

Chapter 7

Notch Signaling in Graft-Versus-Host Disease



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Abstract Graft-versus-host disease (GVHD) refers to a constellation of adverse immune responses resulting in tissue destruction following hematopoietic stem cell or solid organ transplantation. Through a complex network of priming and activation events, immune-competent T cells residing in the transplanted tissue (the graft) become stimulated, migrate into target organs, and mediate immune destruction of the recipient's healthy tissue (the host). Paradoxically, this immune activation can also eradicate residual leukemic cells, when hematopoietic stem cell transplantation occurs in the context of hematological malignancies, resulting in a beneficial graft-versus-leukemia (GVL) effect. The Notch family of transmembrane receptors functions in many aspects of immune responses, including those that mediate GVHD. Here we will review the complex nature of GVHD and how Notch signaling may play a prominent role during the initiation and progression of the disease.

Keywords Notch · Graft-versus-host disease · GVHD · Graft-versus-leukemia · GVL · Immune destruction · Hematopoietic stem cell transplantation · Dll4 · M1 macrophages · Chemokine receptors · Toll-like receptors

7.1 Overview of Notch Signaling

Signaling mediated by Notch receptors is essential to many varied cell processes. Excellent reviews discuss Notch signaling in embryonic tissue and organ development [108], fetal hematopoiesis [13, 104], intestinal cell homeostasis [25], T cell maturation in the thymus [53, 61], and the regulation of numerous immune responses in the periphery [6, 8, 30, 80, 88]. As depicted in Fig. 7.1, in mammals the Notch family of signaling proteins is comprised of four transmembrane receptors (Notch1–4) and five signal-initiating ligands that belong to two distinct groups: Jagged ligands (Jag1,2) and Delta-like ligands (Dll1,3,4). Notch receptors undergo a series

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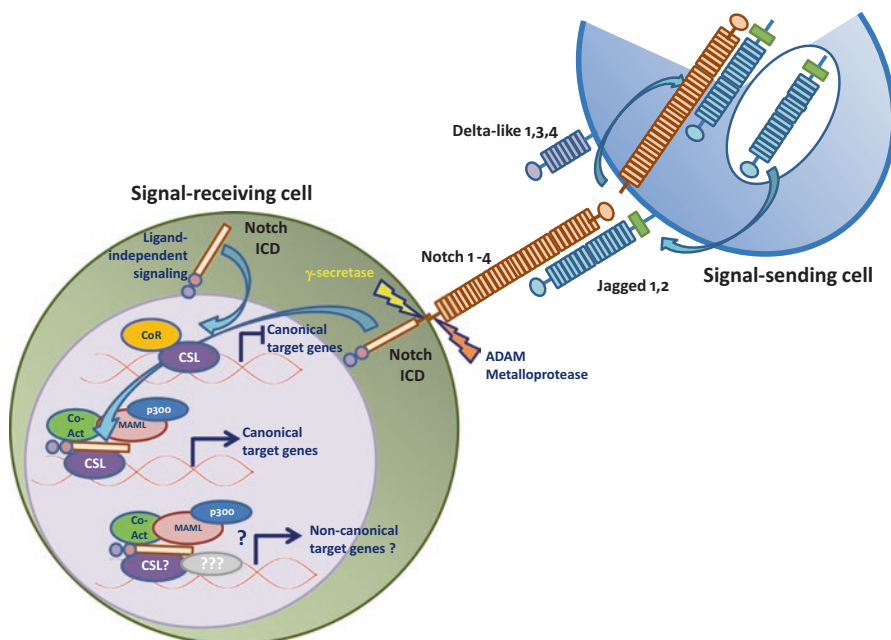


Fig. 7.1 Overview of Notch signaling. Ligand-mediated Notch signaling is initiated when a ligand of the Delta-like (Dll 1,3,4) or Jagged (Jag 1,2) family, present on the surface of a nearby signal-sending cell, binds to a Notch receptor expressed on a signal-receiving cell. Following cleavage of the extracellular domain of Notch by an ADAM protease, the Notch extracellular domain is transendocytosed by the signal-sending cell, together with the bound Notch ligand. Notch ligands can be recycled and re-expressed on the surface of the signal-sending cell. In the signal-receiving cell, following processing by ADAM protease, transmembrane-tethered Notch is cleaved just proximal to the cell membrane to release intracellular domain (ICD) of Notch, which is transcriptionally active. Notch ICD associates with its canonical DNA-binding partner, CSL, which itself resides on DNA in repressor complexes that prohibit transcription of canonical gene targets. Upon association with CSL, additional coactivators including mastermind-like (MAML) and p300 are recruited to the NotchICD-CSL complex, activating its transcriptional capabilities. Transcription of some Notch-regulated genes can proceed in the absence of CSL and is referred to as noncanonical Notch signaling

of posttranslational events that ultimately result in a mature, non-covalently linked heterodimer that is inserted in the plasma membrane of the signal-receiving cell [31]. Upon engaging a cognate ligand, often displayed on the surface of nearby signal-sending cells, Notch receptors undergo conformational change that allows membrane proximal cleavage at the S2 site by ADAM proteases [24]. The extracellular domain of Notch receptors is transendocytosed by the signal-sending cell, through a process that initiates signaling within the ligand-bearing cell [98]. Removal of the extracellular domain leaves only a short transmembrane “stub” protruding from the cell surface, generating a structural substrate for the enzymatic actions of gamma secretase. Gamma secretase, a multiprotein complex comprised of presenilins 1 and 2, APH, and nicastrin, cleaves Notch receptors at the S3 site,

liberating an active, intracellular form of Notch (NIC), capable of translocating to the nucleus to regulate downstream targets [27]. Within the context of canonical signaling, NIC is recruited to preformed, DNA-bound complexes containing its canonical nuclear binding partner CSL/RBPj. Notch binding to CSL/RBPj displaces transcriptional repressors, which are subsequently replaced by transcriptional activators, and allows for gene transcription [56]. In addition to canonical signaling, noncanonical signaling (i.e., CSL/RBPj-independent) and ligand-independent signaling have also been described [10, 73, 78, 135].

7.2 Pathophysiology of GVHD

Hematopoietic stem cell transfer (HSCT) provides the means for full hematopoietic reconstitution following myeloablative therapy commonly used to treat hematologic malignancies, solid tumors, or certain immune-mediated bone marrow failure diseases, such as aplastic anemia [26, 99, 118]. Graft-versus-host disease (GVHD) refers to a constellation of adverse sequelae following HSCT, whereby immunocompetent cells present in the stem cell graft are activated and damage host tissues [109]. Studies suggest GVHD can affect greater than 40% of patients following HSCT, and, as such, it remains a significant barrier to the broader use of HSCT in the clinic. GVHD is classified as acute or chronic, based on how soon after HSCT symptoms appear. Symptoms presenting within the first 100 days post-HSCT are attributed to acute GVHD, while those occurring later than 100 days constitute chronic GVHD. Preventing acute GVHD is thought to decrease the likelihood of chronic GVHD, which also appears to have autoimmune underpinnings.

In cases of hematologic malignancy or transplant in the setting of solid tumors, conventional conditioning in preparation of HSCT is intended to reduce tumor burden and neutralize host immune responses, so as to prevent HSC rejection [67]. However, pre-transplant conditioning can also create the environment for host tissue to be targeted by HSCT-derived T cells, resulting in GVHD [121]. The pathophysiology of GVHD can be divided into three stages that represent a continuum of disease (Fig. 7.2). During the initiation phase, conditioning regimens cause the release of endogenous bacterial lipopolysaccharides (LPS) and damage host cells, resulting in inflammatory cytokine release [43]. The second, inductive phase begins when host, and possibly donor, antigen-presenting cells (APCs) are activated in response to potent LPS stimulation in the presence of pro-inflammatory cytokines, which serves to enhance their ability to present alloantigens to donor CD4 and CD8 T cells [41]. This stage progresses into the effector phase as immunocompetent T cells, resident within the stem cell graft, proliferate and differentiate after encountering major or minor histocompatibility antigens that are mismatched between donor and host. Tissue destruction is mediated during the effector phase of GVHD, which manifests when distinct subsets of differentiated T cells, directed by the chemokine receptors they express, infiltrate and damage specific host tissues [57].

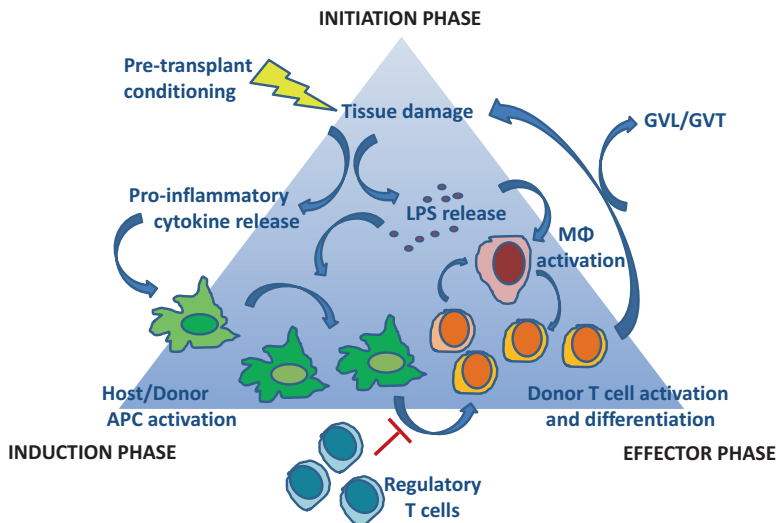


Fig. 7.2 Stages of graft-versus-host disease. Graft-versus-host disease (GVHD) is initiated as the result of host conditioning regimens, especially those that involve total body irradiation, prior to hematopoietic stem cell transplantation. Tissue damaged by pre-transplant conditioning causes the release of lipopolysaccharide (LPS) from gut bacteria, together with induction of pro-inflammatory cytokine secretion. Host/donor antigen-presenting cells (APC) and host macrophages (MΦ) are activated and primed to present alloantigen to donor T cells. Activated, differentiated donor T cells increase expression of pro-inflammatory cytokines and chemokine receptors, migrate to target tissues, and mediate tissue destruction. Regulatory T cells may negatively regulate T cell activation and differentiation, minimizing GVHD pathology

Organ involvement in GVHD can vary and may be linked to the intensity of the conditioning regimen [72, 95]. The major organs targeted for immune attack include the gut, liver, skin, and lungs. Increasingly, evidence suggests distinct populations of T helper (Th) cells mediate organ-specific destruction, likely as a combined result of the cytokines they produce together with the repertoire of chemokine receptors they express [39, 60]. To this end, Th type 1 cells (Th1) appear to be important in GVHD affecting the gut and liver, although these cells are thought to mediate, to some degree, immune attack against all of these tissues. Th2 and Th17 cells exacerbate immune-mediated damage to the skin and lungs, but Th2 cells also can contribute substantially to gut GVHD [131]. Chemokine-directed migration of Th cells has also been implicated in organ-specific trafficking. CCR9 and CCR5 are important in migration to the gut [7, 100]. CCR6 mediates targeting to the skin, while CXCR3 is critical for GVHD of the liver, gut, and skin [40, 120]. Finally, CCR2, CCR4, and CCR6 all can contribute to Th cell trafficking to the lungs [131].

For patients undergoing HSCT following myeloablative therapy for hematologic malignancies, such as leukemia or lymphoma, the allo-specific responses that result in GVHD can also provide a benefit to HSCT recipients through an immune attack directed against residual leukemic cells. This “graft-versus-leukemia” (GVL) or “graft-versus-tumor” (GVT) effect is neither well understood nor well defined, but

studies do suggest it can be separated from the detrimental GVHD responses commonly observed [55, 64, 65, 133]. To preserve GVL/GVT, while selectively abrogating GVHD, is the ultimate therapeutic goal of researchers in this field.

This review will focus on the various stages of GVHD initiation and progression: activation of APCs, differentiation of the major Th subsets and secretion of their signature cytokines, as well as expression of chemokine receptors that direct tissue-specific infiltration, and how Notch signaling may be implicated in each of these.

7.3 Notch Receptors and Ligands During the Initiation and Induction Phase of GVHD

Acute GVHD occurs within 100 days in the post-transplant period and culminates in extensive tissue destruction characterized by apoptosis. Its development is absolutely dependent on the presence and function of alloreactive T cells in the donor inoculum and is closely tied to the curative GVT/GVL effect [64, 65]. However, following HSCT, tissue injury and inflammation, defined by pro-inflammatory cytokine release, are initiated by the conditioning regimen. Both the severity and the incidence of GVHD following HSCT have been associated with the intensity of the conditioning regimen, especially when total body irradiation (TBI) is included [42]. During the initiation phase of GVHD, pro-inflammatory cytokines, together with lipopolysaccharide (LPS), released as a result of conditioning-induced gut damage, converge to craft the inflammatory environment. Subsequent activation of host antigen-presenting cells (APC), including conventional dendritic cells (cDC) and macrophages, occurs during the inductive phase.

The requirement for host APCs to induce GVHD is complicated and controversial. While host APCs may not be required for GVHD to ensue [63], host DCs are sufficient to activate donor T cells [28, 64, 65] and initiate GVHD [58]. Toll receptor 4 (TLR4) is a major pattern recognition receptor capable of responding to LPS, and TLR4 expression on host cDCs exacerbates GVHD [16, 137]. Furthermore, cDC exposure to LPS also enhances expression of the Notch ligand Delta-like 4 (Dll4), likely through a TLR4-mediated process (Fig. 7.3 (1); [68, 111]). Seminal work by Flavell's group suggested engagement of specific Notch ligands can influence T helper cell differentiation, with ligands of the Delta-like family (Dll1,3,4) promoting T helper type 1 responses and those of the Jagged family (Jag1,2) facilitating T helper type 2 differentiation [3]. In the context of GVHD, Dll4 expression on host cDCs has been shown to drive allogeneic T cell responses, including increased production of IFN γ and IL-17, both in vitro and in vivo (Fig. 7.3 (2); [75, 81, 85]). This study coincided with elegant work by Maillard's group showing that administering antibodies specific for Dll1 and Dll4 in a mouse model of GVHD, early after induction, provided durable protection against disease pathology without significantly sacrificing the beneficial effects of GVL/GVT [114]. These studies not only highlight the contribution of Notch signaling to the induction of GVHD during the very

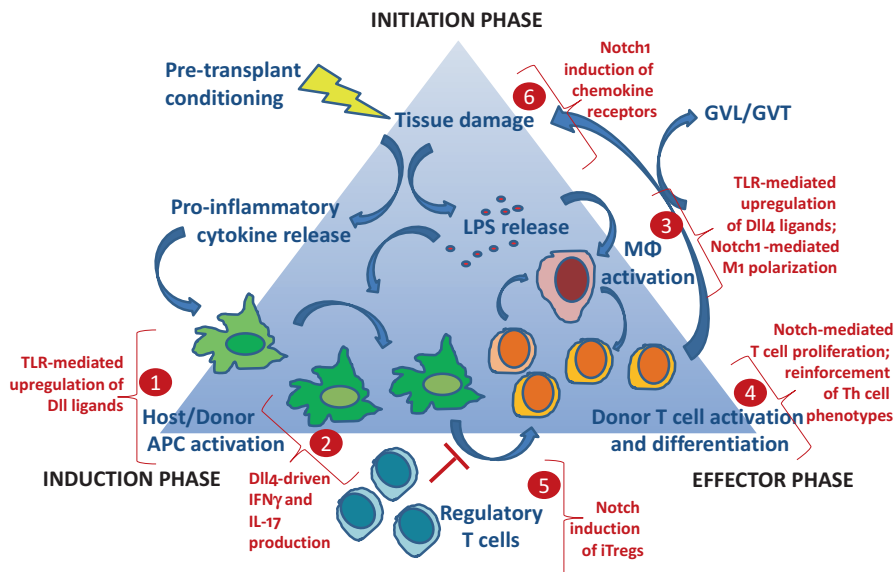


Fig. 7.3 Potential for Notch involvement in graft-versus-host disease. Notch signaling may mediate numerous aspects of GVHD. (1) Toll receptor (TLR) signaling in response to lipopolysaccharide (LPS) release following pre-transplant conditioning upregulates Dll ligands on host/donor APCs; (2) Dll4 expression on donor dendritic cells increases IFN γ and IL-17 secretion by Th1 and Th17 cells, respectively; (3) Notch1 upregulation in activated macrophages (M Φ) promotes a pro-inflammatory “M1” phenotype; (4) Notch receptors play an important role in reinforcing differentiated phenotypes of T helper (Th) CD4 cells, including Th1, Th2, and Th17 cells. (5) Notch signaling may play an important role in the development of induced regulatory T cells (iTregs); (6) Notch signaling may regulate chemokine receptor expression to direct T cell trafficking to GVHD target organs. Please refer to the text for expanded description of Notch signaling at each of these potential stages of GVHD induction and progression

early priming stages, their results suggest that inhibiting the signaling capacity of specific Notch ligands represents a viable therapeutic strategy to prevent GVHD.

The contribution of macrophages, both of host and donor origin, appears to be equally complex, especially in terms of Notch signaling. Macrophages can be polarized toward “M1” or “M2” phenotypes, and a critical involvement of Notch in this process has been described [110, 122, 129]. TLR4 signaling in macrophages increases Notch1 expression which, in turn, promotes their differentiation into pro-inflammatory M1 cells, through interaction with its canonical DNA-binding partner, CSL/RbpJ [45, 128]. Some reports show M1 macrophages exhibit increased capacity for antigen presentation, as well as increased secretion of pro-inflammatory TNF, IL-6, IL-12, and IFN γ , all through Notch1-mediated signaling cascades (Fig. 7.3 (3); [15, 82, 124, 125]). In a model of atherosclerosis, macrophage exposure to LPS was also shown to increase Dll4 expression via TLR4 signaling and promoted an M1 phenotype [34]. Thus, it is attractive to speculate that the Dll4-specific antibodies used in Maillard’s studies may target M1 macrophages, as well

as effectively targeting Dll4+ cDCs, although additional studies are needed to confirm this. Nonetheless, M1 macrophages can accumulate in the skin to mediate cutaneous GVHD, where the number of infiltrating M1 macrophages is directly correlated with the severity of skin pathology [86], and reducing M1 differentiation post-HSCT transfer using dexamethasone palmitate can also mitigate acute damage to the skin [87], although its effects on Notch receptors and ligands were not evaluated.

The expansion and activity of host-derived M2 macrophages can also provide protection against GVHD-mediated tissue destruction, and inhibiting Notch1 signaling in macrophages can induce an M2 cell fate [110]. CSF-1 promotes the M2 phenotype in macrophages, with reduced secretion of pro-inflammatory cytokines, suggesting an increase in M2 macrophages may provide protection from GVHD. This hypothesis was supported in a mouse model by administering CSF-1 prior to GVHD induction. CSF-1 greatly expanded the host M2 macrophage pool, decreased the expansion of donor T cells, and reduced the severity of acute GVHD [38]. Conversely, donor-derived macrophages appear to require CSF-1 to mediate chronic GVHD, since depleting macrophages using an antibody directed against the CSF-1 receptor or transferring macrophages from *csf1* deficient donors resulted in markedly reduced skin and lung GVHD, due to decreased IL-17 production [2].

Collectively, these data suggest that lingering, donor-derived M2 macrophages may contribute to tissue destruction characteristic of chronic GVHD. These confounding data underscore the complexity of temporal GVHD pathology, the diversity of cell types involved, and the potentially conflicting contributions of Notch signaling in acute vs chronic GVHD. Additional studies that specifically address Notch signaling in chronic GVHD will be imperative to clarify these discrepancies.

7.4 Notch, T Helper Cells, and the Effector Phase of GVHD

As the continuum of acute GVHD progresses into the effector phase, activated APCs are now suited to prime immunocompetent donor T cells to differentiate into various T helper (Th) subsets and expand effector CD8 T (Teff) cells. CD4 Th cells, along with CD8 cytolytic T cells (CTL), will infiltrate target organs to mediate tissue damage (Fig. 7.3 (4)).

In response to antigen stimulation, mature T cells integrate myriad external signals derived from the T cell receptor, co-stimulation, and cytokines present in the microenvironment to initiate the genetic programming that will result in their full activation, expansion, and subsequent differentiation [107]. Th type 1 (Th1), Th2, Th17, Th9, and Th22 cells have all been described based on the unique combinations of cytokines that drive their differentiation, the master transcription factor they upregulate, and the signature profile of cytokines they produce. Accumulating evidence suggests that individual Notch receptors and ligands critically influence

Th cell differentiation, both in healthy and aberrant immune responses, including in those that mediate GVHD [130].

Notch signaling in donor CD4 and CD8 T cells augments their activation and expansion and regulates expression of CD25, the high-affinity subunit of the IL-2 receptor [1, 92], supporting the notion that Notch signaling likely acts as a signal amplifier [29]. Engagement of Notch receptors on T helper cells by cognate ligands expressed on APCs was initially thought to instruct T helper cell differentiation: exposure to Dll family ligands promoted a Th1 phenotype, while binding to Jag family ligands directed a Th2 cell fate [3]. A closer examination of the conditions under which T helper cells adopt a specific, differentiated state now supports the notion that Notch signaling provides an unbiased amplification signal to helper T cells, allowing them to proliferate and reinforce a T helper phenotype (i.e., cytokine secretion) acquired as a result of response to the extracellular cytokine milieu [11, 90]. This perspective helps to reconcile the pleiotropic requirements for Notch activation in Th1, Th2, Th17, and induced regulatory T cell (iTreg) differentiation, with different ligands augmenting, rather than inducing Th signature cytokine secretion. Furthermore, redundant functions for some Notch receptors have been described, suggesting strategies aiming to target specific Notch receptors in the management of GVHD may prove ineffective [9].

7.5 Th1 Cells in GVHD

GVHD that manifests in the gut and liver is driven by a strong Th1 response [93] although IFN γ , a signature Th1 cytokine, appears to contribute to tissue damage in nearly all organs affected by GVHD. T cells that adopt a Th1 cell fate exhibit increased and sustained expression of the master transcriptional regulator, T-bet. Th1 cells secrete IFN γ which, together with IL-12, serves to reinforce the Th1 phenotype. First described by Szabo et al. in 2000, T-bet is a T cell-specific transcription factor that is absolutely required for Th1 differentiation [112]. In GVHD, T-bet is also an important determinant of disease pathology. GVHD induction using T-bet-deficient T cells resulted in significantly reduced tissue damage in the gut and liver, compared to disease induction using wild-type or IFN γ -deficient T cells, and regardless of whether the model used was mismatched for major or minor histocompatibility antigens [33]. In this study, although loss of T-bet enhanced Th17 cell differentiation, the Th17 cells were less effective at inducing GVHD. Of note, GVL/GVT effects were also compromised but could be restored following neutralization of IL-17 in hosts. By contrast, selectively inhibiting Th17 development with low-dose halofuginone in a mouse model of GVHD exacerbated Th1-mediated pathology in the liver and gut, although pulmonary GVHD was attenuated with reduced Th17 differentiation [22, 23, 93]. Moreover, in a prospective study of patients with cutaneous GVHD, an increased number of IFN γ -producing Th1, but not Th17, cells were found in skin lesions of

patients with acute GVHD, compared to those without GVHD, suggesting that Th1 cells can also mediate skin-associated tissue damage [17].

The gene encoding T-bet, *Tbx21*, is a direct transcriptional target of Notch1, and inhibiting Notch signaling greatly impairs both the differentiation of Th1 cells and the production of pro-inflammatory cytokines [77, 92, 101]. Canonical Notch signaling in T cells can be compromised when a dominant negative form of a key transcriptional coactivator mastermind-like (DNMAML) is expressed [70]. In the context of GVHD, inducing disease by transferring DNMAML T cells from C57BL/6 donor mice into Balb/c recipients attenuates disease pathology and impairs IFN γ secretion, although T-bet expression remained intact [105, 136]. However, a subsequent report demonstrated that T-bet expression was independent of CSL/Rbpj signaling and, rather, was regulated via a noncanonical signaling pathway [29]. In light of these combined studies, it is likely Notch signaling contributes significantly to Th1-mediated pathology in GVHD, through its regulation of canonical and noncanonical transcriptional targets.

7.6 Th17 Cells in GVHD

Th17 cells are an additional subset of differentiated T cells capable of migrating into the gut, lung, and skin tissues where they can mediate tissue destruction [74, 133]. Th17 cells require ROR γ t for their terminal differentiation, and this process is facilitated by the presence of IL-6 and TGF β [51, 71]. Plasticity between Th1 and Th17 phenotypes has been demonstrated, suggesting that within the appropriate constellation of signaling modifiers, especially those conveyed by the cytokine milieu, Th1 cells can adopt features of Th17 cells and vice versa [18, 37, 69]. Within the complex progression of GVHD, it has been shown that even when the Th1-predominant, IFN γ , and the Th17-signature, IL-17, cytokines are present, the kinetics of their accumulation in target tissues differ. In an MHC minor antigen-mismatched model of GVHD, IFN γ secretion by CD4 and CD8 T cells preceded IL-17 production in the liver and lung, as well as in the spleen and mesenteric lymph nodes. This finding suggests that, in this model of GVHD, Th1 cell differentiation occurred prior to that of Th17 cells [49]. A recent study using a mouse model of colitis suggests that, in the presence of TGF β , Th1 cells can be converted to IFN γ +IL-17+ CD4 T cells, which exacerbate gut pathology [37]. Whether or not a similar conversion can or does occur in the context of gut GVHD remains to be examined. Th1 and Th17 cells share a common cytokine subunit: the IL-12/IL23 p40 subunit. Each of these cytokines contributes to stabilizing their respective associated Th phenotype: IL-12 will reinforce Th1 polarization, while IL-23 increases Th17 cell fate stability. Although neutralization of the common p40 subunit can attenuate GVHD in a mouse model [126], it is not known, definitively, if signaling through this common subunit influences the interconversion of these Th cell subsets in response to the complex array of cytokines in the microenvironment.

Overall, the question of how extensively Th17 cells contribute to GVHD pathology remains unanswered [47, 119]. It is clear that Th17 cells can be found in target tissues during GVHD progression, and disease severity can be attenuated when T cells lacking ROR γ t are used in a model of CD4 T cell-mediated GVHD. The resulting decreased levels of TNF α in the gut, liver, and lung were thought to be significant factors in the reduced pathology noted [138]. Interestingly, in a model of spontaneous type I diabetes, TNF α could be induced in a Th17-dependent manner [62, 66]. Whether or not differentiated Th17 cells act to sustain TNF α secretion produced during the induction and/or initiation phases of GVHD will require further investigation. In a separate report, *in vitro*-generated Th17 cells were found to be sufficient but not required to induce GVHD, and their presence also correlated with higher levels of TNF α [47]. However, ROR γ t-deficient CD4 T cells were also capable of inducing lethal GVHD in this model, suggesting that, although Th17 cells may contribute significantly to GVHD progression, additional subsets of Th cells also are critically important.

One important observation of the study by Iclozan et al. was that Th17 cells showed extensive expansion, *in vivo*, and were highly resistant to activation-induced cell death (AICD). Additional work has demonstrated that human Th17 cells constitute a long-lived memory T cell population [59]. Under various pathological conditions, these cells were shown to be highly proliferative, *in vivo*, resistant to AICD, and preserved their ability to further differentiate into functional Th1 and iTreg cells, although they retained their capacity for IL-17 secretion. Notably, survival of these human Th17 cells was attributed to a Notch-dependent pathway, downstream of HIF1 α , since overexpression of NICD could rescue a significant percentage of Th17 cells from the apoptosis induced when HIF α signaling was blocked. These data add to our understanding of Notch signaling in Th17 cell differentiation. Dll4-Notch signaling between DCs and T cells, in the presence of the Th17-polarizing cytokines, IL-6 and TGF β , resulted in higher percentages of Th17 cells than those polarized under identical conditions in the absence of Notch signaling [85]. Although not expressly examined in their studies, it is also possible that the robust protection from GVHD induction observed by Maillard's group during Dll4-blockade may include reduced differentiation of Th17 cells and the concomitant benefit that provides.

7.7 Th2 Cells in GVHD

A detailed study conducted by Zeng's group would suggest that some of the complexities of GVHD may be attributed to cross-regulation among Th cell subsets which, in turn, influences the severity and organ specificity of the associated pathology [131]. Using a major MHC antigen-mismatched model of GVHD (C57BL/6 donor into Balb/c recipient), they showed that transplanting IFN γ -deficient T cells augmented both Th17- and Th2-mediated tissue destruction in the lung and skin. By contrast, inducing GVHD using T cells that were doubly deficient for IFN γ and

IL-17 led to a massive upregulation and infiltration of Th2 cells into the lungs, resulting in idiopathic pneumonia syndrome (IPS). Thirteen days after GVHD induction, there was a nearly 16-fold increase in the number of CD4 Th2 cells in the lungs of mice that received doubly deficient T cells, compared to mice whose disease was induced using wild-type CD4 T cells. Mice that received IFN γ -only deficient cells had a nearly tenfold increase in Th2 and Th17 cells in the lung, compared to mice receiving wild-type T cells. This study further showed that induction of the immune-modulating ligand, B7-H1, in host lung epithelium required IFN γ for its upregulation, and absence of its expression was a compounding factor leading to IPS. In a complementary study by Yu et al. [133] that employed the same model of GVHD, transferring donor T cells that were doubly deficient for the transcription factors, T-bet and ROR γ t, resulted in overall decreased pathology in the gut, liver, and lung; however, significant increases in IL-4 and IL-5 expression 5 days after GVHD induction were also noted in the lungs of recipient animals [133]. These conclusions were further supported using a model of LPS inhalation-induced acute pulmonary GVHD in the context of HSCT. Transferring T-bet-deficient T cells in this model resulted in more severe lung pathology and pulmonary fibrosis, together with increased IL-13, IL-17, and Th17 cells, suggesting that, in the absence of cross regulation by IFN γ , Th17 cells can contribute significantly to lung GVHD [35].

As with other Th subsets, a role for Notch signaling in Th2 cell differentiation has also been described [4]. Using a model of *Trichuris muris* infection, Pear's group demonstrated a requirement for canonical, MAML-mediated Notch signaling to generate the protective, in vivo Th2 response necessary to clear the parasitic infection [115].

Furthermore, while TCF and β -catenin, components of the Wnt signaling pathway, initiate early, IL-4-independent GATA3 expression via their binding to the *Gata3-1b* enhancer, it would appear that Notch binding to the *Gata3-1a* enhancer leads to sustained Th2 cell differentiation by providing synergistic upregulation of IL-4 [32, 132]. Thus, as with the other Th cell subsets discussed, and which are critically important in mediating GVHD, Notch is also an important regulator of Th2 cell differentiation [5].

7.8 Regulatory T Cells in GVHD

In addition to Th cells, distinct subsets of immunosuppressive regulatory T (Treg) cells also have been identified, including naturally occurring (nTreg) and inducible (iTreg) regulatory T cells that can express either CD4 or CD8 [69, 83, 106]. To date, Tregs exhibiting a CD4⁺ CD25⁺FoxP3⁺ phenotype are perhaps the most well-studied. However, unlike their Th and Teff cell counterparts which drive GVHD pathology, Treg cells have been shown to attenuate the severity of acute GVHD, mitigate the extent of tissue involvement, and prevent chronic GVHD altogether [76, 116].

Naïve T cells will adopt a Treg phenotype when they differentiate in the presence of TGF β and IL-2 and are characterized by increased expression of the master

transcriptional regulator FoxP3. In vivo, the Treg phenotype is considered to be unstable, with reports of interconversion of Tregs to effector T cells in the presence of high levels of inflammatory cytokines, especially IL-6 and IL-17 [54, 127].

Extensive and impressive progress has been made in defining the protective effects of Tregs and their mechanisms of action, both in murine and human studies. Functional Treg suppression has been reported to be faulty in patients with acute GVHD [14]. This may be due to the observed lower surface expression of CCR5 and CXCR3 on donor Tregs, resulting in impaired trafficking to target organs [117]. Studies also suggest that donor-derived, or third-party, Tregs provide far more potent immunosuppression than host Tregs when used as an immunotherapy [94]. Human trials using ex vivo-expanded nTregs as a potential prophylaxis for GVHD were recently concluded [19].

The encouraging results showed that, when administered at the time of HSCT, only 9% of patients who received expanded Tregs exhibited grade II–IV acute GVHD at 100 days post-transplant, compared to a control cohort in which 45% showed symptoms of GVHD, grade II or higher.

Patients in both cohorts received identical conditioning regimens prior to HSCT. Furthermore, while approximately 14% of the non-Treg-treated patients showed signs of chronic GVHD at 1 year post-HSCT, none of the patients who received the ex vivo-expanded Tregs exhibited chronic GVHD. The impressive results of this trial may be due to the co-administration of Tregs at the time of HSC transfer. In complementary studies, Negrin's group utilized a minor antigen MHC-mismatched model to show that the immunosuppressive capacity of transferred Tregs, both donor-derived and third party, is most effective early after their transplantation and suggests that the timing of administering Tregs is a critical factor to be considered [97]. A small study using donor-derived or third-party expanded Tregs in the treatment of established cases of chronic and acute GVHD were less positive [113], lending support to Negrin's findings.

There is a preponderance of evidence supporting a positive role for Notch signaling in iTreg differentiation and which further support the influence of specific Notch ligands in this process (Fig. 7.3 (5)). Notch signaling may generate Tregs through both direct and indirect mechanisms. In an early report, APCs engineered to overexpress Jag1 (*serrate1*) could induce a regulatory phenotype in co-cultured CD4 T cells, and these cells maintained antigen-specific suppressive activity when transferred into naïve hosts prior to immune challenge [44]. Two subsequent studies revealed Notch1 and TGF β can cooperate to induce FoxP3 expression, and this process may involve canonical Notch signaling via CSL/Rbpj binding to the *FOXP3* promoter [89, 91, 103]. Notch3 has also been implicated in *Foxp3* transcription during nTreg generation, and this has been shown to proceed through an NF- κ B-dependent pathway [12, 20]. Furthermore, it was demonstrated that human memory CD4 T cells could adopt a Treg phenotype, including expression of FoxP3 and upregulation of TGF β , when exposed to Dll1 during in vitro culture [84].

Indirect involvement of Notch signaling in Treg-mediated immunosuppression has also been demonstrated. Co-incubating CD4+CD25- T cells with Dll4 or Jag1 increased their responsiveness to Treg-mediated suppression by upregulating

TGF β RII expression [46]. Additionally, Notch signaling is also important for production of the immunosuppressive cytokine, IL-10, in T cells, and this is enhanced when CD4 T cells are co-cultured with Dll4-expressing plasmacytoid DCs [50, 102]. Elegant work by Sarin's group showed that differential localization of Notch1 mediated Treg survival through a membrane-associated NICD/Rictor complex, which was stabilized by interactions with Dll1. This protection was lost when NICD was targeted to the nucleus [96]. Recent work, however, challenges the notion that Notch signaling positively regulates Treg induction [21]. The results of this study showed that when NICD was overexpressed in FoxP3+ Tregs, the Treg phenotype was destabilized, resulting in an autoimmune lymphoproliferative manifestation. Canonical Notch signaling mediated this phenomenon, since loss of *Rbpj* restored the number and frequency of Tregs and protected them from apoptosis in a model of GVHD generated by major histocompatibility antigen mismatch. Reconciling these seemingly disparate data will be important to informing therapeutic strategies aimed at limiting Notch signaling in GVHD.

7.9 Concluding Remarks and Future Directions

Much has been learned about the constellation of alloimmune responses that can be generated after HSCT and that result in the complex pathology of GVHD. However, much less is known about the mechanisms of the beneficial GVT responses that may also come into play following HSCT. Indeed, details regarding this phenomenon are only recently emerging and suggest that factors responsible for mediating this effect will be as complex as those that drive GVHD. It is also likely that multiple cellular subsets contribute to GVT; therefore, approaches that target multiple cell populations may ultimately prove to be the most successful.

Effective *ex vivo* expansion and delivery of donor-derived nTregs or *in vivo* generation of donor-derived iTregs represent viable approaches to attenuating GVDH, although the effect of increased numbers of Tregs on GVT has not been well-characterized. One caveat to this approach is the demonstrated capacity for Tregs to be converted to Th1 or Th17 cells in the context of a robust pro-inflammatory environment and will need to be considered when designing Treg-based therapies. As discussed above, the degree to which Notch signaling positively or negatively regulates Treg development also has not been fully elucidated. Thus, approaches that aim to attenuate GVHD by manipulating Notch signaling may need to be precise and acutely applied.

Manipulating chemokine receptors, which play a vital role in T cell trafficking, may be one means of achieving selective organ targeting to reduce Teff cell infiltration or to increase trafficking of Tregs to specific organs. CXCR3, CCR5, and CCR9 have been identified as chemokine receptors that facilitate T cell migration, and gut infiltration of CCR9+ CD4 T cells is associated with tissue destruction [7, 134]. CXCR3 has been shown to be a transcriptional target of T-bet [48, 52], which itself

is regulated by Notch1 [77, 101]. Furthermore, CCR5 and CCR9 have both been demonstrated to be regulated by Notch1 in T-ALL [79]; however, whether Notch also primes Teff cells for gut infiltration in GVHD is not yet known (Fig. 7.3 (6)). Interestingly, CCR9 also mediates the migration of plasmacytoid DCs (pDC) to the intestines [123], and CCR9+ pDCs show an immature phenotype with a high capacity for suppressing GVHD by reducing the number of IL-17 producing cells and increasing the number of FoxP3+ Treg cells [36]. Further investigation is needed to determine if Notch signaling regulates CCR9 expression in pDC, as it does in CD4+ T cells.

Therapeutic modalities for the treatment and prevention GVHD that aim to limit Notch signaling represent an exciting and active area of investigation. Very early reports suggest that, at least in animal models, this is a promising approach that may serve both to attenuate tissue damage and preserve beneficial GVT effects. Current research in therapies that range from delivering ligand-specific neutralizing antibodies to developing γ -secretase inhibitors designed to selectively modulate Notch signaling, to expanded use of Treg cells as immune modulators, makes for an impressive, and hopefully successful, array of approaches to tackle a complex and complicated disease.

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