cd4-Cre*	Decreased pro-inflammatory cytokines Increased Tregs Decreased alloreactive T cells in the gut	Zhang et al., 2011 [19]
B6→BALB/c	*ROSA26*^{DNMAML1f} x *Cd4-Cre* *Notch1*$^{f/f}$*Notch2*$^{f/f}$ x *Cd4-Cre* γ-secretase inhibitor Anti-Notch1 Ab Anti-Notch2 Ab Anti-Dll1 Ab Anti-Dll4 Ab	Decreased pro-inflammatory cytokines Increased Tregs	Tran et al., 2013 [20]
B6→BALB/c B6→B6 x DBA/2 F1	Anti-Dll1 Ab Anti-Dll4 Ab	Decreased pro-inflammatory cytokines	Mochizuki et al., 2013 [21]
B6→BALB/c B6→BALB/b	*ROSA26*^{DNMAML1f} x *Cd4-Cre* *Rbpj*$^{f/f}$ x *Cd4-Cre*	Acquisition of T cell hyporesponsiveness Decreased pro-inflammatory cytokines Increased Tregs	Sandy et al., 2013 [22]
B6→B6 x BALB/c F1	γ-secretase inhibitor *Notch1*$^{f/f}$ x *Mx-Cre*	Decreased pro-inflammatory cytokines Decreased T-bet and Granzyme B	Roderick et al., *in press* [90]

Ab, antibody; Tregs, regulatory T cells.