Cellular and molecular analysis of Notch signaling in T cells after allogeneic bone marrow transplantation

by

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Dedication

To my loving family, girlfriend, friends, lab members, and PhD mentor. Thank you for your constant support and for understanding that research requires persistent effort and dedication. I am grateful to each and every one of you.

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Abstract

Allogeneic bone marrow transplantation (allo-BMT) is a potentially curative therapy for patients with cancer and hematological disorders. However, its success is limited by graft-versus-host disease (GVHD). During acute GVHD, donor T cells attack host epithelial tissues to trigger intestinal, skin, and thymic damage. Less than 50% of patients who manifest with acute GVHD symptoms respond to corticosteroid therapy, underscoring the need to develop better therapeutic strategies. Notch signaling has emerged as a critical regulator of T cell alloimmunity after allo-BMT. During allo-BMT, Notch signals are mediated by Notch1/2 receptors and Delta-like1/4 ligands. Systemic inhibition of Delta-like1/4 Notch ligands with neutralizing antibodies results in long-term protection from acute GVHD. In this thesis, I first investigated the spatial and temporal regulation of Notch signals during allo-BMT. I found that a single dose of Dll1/4-neutralizing antibodies at the time of allo-BMT was sufficient to confer long-term protection from GVHD mortality. In contrast, delayed administration of antibodies by only two days failed to protect from GVHD. Together, these data identified a critical early period of Notch activity that promotes GVHD pathogenesis. To determine the cellular source(s) that delivered Notch signals to alloreactive T cells during this window of activity, I genetically inactivated *Dll1* and *Dll4* Notch ligands within donor hematopoietic, host hematopoietic, and host nonhematopoietic tissues. Mice lacking *Dll1* and *Dll4* expression within their donor and host hematopoietic tissues remained sensitive to GVHD. In contrast, mice that lacked *Dll1* and *Dll4* expression selectively within $Ccl19^{+}$ fibroblastic cells were profoundly protected from GVHD lethality. Thus, these data revealed that $Ccl19^{+}$ fibroblasts deliver early Notch signals to alloantigen-

specific T cells. Next, I developed a novel model of acute GVHD with alloantigen-specific 4C $CD4^+$ donor T cells to study molecular events during the critical 48-hour period of Notch activity. Notch inhibition in alloantigen-specific T cells preserved early activation marker expression, IL-2 production, and initial proliferation. In contrast, Notch inhibition impaired IFNγ and IL-17 proinflammatory cytokine production and reduced both mTORC1 and Ras/MAPK activity. Transcriptional profiling during T cell priming revealed that Notch inhibition diminished transcription of an assortment of cytokines, cytokine receptors, and a subset of Myc target genes. Collectively, my data demonstrated that fibroblastic niches deliver critical Notch signals to alloantigen-specific T cells during the first two days after allo-BMT. These early Notch signals were essential for acquisition of effector functions but not for initial proliferation. Work in this thesis introduces the novel concept that early interactions between donor alloreactive T cells and host fibroblastic niches dictate the long-term outcome of GVHD. A deeper understanding of these interactions could lead to exciting new therapies for alloimmune and autoimmune disorders.

Chapter 1

Introduction to Notch signaling and T cell alloimmunity[1]

Alloreactive T cell immunity

Allogeneic T cell responses drive immune reactivity against foreign tissue antigens. Alloimmunity plays an essential role in several situations of high medical significance. Host alloreactive T cells mediate immune rejection of implanted organs after solid organ transplantation (e.g. heart, lung, liver or kidney transplantation). With up to 100,000 organ transplants performed each year worldwide, T cell-mediated immune rejection represents a sizable public health problem. Current medical practice involves administration of life-long global immunosuppression (e.g. calcineurin inhibitors) to prevent organ rejection and corticosteroids to treat breakthrough rejection. This strategy is more successful for acute than for chronic rejection. In addition, life-long immunosuppression is associated with high costs and side effects, including increased risk of opportunistic infections and secondary malignancies. As an alternative, researchers have investigated strategies to induce host tolerance to the implanted organ, for example by interference with costimulatory molecules [1] or with non-myeloablative

[1] Excerpts taken from:

Chung J, Maillard I. Notch signaling in alloreactive T cell immunity. Current Topics in Microbiology and Immunology, 360: 135-150, 2012. PMID 22689203

Chung J, Riella LV, Maillard I. Notch signaling in transplant rejection. Am J Transplant. 2016 Apr 1. PMID 27037759

hematopoietic cell transplantation [2, 3]. At this stage, these strategies have not translated into treatments that can be used in routine clinical practice.

After allogeneic bone marrow or hematopoietic cell transplantation (allo-BMT), rejection is rare due to the immunosuppressive effects of the conditioning regimen on the recipient [4]. However, mature donor-derived T cells present in the transplant inoculum drive reactivity to alloantigens in host tissues and host-derived tumors [4-6]. Efficient donor T cell-mediated graft-versus-tumor (GVT) activity results in cancer cell killing [4, 7-9]. This is essential for the success of allo-BMT since the procedure is performed most often for patients with leukemia, lymphoma or other hematological malignancies (>75% of the ~25,000 allo-BMTs performed worldwide each year). Unfortunately, GVT is often associated with reactivity of donor T cells against normal host tissues, leading to graft-versus-host disease (GVHD). GVHD is the most significant complication that limits the success and broad applicability of allo-BMT. Despite the universal use of potent immunosuppression in allo-BMT recipients, acute and chronic GVHD still occur in a substantial fraction of patients. Although intensifying the degree of global immunosuppression decreases the risk of GVHD, it comes at the price of impairing GVT efficiency. As a result, patients experience less GVHD but more tumor relapses, and thus no improvement in their overall survival [4, 10]. Therefore, progress in the field will require identification of new approaches to decrease GVHD severity without eliminating efficient GVT.

Alloantigens can be associated with the major histocompatibility complex (MHC), or independent of it ("minor histocompatibility antigens") [6]. Priming and differentiation ofalloreactive T cells occurs in several steps (**Fig. 1.1**) [5, 6]. Tissue damage and inflammation trigger activation and maturation of APCs through Toll-like receptor signaling and other innate pathways [11]. In allo-BMT, toxic conditioning regimens including total body irradiation and/or

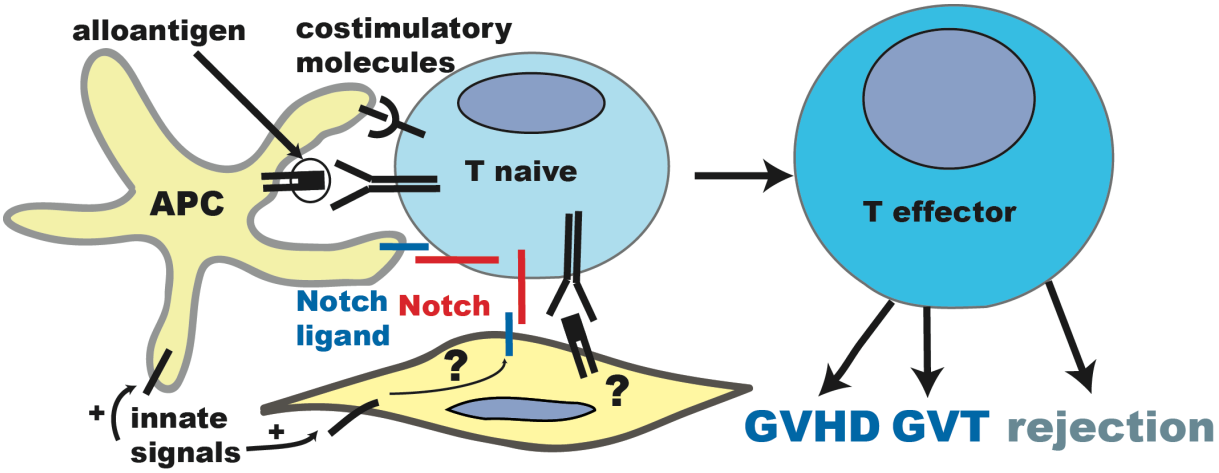

Figure 1.1. Regulation of alloreactive T cell activation and differentiation
Signals from the innate immune system activate antigen-presenting cells (APCs) and enable them to present alloantigens to naïve T cells in the presence of costimulatory signals and/or Notch signaling. Recent literature suggests that nonhematopoietic cells (noted by the question mark) can also serve as APCs. Notch ligands are provided by APCs or other cell types. Upon activation, naïve T cells become effector T cells, and mediate graft rejection, graft-versus-host disease, and/or graft-versus tumor effects.

chemotherapy contribute to APC activation and maturation. Activated APCs expressing costimulatory molecules prime naïve alloreactive T cells. In mouse allo-BMT models, host DCs play a dominant role in stimulating donor-derived alloreactive $CD8^+$ T cells rapidly after transplantation [12-14]. Donor APCs are important in certain settings as well [15]. Interestingly, recent work suggests that nonhematopoietic APCs also play a critical role in driving $CD4^+$ T cell alloreactivity [*81, 82*]. Alloreactive T cells undergo activation, proliferation and expansion in lymphoid tissues. These cells then acquire effector functions through differentiation along various $CD4^+$ and $CD8^+$ T cell lineages. Much work has been performed to understand how effector pathways are regulated in alloreactive T cells, and this will be discussed later as it relates to Notch signaling in these cells. After priming in lymphoid organs, effector T cells undergo chemokine-directed migration into target tissues, mediating rejection (solid organ transplantation), as well as GVHD and GVT (allo-BMT) [16]. Clinically important targets of GVHD include the skin, intestine, liver, lung and thymus. Other immune cells collaborate with T

cells in inducing tissue damage, especially in the chronic phase of the response. Activation of conventional T cells occurs simultaneously with activation and expansion of regulatory T cells, which play a critical role in the outcome of the alloreactive T cell response [4, 17-19].

Overview of Notch signaling

Notch signaling is a highly conserved cell-to-cell communication pathway triggered by Notch ligand-receptor interactions between adjacent cells (**Fig. 1.2**) [20, 21]. In mammals, four Notch receptors (Notch1-4) have been identified in addition to five Notch ligands of the Jagged (Jagged1/2) and Delta-like families (Dll1/3/4). Jagged1/2 and Dll1/4 have agonistic properties, while Dll3 functions as a natural antagonist of the pathway [20]. Notch ligand-receptor interactions induce sequential proteolytic cleavage of the receptor by an ADAM family metalloprotease (ADAM10) and by the γ-secretase complex, ultimately releasing intracellular Notch (ICN) into the cytoplasm [20, 21]. ICN migrates into the nucleus where it interacts with a DNA-binding transcription factor referred to as CSL (CBF1/Suppressor-of-hairless/Lag-1) or RBP-Jκ (encoded by *Rbpj*). Upon Notch activation, CSL and ICN nucleate a large transcription activation complex that recruits a member of the Mastermind-like (MAML) family and other co-activators to mediate transcriptional activation of Notch target genes [20-22].

In the hematopoietic system, Notch was first identified for its oncogenic activity in T cell acute lymphoblastic leukemia [23, 24]. Recent studies in Notch-driven cancer cell lines detected binding of CSL and ICN at thousands of genomic sites [25]. However, only a fraction (<10%) of these sites appeared dynamically regulated by Notch signaling. Regulated sites were enriched for concomitant binding of specific transcription factors, suggesting context-specific regulation of the Notch target gene landscape by cooperating factors. Moreover, the majority of dynamic Notch-binding sites were located in distant elements with superenhancer features, suggesting that

Notch is involved in long-range chromatin regulation [26]. Additional studies about the molecular mechanisms of Notch-mediated transcriptional activation will be essential to understand the context-specific effects of the Notch pathway.

At physiological levels of signaling, Notch is required at early stages of T lineage development in the thymus [22, 27-30]. The effects of Notch are regulated *in vivo* through tight control of signaling intensity [31-33]. Lymphoid progenitors experience a sharp increase in Notch signaling upon entry into the thymus [29, 30] as a result of their exposure to a high density of the Notch ligand Delta-like-4 in the thymic epithelium [34]. High levels of Notch signaling are maintained until progenitors successfully cross the pre-T cell receptor checkpoint, after which signaling intensity is rapidly downregulated [30, 35-39]. Downstream of this checkpoint, $CD4^+CD8^+$ double positive thymocytes experience little if any Notch signaling when undergoing positive and negative selection. Thus, unlike forced induction of Notch signaling, Notch blockade in DP thymocytes does not interfere with T cell development [40-44].

In addition to the effects of Notch signaling in T cell development, increasing attention is being devoted to its role in peripheral T cell immunity [32, 33, 45-47]. Mature T cells predominantly express Notch1 and Notch2 receptors, which can engage Notch ligands during immune responses [48-50]. Toll-like receptor-mediated signals induce expression of Delta-like ligands in dendritic cells (DCs) and other antigen-presenting cells (APCs) [51-54]. Additional sources of Notch ligands may be available to T cells in tissues (e.g. from stromal cells, vascular structures or epithelial elements). Altogether, this sets the stage for a highly regulated context-

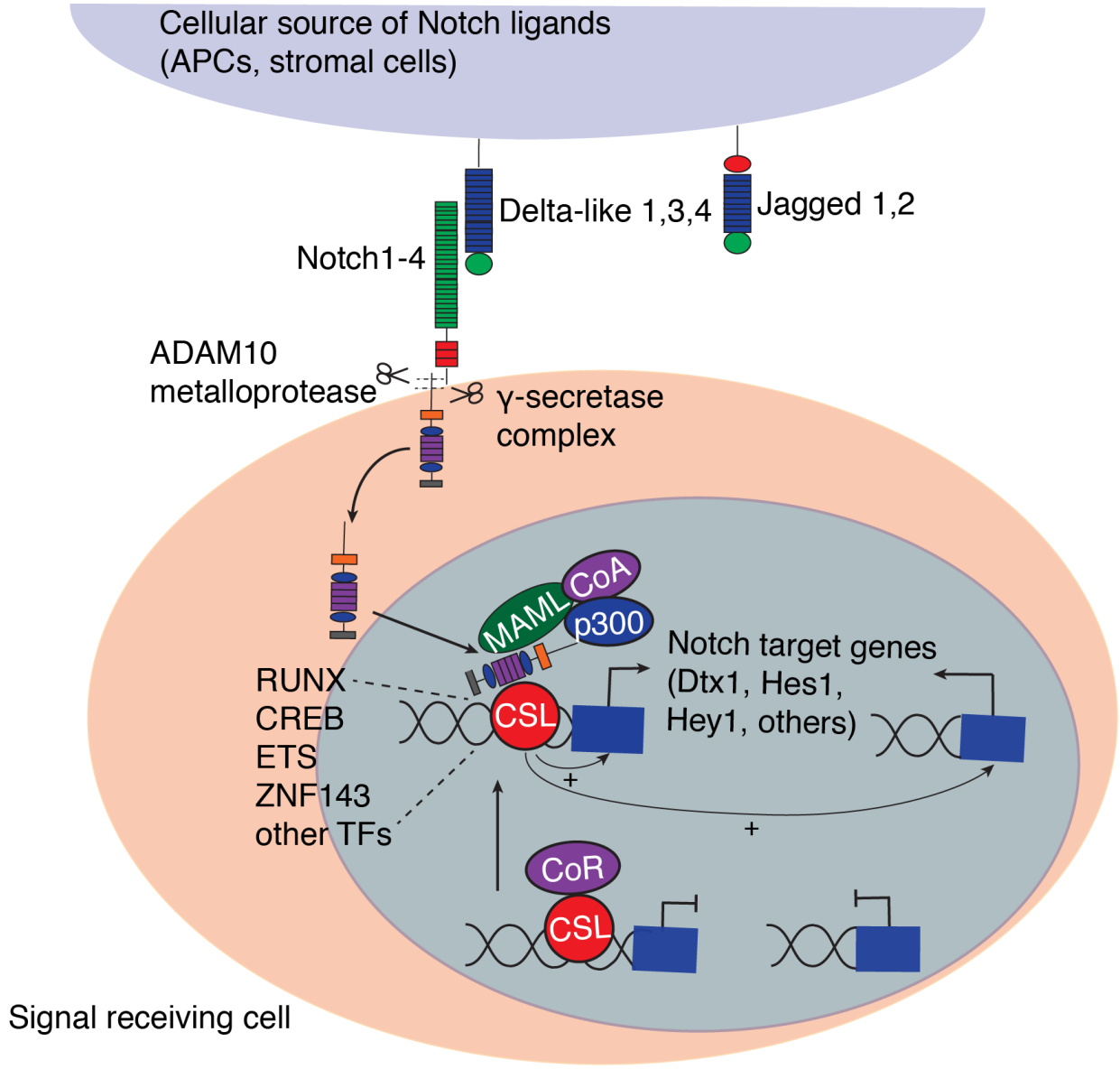

Figure 1.2. Overview of Notch signaling

Mammalian Notch signaling is initiated by interactions between Notch receptors (Notch1-4) and Notch ligands (Delta-like 1,3,4; Jagged 1,2). Ligand-receptor binding triggers two sequential proteolytic cleavages of the Notch receptor, releasing the intracellular domain of Notch (ICN) into the cytoplasm. Upon entry into the nucleus, ICN forms a transcriptional activation complex with the transcription factor [55] CSL (CBF1/Suppressor-of-hairless/Lag-1), a member of the Mastermind-like (MAML) family, and other coactivators such as p300. ICN/CSL transcriptional complexes often assemble adjacent to other TFs, and can regulate Notch target gene expression proximally through promoter binding or distally through enhancer binding and long-range interactions.

dependent exposure of T cells to Notch signaling in different immune responses, with specific roles for individual Notch ligands and receptors.

Complementary gain-of-function and loss-of-function experiments have been used extensively to study Notch in lower organisms and mammals. Although this approach has often proven to be fruitful, caution is required when Notch signaling intensity is tightly regulated. For example, Notch stimulation and Notch blockade may not have opposite effects in cells experiencing a low intensity of Notch signaling, as shown in DP thymocytes or in adult hematopoietic stem cells [40, 41, 44, 56-60]. In addition, forced exposure to Notch ligands or forced signaling through individual Notch receptors may have functional consequences that do not represent the *in vivo* function of the pathway. These considerations apply to the study of Notch in mature T cells. Although *in vitro* studies and gain-of-function experiments can generate useful working hypotheses, we believe that optimal experimental systems should involve stringent Notch loss-of-function systems applied *in vivo*.

Early work on Notch signaling in T cell alloreactivity and tolerance

Initial studies exploring a potential role for Notch signaling in mature T cell function and alloreactivity relied heavily on gain-of-function strategies. Lamb and coworkers were the first to spark interest in a role for Notch as an inducer of tolerance [61]. While studying T cell responses against a house dust mite protein, they engineered mouse dendritic cells (DCs) to overexpress the Notch ligand Jagged1. Adoptive transfer of antigen-pulsed Jagged1-overexpressing DCs led to antigen-specific hyporesponsiveness. Building on this concept, the Brenner group tested the ability of Jagged1-overexpressing antigen-presenting cells to modulate responses to alloantigens or viral antigens [62, 63]. Using Jagged1-transduced Epstein Barr Virus-transformed

lymphoblastoid cell lines, they observed decreased T cell reactivity and evidence for transferrable suppressive effects. Similar outcomes were described in an *in vivo* cardiac allograft model, when Dallman and colleagues adoptively transferred mouse L cell fibroblasts engineered to overexpress MHC alloantigens and the Notch ligand Dll1 [64]. Although Dll1-overexpressing L cells delayed allograft rejection in a $CD8^+$ T cell-dependent manner, it is unclear whether the effects were the result of direct engagement of Dll1 with Notch receptor in T cells. Similar observations were reported recently upon *in vivo* transfer of a Jagged1-transduced dendritic cell line in combination with CD40 blockade [65]. Altogether, these studies suggested that inducing artificially high Notch signals in T cells could generate a state of antigen-specific tolerance.

In parallel, other laboratories observed that expression of specific Notch ligands could be induced by innate stimuli in professional antigen-presenting cells (APCs) [51, 53]. In coculture systems, Delta-like or Jagged Notch ligands within APCs was reported to promote skewing of T cell differentiation towards the T helper 1(Th1) vs. Th2 lineage, respectively [51, 66], although dichotomous inductive effects of Delta-like and Jagged Notch ligands were not detected in subsequent studies [67]. Collectively, while helpful for recognizing an important role for Notch in T cell alloreactivity, the use of artificial *ex vivo* conditions and overexpression models led to conclusions that were contradictory and have to be interpreted with caution. Subsequent *in vivo* loss-of-function studies on mature T cells identified an even broader range of effects of Notch on both $CD4^+$ and $CD8^+$ T cell reactivity and function [68-70], many of which are discussed later in the "**Mechanistic Considerations**" section of this chapter.

In vivo studies of Notch signaling in allograft rejection

In recent years, several groups have used genetic and pharmacological loss-of-function approaches to evaluate the *in vivo* effects of Notch signaling in alloreactive T cell responses, both in the setting of allograft rejection **(Table 1.1A)** and in graft-versus-host disease (GVHD) (**Table 1.1B**) [71-78]. An emerging consensus across these studies indicates that Notch signaling is a major pro-inflammatory pathway in T cell alloimmunity, and that Notch inhibition can dampen both allograft rejection and GVHD. Thus, the true *in vivo* functions of Notch signaling appear to be diametrically different from the tolerogenic effects first detected using artificial gain-of- function strategies [61-64]. Furthermore, these studies identify Notch inhibition as a new promising therapeutic approach to mitigate the damaging consequences of T cell alloreactivity.

Riella and coworkers used monoclonal antibodies to target the Notch ligand Dll1 in a MHC-mismatched cardiac allograft transplantation model [75]. Systemic anti-Dll1 antibodies delayed allograft rejection when provided in conjunction with costimulatory blockade in *Cd28*-deficient mice or in recipients treated with CTLA4-Ig. Protection was associated with decreased production of IL-2, interferon gamma (IFNg), IL-6 and IL-17 by donor-specific T cells, but with increased production of the Th2 cytokines IL-4 and IL-5. In this model, the protective effects of anti-Dll1 antibodies were lost when transplantation was performed in STAT6-deficient recipients or upon concomitant IL-4 neutralization, indicating that increased IL-4 production was important to delay rejection. This paper was the first to demonstrate a pathogenic effect of Notch signaling and a therapeutic benefit of Notch inhibition in allograft rejection *in vivo*, in stark contrast to earlier literature using artificial gain-of-function systems. Because this study was performed in the presence of costimulatory blockade and only examined the effect of Dll1 inhibition but not

other Notch ligands, it was unclear if similar outcomes would be observed without costimulatory blockade or upon more complete Notch inhibition. Due to the systemic nature of Dll1 inhibition, it could not be determined if the protective effects of anti-Dll1 antibodies were related to their direct effects on T cells and/or on other cell types.

To achieve a higher level of Notch inhibition in alloreactive T cells, Wood et al. studied a MHC-mismatched heart allograft model in mice expressing the pan-Notch inhibitor dominant negative Mastermind-like1 (DNMAML) specifically in T cells [74]. DNMAML blocks transcriptional activation downstream of all Notch ligands and receptors [22]. In *Cd4-Cre x* $ROSA26^{DNMAML}$ mice, DNMAML expression first arises in $CD4^+CD8^+$ double positive [79] thymocytes without interfering with early Notch-dependent stages of T cell development [42]. Thus, mature $CD4^+$ and $CD8^+$ T cells develop normally from DP thymocytes in these mice, but cannot respond to Notch signals during subsequent T cell responses due to DNMAML expression. This strategy is highly effective in capturing the overall effects of Notch signaling in T cell immunity, irrespectively of the individual Notch ligands and receptors involved [71, 73]. DNMAML mice rejected MHC-mismatched hearts in a delayed fashion [74]. Although the delay was relatively modest in the absence of other interventions, it was observed in the absence of costimulatory blockade, suggesting that complete Notch inhibition in T cells could achieve higher protection from rejection than the level of protection seen upon partial Notch inhibition with anti-Dll1 antibodies [74, 75]. Importantly, upon concomitant CD8 depletion prior to transplantation, DNMAML expression led to markedly enhanced protection, with a median allograft survival of >40 days. These findings suggested that $CD4^+$ alloreactive T cells were particularly sensitive to Notch inhibition. Mechanistically, DNMAML led to decreased production of both IFNg and IL-4 by donor-reactive T cells, decreased immune cell infiltration

and an increased regulatory T cell (T_{reg})/effector T cell [80] ratio within the graft. DNMAML recipients also showed delayed appearance of graft-specific alloantibodies, suggesting a role for Notch in T cell help to allospecific B cell responses.

Building on their observations from studies using genetic pan-Notch inhibition in T cells, Wood et al. assessed the impact of humanized anti-Dll1 and anti-Dll4 antibodies, alone or in combination, on allograft rejection [74]. This approach was chosen given the effects of Dll1 in transplant rejection [75] and the dominant role of Dll1/4 Notch ligands in GVHD [72, 76]. Anti-Dll1/4 antibodies had high therapeutic activity in allograft rejection. Both anti-Dll1 and anti-Dll4 antibodies by themselves induced significant protection, indicating that these two Notch ligands were involved non-redundantly in the rejection process. Combined administration of anti-Dll1 and anti-Dll4 antibodies was the most effective strategy tested, enabling long-term engraftment in CD8-depleted recipients and markedly delayed rejection even in CD8-replete hosts. Surprisingly, systemic Dll1/4 blockade provided a higher degree of protection from allograft rejection than DNMAML-mediated pan-Notch inhibition in T cells. Enhanced protection was associated with a persistent decrease in donor-specific alloantibody titers, plasma cell numbers and complement deposition in the graft. These findings suggest that the therapeutic activity of anti-Dll1/4 antibodies is related both to their effects on T cells (preventing acute cellular rejection) and to their effects on the B cell response (preventing chronic rejection at least in part through humoral mechanisms). Furthermore, long-term protection was observed upon short-term Dll1/4 blockade in the peri-transplant period, similar to findings in allogeneic bone marrow transplantation and GVHD [72, 74].

Together, Dll1/4 Notch ligands play dominant roles in the regulation of alloimmunity, but the role of Jagged ligands remains unclear. To start addressing this question, Riella and coworkers

A

Allograft rejection model **Modulation of Notch**	**Outcome** **Suggested mechanism**	**Citation**
MHC-mismatched heart BALB/c → B6 systemic neutralizing Dll1 antibodies	Delayed allograft rejection Decreased IFNγ, IL-2, IL-6 production Increased IL-4, IL-5, IL-13 production	[75]
MHC-mismatched heart BALB/c → B6 *Cd4-cre;ROSA*^{DNMAML} hosts systemic neutralizing Dll1/Dll4 antibodies	Delayed allograft rejection Decreased IFNγ production Decreased serum antibodies and complement deposition with systemic neutralization of Dll1/4	[74]

B

Graft-versus-host-disease model **Modulation of Notch**	**Outcome** **Suggested mechanism**	**Citation**
B6 → BALB/c major mismatch B6 → B6xDBA/2 F1 *Cd4-cre; ROSA*^{DNMAML} donors *Cd4-cre;rbpj*$^{fl/fl}$ donors	Protection from GVHD-induced mortality Decreased IFNγ, TNFα, IL-17, IL-2 production Increased T_{reg} numbers	[71]
B6 → BALB/c major mismatch *Cd4-cre;notch1*$^{fl/fl}$*;notch2*$^{fl/fl}$ donors gamma secretase inhibitors (GSIs) systemic neutralizing N1/N2 antibodies systemic neutralizing Dll1/4 antibodies	Protection from GVHD-induced mortality Decreased IFNγ, IL-2 production Increased T_{reg} numbers	[72]
B6 → BALB/c major mismatch B6 → BALB/b minor mismatch *Cd4-cre; ROSA*^{DNMAML} hosts *Cd4-cre;rbpj*$^{fl/fl}$ hosts	Protection from GVHD-induced mortality Blunted MAPK and NFκB signaling Increased expression of negative regulators of T cell signaling	[73]
B6 → B6xBALB/c F1 major mismatch *Mx1-cre;notch1*$^{fl/fl}$ donors gamma secretase inhibitors (GSIs)	Protection from bone marrow failure Decreased IFNγ, Gzmb production Decreased expression of T-bet	[77]
B6 → BALB/c major mismatch B6 → B6xDBA/2 F1 systemic neutralizing Dll4 antibodies	Protection from GVHD-induced mortality Decreased IFNγ, IL-17 production	[76]
B6 → BALB/c major mismatch *Foxp3-cre;rbpj*$^{fl/fl}$ donors *Foxp3-cre;notch1*$^{fl/fl}$ donors	Protection from GVHD-induced mortality Increased T_{reg} survival, numbers Decreased IFNγ production by Teff	[78]

Table 1.1. Summary of published work using *in vivo* loss-of-function approaches to evaluate the role of Notch signaling in T cell alloimmunity

A. Allograft rejection

B. Graft-versus-host disease

used a Jagged2-specific antibody in mouse heart allograft rejection models [81]. This antibody was previously shown to specifically bind Jagged2, but was suggested to facilitate forward Notch signaling in an *in vitro* coculture system through unknown mechanisms [82]. Jagged2-specific antibodies induced accelerated rejection in two heterotopic heart transplantation models. Accelerated rejection was associated with complex immunological changes, including increased production of IL-6 and Th2 cytokines, and increased T_{reg} expansion. These findings suggest that Jagged2 can have a proinflammatory role in allograft rejection, but interpretation is challenging, as the biochemical impact of the Jagged2-specific antibody used in these studies is not fully characterized. Future work using genetic approaches and other pharmacological reagents could clarify the role of Jagged ligands in transplant rejection.

Although there are differences in experimental approaches, particularly in terms of global Notch inhibition in T cells vs. selective systemic targeting of Notch pathway components, studies of allograft rejection and *in vivo* Notch inhibition delineate elements of an emerging consensus: 1) Notch signaling is a major pathway that promotes inflammation and opposes tolerance in allograft transplantation; 2) Notch signaling controls alloreactive T cell immunity, but may also regulate non-T cell subsets that contribute to the pathogenesis of organ rejection; 3) Targeting the Notch pathway has therapeutic potential to prevent allograft rejection, with short-term blockade of Delta-like Notch ligands in the peri-transplant period capable of inducing long-term effects.

In vivo studies of Notch signaling in graft-versus-host disease

Table 1.1B highlights the studies that employed loss-of-function approaches to interrogate the role of Notch signaling in GVHD [71-73, 76, 77]. Using DNMAML expression or *Rbpj*

inactivation to block all canonical Notch signals in T cells, Zhang et al. first reported major protective effects of Notch inhibition in mouse models of acute GVHD [71]. Notch inhibition led to markedly increased survival of transplant recipients. Notch-deprived alloreactive T cells showed decreased production of multiple inflammatory cytokines (including IFNγ, TNFα, IL-17 and IL-4) and increased expansion of preexisting T_{regs} [71, 73]. Decreased cytokine production was observed in both $CD4^+$ and $CD8^+$ T cells, and was associated with features of acquired hyporesponsiveness in alloreactive T cells [73]. Individual T cell effector functions were affected to a variable extent by Notch inhibition, as *in vivo* T cell proliferation and expansion were preserved in irradiated recipients. T cell cytotoxic functions were also largely maintained in the absence of Notch signaling, leading to the preservation of potent graft-versus-tumor effects. Using a genetic strategy to inactivate the *Notch1* or the *Rbpj* gene only in T_{regs}, Chatila's group reported that Notch negatively regulates T_{reg} numbers and function *in vivo*, and that Notch inhibition in Tregs alone conferred therapeutic benefits in acute GVHD [78]. Thus, Notch inhibition may exert beneficial immunomodulation in conventional $CD4^+$ and $CD8^+$ T_{eff} as well as in T_{regs}.

Therapeutically, γ-secretase inhibitors (GSI) were shown to be effective in a mouse model of alloimmune bone marrow injury [77]. However, in acute GVHD models involving lethal irradiation, systemic pan-Notch inhibition with GSI was poorly tolerated because of on-target toxicity in the gut [72]. To bypass this toxicity, the role of individual Notch ligands and receptors was investigated using genetic models and paralog-specific monoclonal antibodies [72, 76]. Notch1/Notch2 receptors and Dll1/4 Notch ligands accounted for all the effects of Notch signaling in alloreactive T cells during GVHD, with dominant roles for Notch1 and Dll4. Dll1/4 blockade emerged as the most promising therapeutic approach to prevent GVHD while avoiding

system side effects of pan-Notch inhibition. Interestingly, transient early Dll1/4 inhibition was essential and sufficient to confer long-term GVHD protection [72]. Altogether, clear parallels are emerging between the functions of Notch signaling in acute GVHD and allograft rejection. In both cases, early Dll1/4-mediated Notch signals exert profound and durable pro-inflammatory effects, such that transient Dll1/4 inhibition provides long-lasting therapeutic benefits.

Mechanistic considerations

The molecular mechanisms of Notch action in mature T cells remain under active investigation. The most relevant observations are and will continue to be derived from *in vivo* experiments that evaluate physiological levels of Notch signaling in defined immunological contexts. Along these lines of investigations, Notch was recently reported to regulate specific functions of $CD4^+$ and $CD8^+$ T cells, including *in vivo* survival and metabolism, responsiveness to CD28-mediated costimulatory signals, and $CD8^+$ T cell differentiation [66, 68, 70, 83-85]. An important overarching theme is that Notch does not appear to function as a lineage-specific regulator, but instead as a regulator of T cell reactivity and function. In T cell alloimmunity, multiple investigators observed that Notch inhibition tips the balance between inflammatory T_{eff} and suppressive T_{reg} functions (**Fig. 1.3**) [71, 74, 78]. Notch-deficient T_{eff} cells appear defective in their production of multiple inflammatory cytokines, while Notch-deficient T_{regs} accumulate in higher absolute or relative numbers and may have enhanced suppressive ability. The dual effects on both T_{eff} and T_{regs} likely account for the prolonged impact of transient Notch inhibition. Of note, key downstream effects of Notch signaling are likely to be mediated by canonical

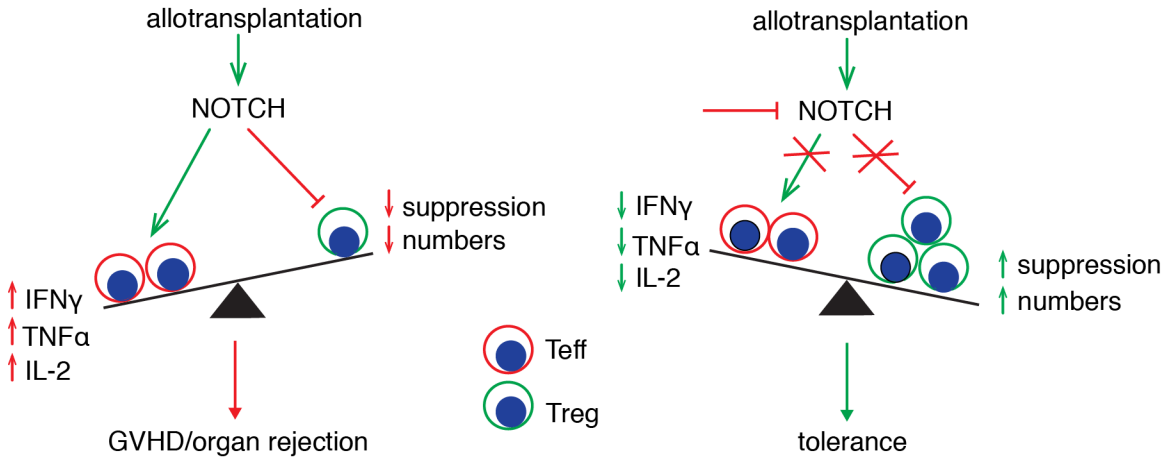

Figure 1.3. Emerging model of Notch signaling as a central regulator of alloreactivity vs. tolerance.
Notch drives T cell pathogenicity during allotransplantation by enhancing pathogenic functions in effector T cells, while decreasing numbers and beneficial immunosuppressive functions of FoxP3$^+$ regulatory T cells (T_{regs}). Interfering with the Notch pathway can reverse this imbalance by dampening proinflammatory cytokine production by T_{eff} cells and enhancing both T_{reg} function and numbers. Importantly, short-term Notch inhibition in the peri-transplant period can confer long-lasting immunological benefits.

CSL/MAML-dependent transcriptional mechanisms [71, 78], but the functionally essential targets of Notch signaling in T_{eff} and T_{regs} remain to be identified.

Therapeutic implications and future directions

Based on available preclinical data, we propose that Notch signaling is an attractive new therapeutic target to prevent allograft rejection. Short-term inhibition of Notch signaling exerts a longstanding beneficial impact by dampening the alloimmune response, highlighting the promise of transient Notch inhibition strategies in the peritransplant period [72, 74]. Beyond allograft rejection, Notch inhibition could also be beneficial in other T cell-mediated immune disorders, including GVHD and autoimmunity [72, 86, 87]. In practice, targeting individual Notch ligands and receptors with specific monoclonal antibodies currently appears to be the most promising therapeutic approach to target Notch signaling in alloimmune cells, while avoiding the systemic

side effects of pan-Notch inhibition [72, 74, 75]. As for other strategies, an important challenge will be to translate these findings from preclinical mouse models into more advanced preclinical models (e.g. non-human primates) and into humans. Given that Notch is an ancient and highly conserved signaling pathway, it is tempting to speculate that key features of its effects will be conserved, although this needs to be investigated systematically. Advances in our understanding of Notch's immunobiological effects and carefully designed translational investigations could unravel the full therapeutic potential of Notch inhibition in allograft rejection and other immune-mediated disorders.

Chapter 2

Fibroblastic Reticular Cells in Immunity

Secondary lymphoid organs (SLOs), which include lymph nodes, spleen, Peyer's patches and lymphoid aggregates associated with mucosal tissues, are key organizational hubs that ensure efficient immune responses against foreign antigens. Rare antigen-specific cells utilize the unique organization and strategic location of SLOs to encounter their cognate antigens. Consequently, SLOs also serve as major enablers of inappropriate responses against foreign tissue antigens or self-antigens.

One important feature of SLOs is their highly compartmentalized nature – antigen-responsive effector cells, such as T cells and B cells, are restricted to distinct microanatomical locations. This feature not only helps rare antigen-specific effector cells encounter their cognate antigen to promote their activation, but also serves as a mechanism for preventing inappropriate immune responses. Much of the structural organization of SLOs is determined by nonhematopoietic stromal cells, which serve as the cellular backbone. Additionally, recent literature has revealed that nonhematopoietic stromal cells can play active roles in immune responses. In this chapter, I will review the diverse functions of stromal cells with a special focus on fibroblastic reticular cells (FRCs), and how they contribute to appropriate immune system function.

Diversity of stromal cells within SLOs

Within SLOs, radioresistant stromal cells are a heterogeneous population, and include blood endothelial cells (BECs), lymphatic endothelial cells (LECs), follicular dendritic cells (FDCs), marginal reticular cells (MRCs), and fibroblastic reticular cells (FRCs). These cells can be distinguished from each other by their anatomical location, morphology, and surface marker expression.

Vascular blood endothelial cells (BECs)

BECs play a critical role in providing SLOs with vascular support. Among BECs, high endothelial venules [88] are a small subset of specialized postcapillary venules that are absolutely critical for the entry of naïve lymphocytes into the LN parenchyma. HEVs transcytose and present key recruitment chemokines on their cell surface to attract T cells [89, 90]. BECs can be distinguished from other SLO-resident stromal cells by their expression of the surface markers CD31/PECAM-1, and the lack of expression of the surface markers gp38/podoplanin and CD45.

Lymphatic endothelial cells (LECs)

LECs line the afferent and efferent lymphatic vessels that transport interstitial fluid into and away from LNs, respectively. Aside from facilitating the delivery of antigens, several reports have identified LECs as enforcers of peripheral tolerance through the cross-presentation of self-antigen [91]. LECs can be distinguished from other SLO-resident stromal cells by their expression of the surface markers gp38/podoplanin, CD31/PECAM-1, and LYVE-1, and their lack of expression of CD45.

Follicular dendritic cells (FDCs)

FDCs form the structural backbone on which B cells traffic and encounter antigen [92]. They are important cellular sources of the B cell chemoattractant CXCL13. During germinal center responses, FDCs and T follicular helper cells (Tfh) engage with maturing B cells to undergo positive selection of the highest affinity clones [93]. FDCs can be distinguished from other SLO-resident stromal cells by their expression of the surface markers gp38/podoplanin, CD21/35, and FcγR, and their lack of expression of CD45 and CD31/PECAM-1.

Marginal reticular cells (MRCs)

First identified in 2008, MRCs are physically located within the outer ridge of B cell follicles, underneath the subcapsular sinus [94]. Lineage tracing studies suggest that MRCs are precursors of FDCs, and can be induced to develop into FDCs with inflammation [95]. MRCs can be distinguished from other SLO-resident stromal cells by their expression of the surface markers gp38/podoplanin, MAdCAM-1, and RANKL, and their lack of expression of CD45 and CD31/PECAM-1.

Fibroblastic reticular cells (FRCs)

Constituting 20-50% of the stromal cells within the LN, FRCs are a heterogeneous population of mesenchymal origin. Like myofibroblasts, FRCs exhibit high contractile activity, and generate physical tension throughout the LN. FRCs are derived from lymphoid tissue inducer cells (LTo), and require lymphotoxin (LT) signaling for their appropriate development. FRCs can be distinguished from other SLO-resident stromal cells by their expression of the surface markers gp38/podoplanin and CD140α/platelet-derived growth factor receptor α, and their lack of expression of CD45 and CD31/PECAM-1. Recent characterization of FRC subsets

have identified at least 3 different subtypes: 1) T zone FRCs; 2) Medullary FRCs; 3) Pericytic FRCs. Subsets of FRCs are distinguished from each other by their anatomical location within the LN.

FRCs as key structural components

The potential structural importance of stromal cells, particularly FRCs, was first appreciated in anatomical studies of LN sections [96]. Silver staining identified a large network of collagen fibrils and extracellular matrix (ECM) that was completely ensheathed by FRCs; together, this structural backbone of ECM, collagen, and FRCs was termed the reticular network. T and B lymphocytes were completely excluded from this 'labyrinthine cavity,' suggesting that FRCs served as a physical barrier that prevented lymphocytes from directly encountering the structural backbone [97]. Later work revealed that FRCs play a far more active role than simply as physical barriers. Immunofluorescence staining with the monoclonal antibody ER-TR7, which binds a yet unidentified epitope on FRCs, demarcated the LN into four major subanatomic regions: 1) Subcapsule-associated reticular network; 2) B cell-associated reticular network; 3) T cell-associated reticular network; 4) Medullary reticular network [98]. ER-TR7 staining also enabled the first identification of the cortical ridge as a unique anatomical structure that was highly enriched for both DCs and HEVs.

FRCs form conduits for the rapid transit of antigen and chemokines

Elegant studies with fluorescently labeled model antigen determined that dendritic cells acquire subcutaneously delivered antigen in two temporally distinct phases [99]. While the first wave occurs within minutes after antigen injection, a second wave of antigen acquisition occurs

4-14 hours later. The earlier phase of antigen delivery is restricted to smaller antigens, as only low molecular weight particles (<70 kDa) are able to enter the LN [100].

3D reconstruction studies of LN sections immediately after subcutaneous delivery of fluorescently labeled tracers revealed that the FRC-ensheathed reticular network is responsible for the first delivery phase of antigen [101]. Upon entering the sinuses of the draining lymph node, antigen was found to rapidly travel through a multilayered conduit that consisted of a collagen I/collagen III core surrounded by an ECM layer of fibrillin-1/fibrillin-2, a basement membrane, and a layer of FRCs. Fluorescently labeled tracer was restricted to the luminal side of the basement membrane, suggesting that it was self-contained within the conduit. Systematic immunofluorescence staining of the conduit's basement membrane revealed that it consisted of laminin 10, laminin 8, nidogen, perlecan, collagen IV, and fibronectin. Immature $CD11b^+$ resident DCs, but not migratory mature DCs, closely associated with the basement membrane of the conduits through β1 integrin-mediated adhesion to laminin 10/laminin 8. Thus, it was concluded that FRCs form a self-enclosed conduit that delivers antigen to uniquely positioned resident DCs, allowing for rapid antigen acquisition.

FRCs as scaffolds for naïve T cell trafficking

Two-photon excitation microscopy led to the direct visualization of mature T trafficking within SLOs [102, 103]. In these studies, while naïve T cells were generally found to move in a random fashion, they often displayed tendencies to abruptly change direction. Thus, it was hypothesized that T cells were being "guided" along scaffolds, perhaps along the reticular network formed by FRCs.

Consistent with this hypothesis, quantification of images from fixed tissue sections demonstrated that naïve T cells nearly always colocalized with the FRC-specific antigen ER-TR7 [92]. Furthermore, intravital imaging studies of popliteal LNs from live BM chimeras that expressed GFP only within nonhematopoietic tissues (WT→UBI-GFP) revealed that fluorescently labeled T cells actively crawled along the fibers of GFP^+ FRCs. Notably, there was a very high correlation (>90%) between changes in T cell direction and corresponding changes in FRC fiber direction. Thus, it was concluded that FRC fibers provide a directional scaffold along which naïve T cells migrate.

In the same study, confocal imaging of static sections from WT→UBI-GFP chimeras identified a population of $desmin^+$ GFP^+ $ER\text{-}TR7^+$ FRCs that completely surrounded $PNAd^+$ HEVs. Thus, it was hypothesized that this specialized subset of FRCs was important for regulating the entry of lymphocytes into the LN parenchyma via the bloodstream. Indeed, adoptively transferred naïve T cells traversed across HEVs to enter the LN parenchyma via discrete spaces between adjacent FRCs. Interestingly, these "exit ramps" were not uniformly distributed throughout the length of the blood vessel, but instead located in fixed locations. Thus, it was concluded that FRCs not only provide guidance cues for trafficking within the lymph node, but also actively regulate the entry of naïve T cells into the LN cortex by forming discrete, fixed access points.

FRCs as important sources of chemokines and cytokines

CCL19/Epstein-Barr virus-induced molecule-1 ligand chemokine (ELC) and CCL21/Secondary Lymphoid organ chemokine (SLC) recruit naïve lymphocytes and DCs into SLOs through ligation of their cognate receptor, CCR7. *Paucity of lymph node T cells (plt)/plt*

mice, which harbor autosomal recessive mutations in both *ccl19* and *ccl21*, display defective migration of lymphocytes and DCs into SLOs, leading to high susceptibility to viral infections [104]. Early *in situ* hybridization studies identified $gp38^+$ radioresistant stromal cells as the major sources of both *ccl19* and *ccl21* [105]. Consistent with this notion, transfer of WT BM into *plt/plt* mice was insufficient to rescue the defects in expression of either chemokine. Thus, these data identified an essential role for FRCs in the recruitment of lymphocytes and DCs into the LN. Subsequent studies demonstrated that FRCs also secrete the prosurvival cytokine IL-7 to ensure naïve T cell homeostasis [106]. Inhibition of naïve T cell access to LNs with anti-CD62L neutralizing antibodies resulted in decreased T cell numbers in the spleen and blood. Furthermore addition of *ex vivo*-isolated FRCs to naïve T cell cultures enhanced their survival in an IL-7/Ccl19-dependent manner.

Unbiased transcriptional profiling confirmed that FRCs expressed high levels of chemokines and ECM components [107]. Consistent with the previously mentioned functional studies, FRCs produced significantly high levels of for *ccl19, ccl21a,* and *il7* transcripts. FRCs also expressed high levels of vascular trophic factors, metalloproteases, and cell adhesions molecules. Interestingly, *in vivo* exposure of FRCs to an active immune response upregulated many components of the MHC class II presentation machinery, suggesting they could be involved in antigen processing during inflammatory conditions. FRCs transcriptionally upregulated both alpha and beta chains of MHC class II and several intracellular components of the antigen presentation pathway (invariant chain, cathepsin S, H2-DM). FRCs also upregulated surface levels of MHC class II.

FRCs mediate contraction and expansion of the lymph node

Upon initiation of an immune response, the lymph node must expand and swell to accommodate the influx of new immune cells. This expansion is mediated by two general mechanisms: 1) proliferation and expansion of FRCs [108]; 2) abrogation of FRC contractile activity [109, 110]. Mechanistically, the contractile activity of FRCs is controlled by receptor/ligand interactions between FRC-derived podoplanin/gp38 and DC-derived C-lectin receptor CLEC-2. During homeostatic conditions, the low availability of CLEC-2 ligand allows for unhindered podoplanin signaling, resulting in the recruitment and phosphorylation of the ezrin/radixin/moesin (ERM) complex. Phosphorylation of ERM results in the phosphorylation of myosin light chain and subsequent activation of RhoA to promote contractile activity. During inflammatory conditions, DCs upregulate CLEC-2, and thus inhibit the signaling capability of podoplanin. Consequently, ligation of podoplanin by CLEC-2 inhibits ERM phosphorylation and RhoA-mediated contractile activity. Accordingly, genetic inactivation of *podoplanin* on FRCs or *clec2* on DCs results in persistent LN contractile activity, and subsequent failure to accommodate the influx of new cells. Therefore, direct signaling between FRCs and DCs drives the contractile activity of LN.

FRCs as enforcers of peripheral tolerance

Tolerance to self-antigens, or the decision to not undergo a full inflammatory response, is an important aspect of immunity. Immune tolerance is enforced through two general mechanisms, first through negative selection of autoreactive developing T cell clones within the thymus (termed central tolerance), and second through deletion or disabling of self-antigen responders that escape negative selection within the thymus (termed peripheral tolerance). Maintenance of

tolerance to self-antigen is of paramount importance, as failure to enforce central or peripheral tolerance results in autoimmunity and exaggerated immune responses. During peripheral tolerance of CD8+ cells, self-antigen responsive CD8+ T cells proliferate in response to antigen initially, but undergo clonal deletion through apoptotic mechanisms.

It was widely believed that circulating tolerogenic DCs played a key role in enforcing peripheral tolerance. However, this notion was challenged by a series of *in vivo* experiments in a mouse model of peripheral tolerance, in which ovalbumin (OVA) is expressed by intestinal epithelial cells (IECs) within the small bowel (iFABP-tOVA) [111]. When transferred into iFABP-OVA mice, OVA-specific TCR transgenic $CD8^+$ OT-I cells proliferated not only within the site of antigen expression (mesenteric LN, inguinal LN), but also in the periphery (inguinal, brachial, renal LNs). Interestingly, inhibition of T cell egress with the sphingosine 1-phosphate agonist FTY720 did not prevent the detection of proliferated OT-I cells in the periphery, thus ruling out T cell migration as an explanation and rather suggesting that OVA was presented in the periphery by an alternative cell source. This cellular source of OVA antigen was nonhematopoietic, as bone marrow (BM) chimeras lacking MHC I specifically within hematopoietic cells (but not nonhematopoietic cells) remained capable of promoting OT-I proliferation in the periphery. Careful quantitative PCR (qRT-PCR) analysis of various LN-resident nonhematopoietic cell subsets, including FRCs, BECs, and LECs, demonstrated that FRCs expressed not only OVA, but also other self-antigens. Indeed, *in vitro* coculture of FRCs from iFABP-tOVA mice with OT-I T cells, in the absence of classical APCs such as DCs, resulted in their intense proliferation and upregulation of CD25 [112].

FRCs as negative regulators of T cell proliferation

In 2011, three groups independently published the surprising finding that FRCs suppress T cell proliferation in a dose-dependent manner when added to T cell/antigen-pulsed DC cocultures [113-115]. These results were quite surprising, as it had been previously published that FRCs themselves were capable of stimulating T cell proliferation in the absence of classical APCs. Similar suppressive effects were observed when FRCs were cultured with CD3/CD28-activated OT-I cells. Suppression by FRCs occurred in an IFNγ-dependent manner, as genetic deletion of IFNγR within FRCs abrogated their ability to suppress OT-I proliferation. IFNγ signaling-mediated suppression occurred independently of the classic inhibitory molecule PD-L1, and instead was dependent on the production of NOS2 and COX1/2-dependent factors by FRCs. Pharmacologic inhibition of NOS2 or COX1/2, but not inhibition of IDO-1 or Arg1, impaired the ability of FRCs to suppress OT-I proliferation *in vitro*. Consistent with this notion, mice genetically deficient in *nos2* engendered exuberant immune responses to antigen. Taken together, these data suggested a model in which FRCs, upon sensing T cell activation through their production of IFNγ, upregulate soluble inhibitory factors to serve as a brake for excessive T cell activation.

FRC niches as nonredundant sources of Delta-like Notch ligands during Notch-dependent immune responses

In 1999, it was reported that *Mx1-Cre*-mediated genetic inactivation of *notch1* in hematopoietic tissues of neonatal mice resulted in a cell-autonomous block in T cell development (see **Chapter 1, "Overview of Notch signaling"** for more detailed review of Notch signaling within the hematopoietic system) [27]. This was the first study that identified a

role for mammalian Notch1 within the hematopoietic system. Mice that lacked *notch1* within their hematopoietic tissues displayed a decrease in the frequencies of single positive (SP) and double positive [79] developing T cells, with a concomitant increase in the frequency of $B220^+$ B cells. Other hematopoietic lineages were unaffected. Subsequently, it was identified that while overexpression of either Delta-like1 (Dll1) or Delta-like4 (Dll4) Notch ligands within OP9 stromal cells could sustain T cell development *in vitro [116, 117],* only Dll4 was expressed within thymic epithelial cells. Genetic inactivation of *Dll4* with the thymic epithelial cell (TEC)-restricted Cre recombinase *Foxn1-Cre* phenocopied the thymic defect that was first identified in mice with conditional *notch1* inactivation. Thus, it was concluded that developing thymocytes received their Notch1-mediated signals exclusively from Dll4-expressing nonhematopoietic $Foxn1^+$ TECs [34, 118].

Similar loss-of-function studies subsequently identified three other hematopoietic cell types that were dependent on Notch signaling for their development and/or maintenance: 1) splenic marginal zone B cells (MZBs), which exclusively utilized Notch2-mediated signals from Dll1 [117, 119]; 2) splenic $Esam^{hi}$ $CD11c^+$ DCs, which exclusively utilized Notch2-mediated signals from an unknown Notch ligand [120, 121]; 3) spleen/LN-resident Tfh cells, which received their Notch signals through both Notch1 and Notch2 receptors from an unknown Notch ligand [122].

Seminal work from the Radtke and Luther labs determined the identity and cellular source of Notch ligands that were necessary for the development/maintenance of the three aforementioned Notch-dependent hematopoietic cell types [123]. To this end, Notch ligands were conditionally inactivated with tissue-specific Cre recombinases in a systematic fashion. Although DCs and BECs had been previously reported to express high levels of Delta-like Notch ligands [51, 124, 125], conditional inactivation of *Dll1* or *Dll4* with the DC-specific *Itgax-Cre* or the BEC-specific

Pdgfβ-CreERT had no impact on the development of MZBs, Esam^{hi} DCs, or Tfh cells. Additionally, BM chimeras that lacked *Dll1, Dll4, Jag1, or Jag2* specifically within their hematopoietic tissues ($Tg^{Mx1\text{-}Cre+}$;$Dll1^{\Delta/\Delta}$ → CD45.1, $Tg^{Mx1\text{-}Cre+}$;$Dll4^{\Delta/\Delta}$ → CD45.1, $Tg^{Mx1\text{-}Cre+}$;$Jag1^{\Delta/\Delta}$ → CD45.1, $Tg^{Mx1\text{-}Cre+}$;$Jag2^{\Delta/\Delta}$ → CD45.1) remained proficient in generating robust Tfh responses. Instead, all three hematopoietic Notch-dependent cell types were found to encounter their Notch ligands on secondary lymphoid organ-resident mesenchymal stromal cells, which could be selectively targeted with a *Ccl19-Cre* BAC transgene *[126]*. Conditional deletion of *Dll1* with *Ccl19-Cre* resulted in complete loss of MZB and Esam^{hi} DCs, while conditional deletion of *Dll4* with *Ccl19-Cre* resulted in markedly decreased Tfh frequencies. Consistent with these findings, $Tg^{Ccl19\text{-}cre+}$;$Dll1^{\Delta/\Delta}$ mice were partially defective in supporting antigen-dependent CD4$^+$ T cell proliferation due to their lack of Esam^{hi} DCs, while $Tg^{Ccl19\text{-}cre+}$;$Dll4^{\Delta/\Delta}$ mice displayed blunted germinal center responses when challenged with infectious stimuli due to their lack of Tfh cells.

Immunofluorescence microscopy, flow cytometry, and qRT-PCR analysis of Dll1 and Dll4 expression within secondary lymphoid tissues of $Tg^{Ccl19\text{-}cre+}$;ROSA26^{eYFP} reporter mice identified three major secondary lymphoid-resident stromal cell types as key cellular sources of Notch ligands – FRCs, MRCs, and FDCs. Importantly, *Ccl19-Cre* displayed no activity within hematopoietic tissues or BECs. Thus, these seminal studies identified a completely unexpected, novel role for nonhematopoietic LN/spleen-resident stromal cells, in particular FRCs, in fostering efficient immune responses through the delivery of critical Notch signals to developing immune cells. Mice that lacked Delta-like Notch ligand expression within their mesenchymal stromal cells were unable to form MZBs, Esam^{hi} DCs, and Tfh cells.

FRCs in allotransplantation

As the field of experimental GVHD has focused much of its attention on the role of hematopoietic cells in mediating disease, very little is known about the role of FRCs during allotransplantation. Recently, it was reported that FRCs are cellular targets of allogeneic T cells during GVHD [127]. In both major and minor-mismatched CD8-dependent models of GVHD, several nonhematopoietic stromal cell types, including $CD157^+$ FRCs and $PNAd^+$ HEVs, were selectively depleted from LNs within 14 days post-transplantation. This effect was independent of irradiation, and was dependent on FasL-mediated cytotoxicity. Functionally, mice that received allogeneic transplants displayed long-term humoral defects in response to immune challenge. Thus, these results identified LN-resident FRCs as an additional cellular target of GVHD.

Chapter 3

Fibroblastic niches prime T cell alloimmunity through Delta-like Notch ligands[2]

Abstract

Alloimmune T cell responses induce graft-versus-host disease (GVHD), a serious complication of allogeneic bone marrow transplantation (allo-BMT). Although Notch signaling mediated by Delta-like1/4 (Dll1/4) ligands emerged as a major regulator of GVHD pathogenesis, little is known about the timing of Notch signals and the cellular source of Notch ligands after allo-BMT. Here, we show that Dll1/4-mediated Notch signals are delivered to donor T cells during a critical 48-hour window after transplantation. Surprisingly, stromal but not hematopoietic cells were the essential source of Notch ligands. Selective *Dll1/Dll4* inactivation in host chemokine *Ccl19*$^{+}$ fibroblastic reticular cells prevented GVHD. Neither T cell recruitment nor initial activation were affected, indicating selective effects of stromal Dll1/4 ligands on alloimmune tolerance and reactivity. Our results reveal a previously unrecognized Notch-mediated

[2] Taken from:

Chung, J., Ebens C.L., Radojcic, V., Perkey E., Koch U., Scarpellino, L., Tong, A., Allen, F., Wood, S., Feng, J., Friedman A., Granadier, D., Tran, I.T., Chai, Q., Onder, L., Yan M., Reddy P., Blazar, B.R., Huang, A.Y., Brennan, T.V., Bishop, D.K., Ludewig, B., Siebel, C.W., Radtke, F., Luther, S.A., Maillard, I. *Fibroblastic niches prime T cell allimmunity through Delta-like Notch ligands.* Submitted

immunopathogenic role for stromal cell niches in secondary lymphoid organs, thus defining a new framework of early cellular and molecular interactions that regulate T cell alloimmunity.

Introduction

Allogeneic bone marrow transplantation (allo-BMT) can cure hematological malignancies and other blood disorders. However, alloimmune T cell responses arising against foreign tissue antigens can trigger major complications after allo-BMT, such as graft-versus-host-disease (GVHD) [5, 128]. At the onset of GVHD, donor T cells are exposed to host tissue alloantigens in a highly inflammatory environment, inducing potent T cell immunoreactivity and subsequent pathogenicity. Current GVHD prophylactic and therapeutic strategies act through global immunosuppression, and thus diminish both beneficial and detrimental aspects of T cell alloreactivity. Efforts to develop new selective therapies to dampen GVHD have focused on early microenvironmental signals to donor alloreactive T cells [129]. Many of these signals, which include alloantigens, costimulatory ligands and local inflammatory mediators, have been assumed to derive from hematopoietic antigen-presenting cells (APCs). However, recent work demonstrated that $CD4^+$ T cell-mediated alloresponses can occur in the absence of hematopoietic APCs as a source of alloantigens [130-132], suggesting that our current understanding of key early cellular and molecular events that drive donor T cell-mediated GVHD is incomplete.

The Notch pathway has emerged as a new attractive therapeutic target to control deleterious effects of T cell alloimmunity [71-75, 77, 78]. Notch signaling is a conserved cell-to-cell communication pathway mediated by interactions between Notch1-4 receptors and its ligands Delta-like1/3/4 (Dll1/3/4) or Jagged1/2 [20, 21]. During GVHD, Dll1/4 ligands in the host engage Notch1/2 receptors in T cells, and transient systemic blockade of Dll1/4 Notch ligands with neutralizing antibodies results in long-term protection from GVHD [72]. Despite the central

role of Notch signaling in alloreactivity, the timing of critical Notch signals, the cellular source of Notch ligands and the microanatomical context in which alloreactive T cells are exposed to Notch signaling *in vivo* remain unknown.

Early studies showed that hematopoietic APCs such as dendritic cells (DCs) can express Dll1 and Dll4 ligands in a Toll-like receptor (TLR)-inducible manner [51, 124]. These observations led to the widely accepted concept that hematopoietic APCs can simultaneously deliver antigen and Notch ligands to modulate T cell function. *In vitro* studies supported this model, as TLR agonist-stimulated antigen-pulsed DCs induced naïve T cells to differentiate in a Notch-regulated manner [51, 53]. Similarly, a subpopulation of $CD11c^{+}Dll4^{hi}$ DCs was capable of delivering Notch signals to alloreactive T cells in mixed lymphocyte reactions when purified from GVHD animal models [76]. However, the *in vivo* relevance of APC-derived Notch signals has not been rigorously tested, and their importance has been inferred indirectly based on their capacity to modulate T cell responses *in vitro*. Non-hematopoietic cells also express Notch ligands in multiple contexts, including in primary and secondary lymphoid organs (SLOs). In the thymus, $Foxn1^{+}$ thymic epithelial cells act as non-redundant transducers of Dll4-mediated signals during T cell development [34, 118, 133]. Blood and lymphatic endothelial cells (BECs/LECs) express high levels of Dll1 and Dll4 [125, 134-137]. Finally, genetic studies identified fibroblastic reticular cells (FRCs) in SLOs as non-redundant sources of Dll1-mediated Notch signals to marginal zone B cells and $Esam^{hi}$ DCs, as well as Dll4-mediated signals to follicular helper T cells [123]. Thus, multiple cellular sources have the potential to deliver Notch signals to T cells *in vivo* after allo-BMT, making it unclear if critical signals are delivered in a defined microanatomical niche, and by hematopoietic or stromal cells.

To address these questions, we employed a combination of systemic neutralizing antibodies and loss-of-function genetics to interrogate the *in vivo* spatial and temporal requirements for Dll1/4-mediated Notch signaling during GVHD. Surprisingly, we found that all essential Notch signals were delivered to incoming T cells within 2 days after allo-BMT, and that both donor and host hematopoietic cells were dispensable as a source of Notch ligands that drives acute GVHD. In contrast, a defined subset of non-hematopoietic FRCs lineage-traced with a *Ccl19-Cre* transgene functioned as the essential non-redundant cellular source of Delta-like Notch ligands after allo-BMT. Interference with Notch ligands in FRCs had selective effects on T cell alloreactivity and did not impair other functions of these cells in immune homeostasis. These findings change our understanding of the key early cellular and molecular events that condition the outcome of T cell alloimmunity. In addition, they pave the way towards development of targeted therapeutic approaches to block Notch signaling and other stromal niche-derived pathogenic signals in GVHD and other T cell-mediated immune disorders.

Results

Early Delta-like1/4-mediated Notch signals drive T cell alloreactivity during acute GVHD

To understand the temporal requirement for Notch signaling after allo-BMT, we used neutralizing antibodies against Dll1 and Dll4 Notch ligands in an irradiation-dependent major histocompatibility complex (MHC)-mismatched mouse allo-BMT model (**Fig. 3.1A**). A single injection of Dll1 and Dll4-neutralizing antibodies before allo-BMT was sufficient to confer long-term protection from GVHD lethality and morbidity, while decreasing production of T cell proinflammatory cytokines and expanding $FoxP3^+$ regulatory T cells (Tregs) (**Fig. 3.1B-D, Fig. 3.2A**). In contrast, delayed initiation of antibody treatment by only two days resulted in loss of clinical protection, persistent IFNγ and TNFα production, and no increase in Tregs. Dll1/4

blockade inhibited Notch target gene expression in donor-derived alloreactive T cells (**Fig. 3.2B-C**). These data identify an early pulse of Notch signaling delivered within two days after allo-BMT that programs T cells to a pathogenic state of reactivity.

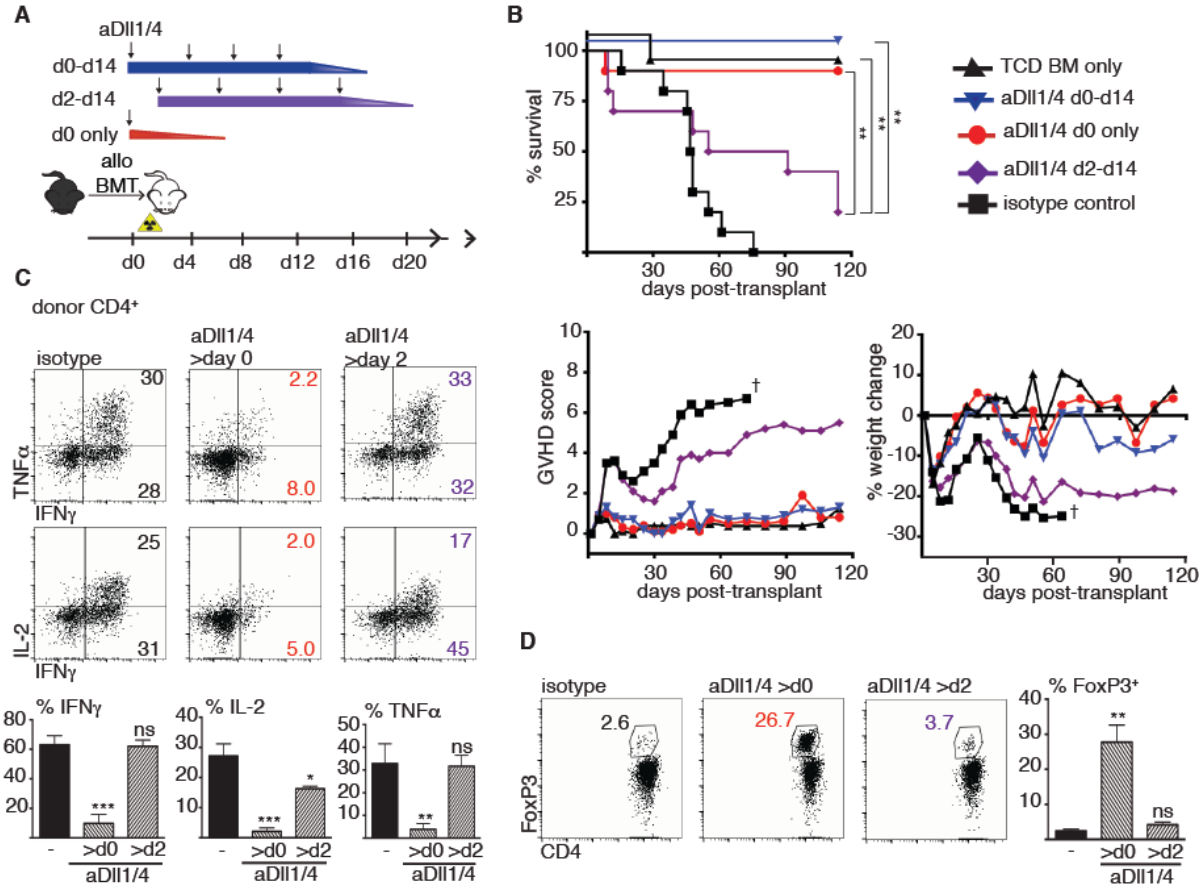

Figure 3.1. An early pulse of Notch signaling is critical to drive pathogenic T cell alloreactivity after bone marrow transplantation

A. Dosing schedule of systemic neutralizing antibodies against Dll1 and Dll4 Notch ligands. **B.** Survival, GVHD score and weight of lethally irradiated (8.5 Gy) BALB/c mice transplanted with $5x10^6$ T cell-depleted (TCD) B6 BM or $5x10^6$ TCD B6 BM + $5x10^6$ allogeneic B6 splenocytes. Isotype control vs. anti-Dll1/4 antibodies were injected i.p, as shown in **A** (n = 10 mice/group). **C.** Intracellular cytokine production by donor $CD4^+$ T cells after anti-CD3/CD28 restimulation at day 6 post-transplantation (n = 5 mice/group). **D.** Intracellular FoxP3 in donor $CD4^+$ T cells at day 6 (n = 5 mice/group). *P<0.05, **P<0.01, ***P<0.001. Data are representative of at least 4 experiments, with error bars indicating SD.

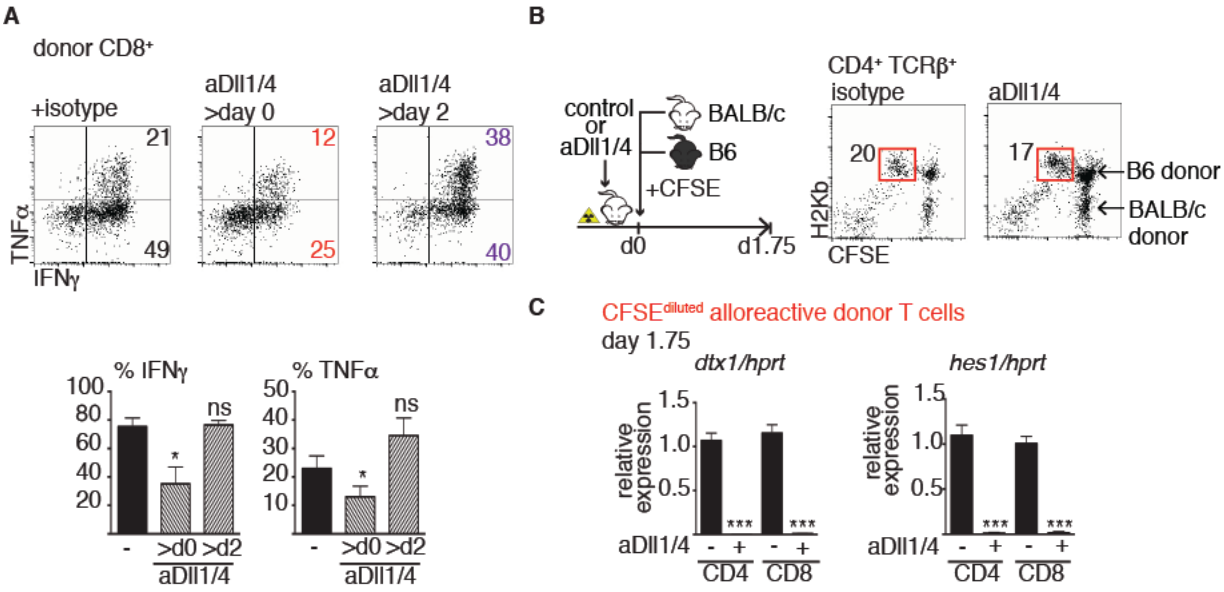

Figure 3.2. Impact of Notch blockade on donor CD8$^+$ proinflammatory cytokine production and Notch target gene expression

A, Detection of intracellular cytokines in donor CD8$^+$ T cells after anti-CD3/anti-CD28 restimulation at day 6 post-transplantation (flow cytometry) (n = 5 mice/group). **B,** Experimental strategy and flow cytometry plots to isolate alloreactive donor CD4$^+$ and CD8$^+$ T cells at day 1.75 post-transplantation. Syngeneic BALB/c and allogeneic B6 splenocytes were simultaneously labeled with CFSE and co-injected into lethally irradiated (8.5 Gy) BALB/c mice. Divided CFSE^{low} B6 cells identified alloreactive T cells and were sort-purified. **C.** Abundance of *Dtx1* and *Hes1* Notch target gene transcripts (qRT-PCR) in sort-purified donor-derived CFSE^{low} CD4$^+$ and CD8$^+$ T cells (n = 6 mice/group). Cell isolation was performed as described in (**B**). Data are representative of at least 4 experiments, with error bars indicating SD.

Donor and host hematopoietic cells are dispensable sources of Notch ligands during GVHD

The early window of sensitivity to Notch inhibition suggested that T cells might receive Notch signals from residual host hematopoietic antigen-presenting cells (APCs), which can express both alloantigens and Notch ligands [51, 53, 70, 76, 84, 138]. To test if hematopoietic APCs were responsible for delivering Notch signals to donor alloreactive T cells, we generated bone marrow (BM) chimeras lacking Dll1/4 only in hematopoietic cells with poly(I:C)-induced $Tg^{Mx1\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ BM (designated as $Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$→B6-CD45.1) (**Fig. 3.3A**). This strategy led to efficient Cre-mediated recombination and high donor chimerism in all

hematopoietic APCs (**Fig. 3.3B-C**). Allo-BMT into *Dll1*$^{\Delta/\Delta}$;*Dll4*$^{\Delta/\Delta}$→B6-CD45.1 chimeras as compared to control BM chimeras did not protect from GVHD mortality or morbidity (**Fig. 3.3D**). In contrast, systemic antibody-mediated Dll1/Dll4 blockade protected both *Dll1*$^{\Delta/\Delta}$;*Dll4*$^{\Delta/\Delta}$→B6-CD45.1 and control BM chimeras from GVHD lethality. Consistent with persistent exposure to Dll1/4 ligands in these mice, donor-derived T cells expressed equivalent amounts of Notch target gene transcripts in *Dll1*$^{\Delta/\Delta}$;*Dll4*$^{\Delta/\Delta}$→B6-CD45.1 and control chimeras, but transcript abundance remained sensitive to systemic anti-Dll1/4 antibodies (**Fig. 3.3E**). Thus, host hematopoietic cells were dispensable as a source of Notch ligands after allo-BMT.

To rule out redundant Dll1/4 expression in donor and host hematopoietic cells, we backcrossed *Tg*$^{Mx1\text{-}cre}$;*Dll1*$^{f/f}$;*Dll4*$^{f/f}$ mice to the BALB/c background, and generated BM chimeras with poly(I:C)-induced *Tg*$^{Mx1\text{-}cre+}$;*Dll1*$^{\Delta/\Delta}$;*Dll4*$^{\Delta/\Delta}$ donors (*Dll1*$^{\Delta/\Delta}$;*Dll4*$^{\Delta/\Delta}$→BALB/c) (**Fig. 3.4A**). Transplantation of *Dll1*$^{\Delta/\Delta}$;*Dll4*$^{\Delta/\Delta}$→B6-CD45.1 donor cells into *Dll1*$^{\Delta/\Delta}$;*Dll4*$^{\Delta/\Delta}$→BALB/c recipients (designated as *Dll1*$^{\Delta/\Delta}$;*Dll4*$^{\Delta/\Delta}$→*Dll1*$^{\Delta/\Delta}$; *Dll4*$^{\Delta/\Delta}$) had no impact on T cell cytokine production and Treg frequency (**Fig. 3.4B-D**), despite sensitivity to systemic Dll1/Dll4 blockade. Donor-derived T cells from *Dll1*$^{\Delta/\Delta}$;*Dll4*$^{\Delta/\Delta}$→*Dll1*$^{\Delta/\Delta}$;*Dll4*$^{\Delta/\Delta}$ recipient mice retained abundant Notch target gene transcripts (**Fig. 3.4E**). Therefore, both donor and host hematopoietic cells were dispensable sources of Dll1/4 Notch ligands during GVHD.

Ccl19*$^{+}$ *stromal cells are critical sources of Notch ligands during GVHD

Among multiple radioresistant non-hematopoietic cells that express Notch ligands in SLOs, FRCs act as a non-motile source of Notch ligands to immune cells in steady-state conditions [123]. To assess the importance of these cells during allo-BMT, we conditionally inactivated

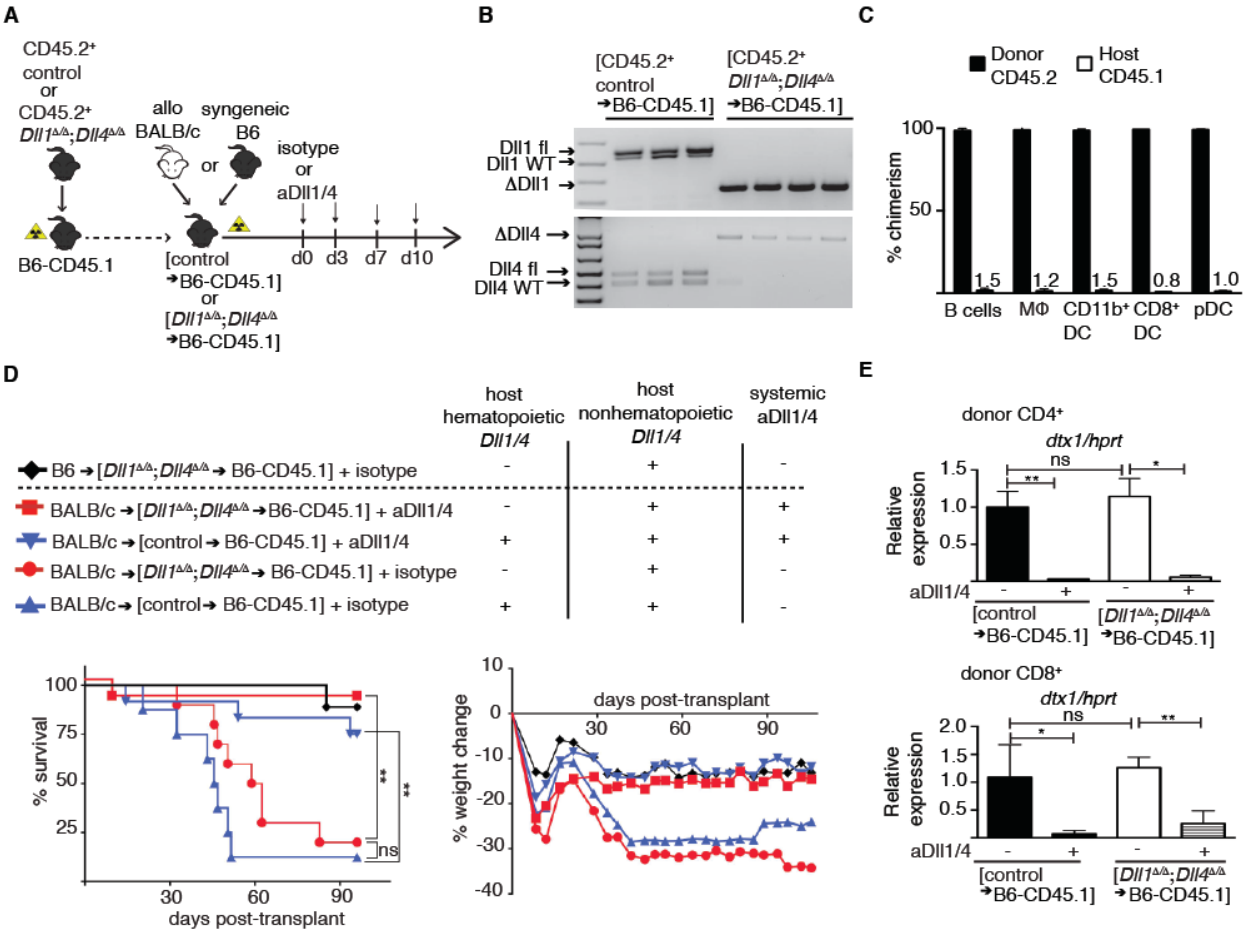

Figure 3.3. Host hematopoietic cells are dispensable as cellular sources of Delta-like1/4 Notch ligands in acute GVHD

A. Experimental strategy. Bone marrow (BM) chimeras were generated via transplantation of syngeneic B6-CD45.2$^+$ poly(I:C)-induced $Tg^{Mx1\text{-}cre-}$ littermate controls or $Tg^{Mx1\text{-}cre+}$;$Dll1^{\Delta/\Delta}$;$Dll4^{\Delta/\Delta}$ BM into irradiated B6-CD45.1 recipients. After reestablishment of steady-state hematopoiesis 12 weeks later, BM chimeras were subjected to a second syngeneic or allogeneic transplant, with or without systemic anti-Dll1/4 blockade. **B.** Quantification of *Dll1* and *Dll4* inactivation in sort-purified Gr1$^+$CD11b$^+$ blood myeloid cells from BM chimeras 12 weeks after transplantation (PCR). In this particular experiment, control BM chimeras were generated from poly(I:C)-induced $Tg^{Mx1\text{-}cre-}$;$Dll1^{fl/+}$;$Dll4^{fl/+}$ donor mice. Separate lanes represent individual mice. **C.** Donor chimerism (frequency of CD45.2$^+$ donor cells) in indicated spleen populations 12 weeks after transplantation. MΦ, macrophage; DC, dendritic cell; pDC, plasmacytoid DC. **D.** Survival and weight loss of lethally irradiated (11 Gy) BM chimeras transplanted with 8x10^6 TCD BM + 30x10^6 B6 splenocytes (syngeneic control) or 30x10^6 allogeneic BALB/c splenocytes (allo-BMT). Isotype control or anti-Dll1/4 antibodies were injected i.p. on days 0, 3, 7, 10 (n = 10 mice/group). **E.** Abundance of *Dtx1* Notch target gene transcripts (qRT-PCR) in sort-purified donor CD4$^+$ T cells and CD8$^+$ cells at day 6 (n = 5 mice/group). *P<0.05, **P<0.01. Data are representative of at least 2 experiments, with error bars indicating SD.

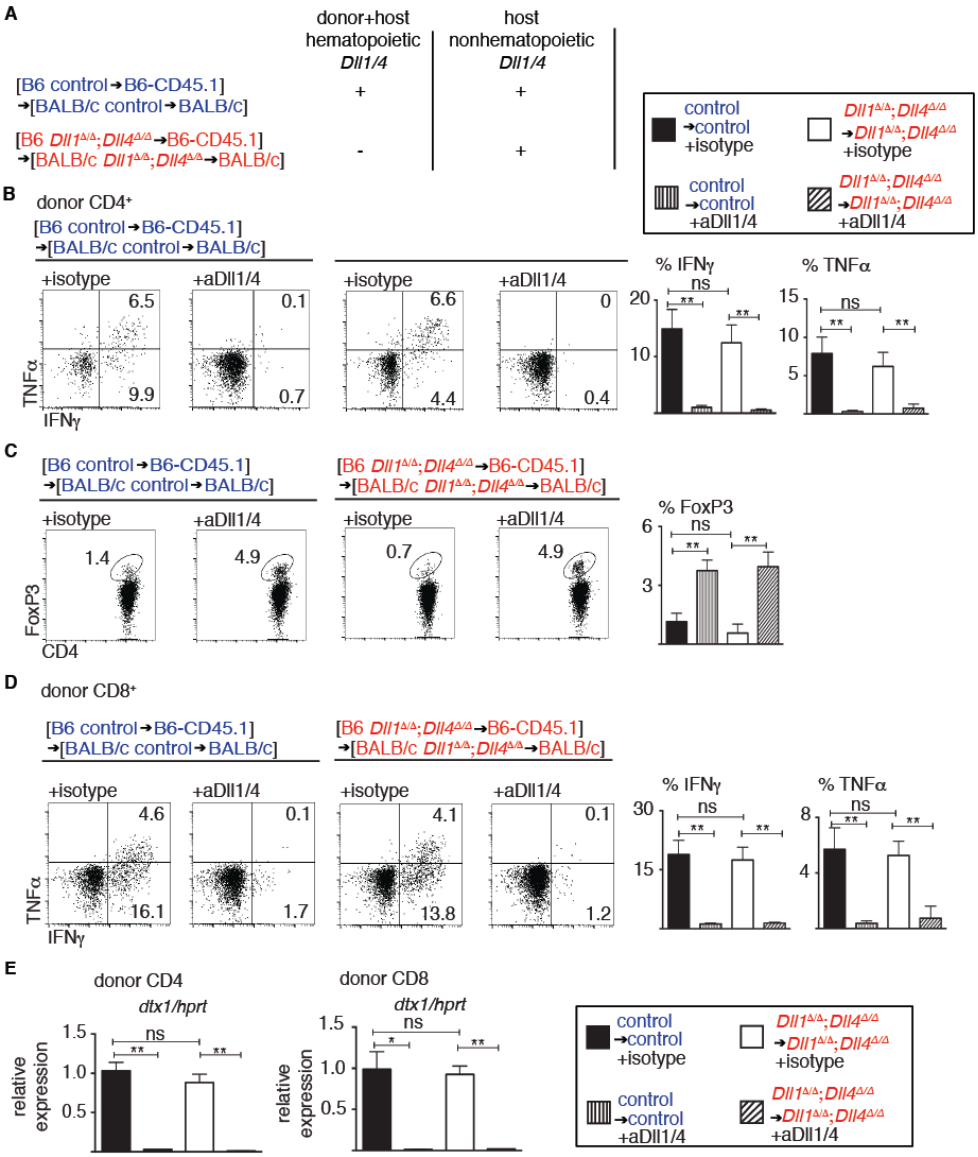

Figure 3.4. Both donor and host hematopoietic cells are dispensable as cellular sources of Delta-like Notch ligands

A, Experimental strategy. BALB/c bone marrow (BM) chimeras were generated by syngeneic transplantation of BALB/c poly(I:C)-induced control or $Tg^{Mx1\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ T cell-depleted (TCD) BM into irradiated BALB/c recipients (with T cell depletion performed to remove preexisting mature T cells that may escape *Mx1-Cre*-mediated target gene excision). B6 BM chimeras were generated by syngeneic transplantation of B6 poly(I:C)-induced control or $Tg^{Mx1\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ BM into irradiated B6-CD45.1 recipients. $5x10^6$ TCD BM + $5x10^6$ allogeneic splenocytes from B6 BM chimeras were transplanted into lethally irradiated (8.5 Gy) BALB/c BM chimeras. Recipient BALB/c BM chimeras were injected i.p. with isotype control or anti-Dll1/4 antibodies. **B.** Intracellular cytokines in donor $CD4^+$ after anti-CD3/anti-CD28 stimulation at day 7 post-transplantation (n = 5 mice/group). **C.** Intracellular FoxP3 in donor $CD4^+$ T cells at day 7 post-transplantation (n = 5 mice/group). **D.** Intracellular cytokines in donor $CD8^+$ T cells after anti-CD3/anti-CD28 stimulation at day 7 post-transplantation (n = 5 mice/group). **E.** Abundance of *Dtx1* Notch target gene transcripts (qRT-PCR) in sort-purified donor $CD4^+$ T cells or donor $CD8^+$ T cells at day 7 post-transplantation (n = 5 mice/group). *P<0.05, **P<0.01. Data are representative of at least 3 experiments, with error bars indicating SD.

Dll1 and *Dll4* with *Ccl19-Cre* (thereafter $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$) [126, 139, 140]. After allo-BMT, *Ccl19-Cre* lineage traced FRCs and a small fraction of LECs, but not BECs or any hematopoietic cells, including professional APCs (**Fig. 3.5A**, **Fig. 3.6A-B**). $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice were highly protected from GVHD lethality and morbidity (**Fig. 3.5B**). Markedly fewer donor T cells from $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta}Dll4^{\Delta/\Delta}$ recipients produced proinflammatory cytokines, as observed with systemic Dll1/4 blockade (**Fig. 3.5C**, **Fig. 3.7**). Notch target gene transcripts were profoundly decreased in donor-derived T cells isolated from $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ recipients (**Fig. 3.5D**). T cell cytokine production was preserved after allo-BMT into $Tg^{Ccl19\text{-}cre+};Dll1^{+/+};Dll4^{+/+}$ recipients, ruling out Cre toxicity (**Fig. 3.8**). Thus, *Ccl19-Cre*-expressing FRCs were the dominant source of Dll1/Dll4 Notch ligands encountered *in vivo* by donor T cells after allo-BMT.

Genetic inactivation of Dll1/4 ligands within Ccl19$^+$ stromal cells preserves immune homeostasis and classical FRC functions

FRCs provide survival cues to naïve T cells [106], form conduits for antigen trafficking [101], and support the overall SLO infrastructure [97, 141]. Physical loss of FRCs disrupts immune homeostasis, leading to profoundly impaired immune responses [139]. To determine if these FRC functions were preserved in $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice, we studied these mice during steady-state and in the peri-transplant period. SLOs from unirradiated $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice had normal lymphocyte numbers, naïve/memory T cell distribution and *Il7* expression in FRCs (**Fig. 3.9A-D**). After allo-BMT, normal numbers of donor T cells were recovered upon *Dll1*/*4* inactivation in FRCs, showing no impact on T cell homing (**Fig. 3.10A**). Donor T cell proliferation was maintained in $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ recipients (**Fig. 3.10B,E**),

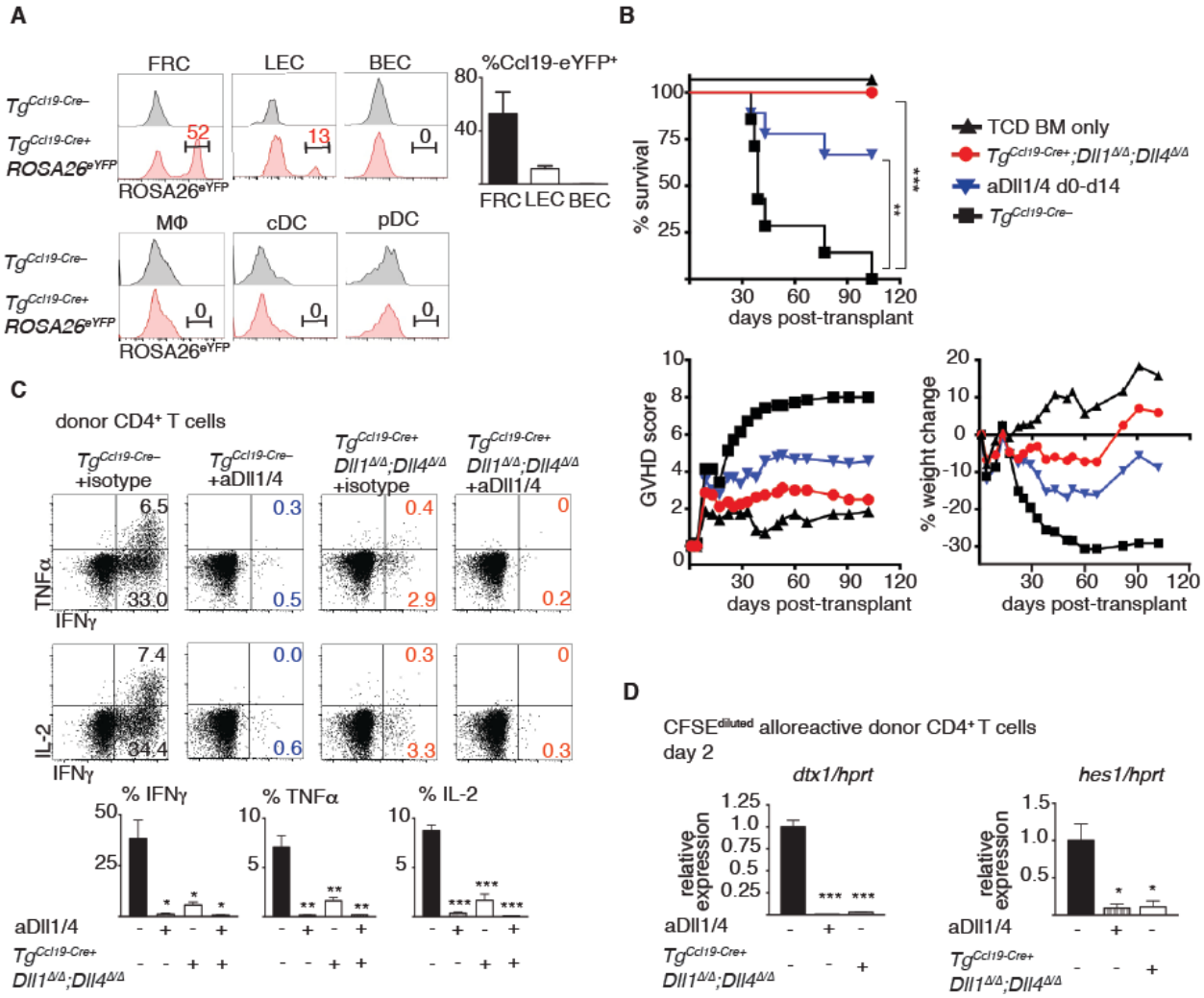

Figure 3.5. *Ccl19-Cre*$^{+}$ lineage-traced stromal cells are the critical cellular source of Delta-like1/4 Notch ligands during acute GVHD

A. eYFP expression in LN-resident fibroblastic reticular cells (FRCs), lymphatic endothelial cells (LECs), blood endothelial cells (BECs), macrophages (MΦ), conventional DCs (cDCs), and plasmacytoid DCs (pDCs) from lethally irradiated $Tg^{Ccl19\text{-}cre+};ROSA26^{eYFP}$ mice receiving allogeneic BALB/c splenocytes. LNs were collected at day 1.5 post-transplantation. **B.** Survival, GVHD score and weight of lethally irradiated (12 Gy) control $Tg^{Ccl19\text{-}cre-}$ or $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice that were transplanted with $10x10^6$ TCD BM only or $10x10^6$ TCD BM + $20x10^6$ allogeneic BALB/c splenocytes. Isotype control or anti-Dll1/4 neutralizing antibodies were injected i.p. on days 0, 3, 7, and 10 (n = 10 mice/group). **C.** Intracellular cytokines in donor CD4^{+} cells after anti-CD3/CD28 restimulation at day 6 (n = 5 mice/group). **D.** Abundance of *Dtx1* and *Hes1* Notch target gene transcripts in donor CD4^{+} T cells sort-purified from $Tg^{Ccl19\text{-}cre-}$+ isotype control, $Tg^{Ccl19\text{-}cre-}$+ anti-Dll1/4 or $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ recipient mice at day 2 post-transplantation (n = 5 mice/group). *P<0.05, **P<0.01, ***P<0.001. Data are representative of at least 5 experiments, with error bars indicating SD.

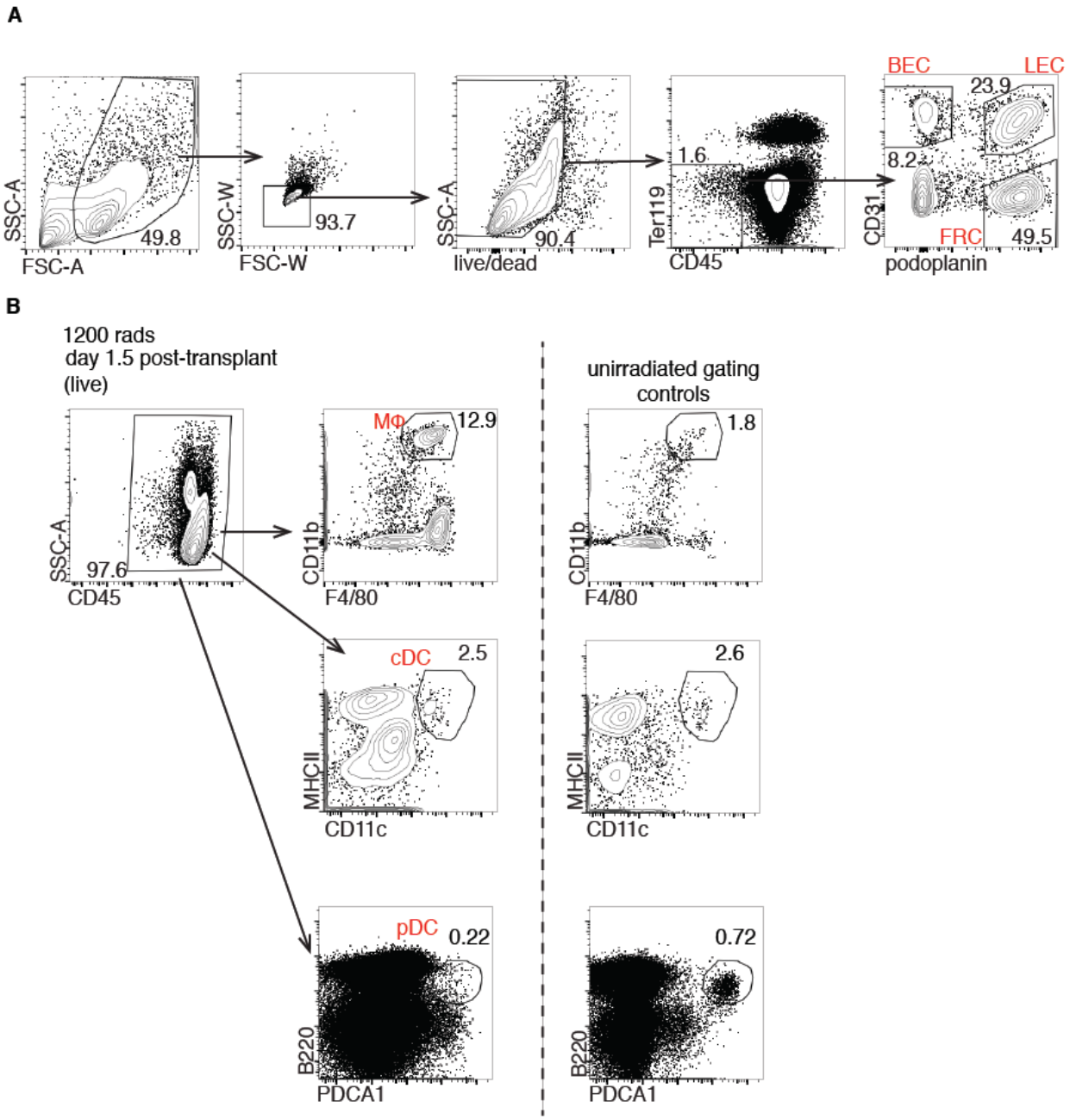

Figure 3.6. Gating strategy for flow cytometric analysis of lymph node stromal cells and hematopoietic antigen-presenting cells post-irradiation

Peripheral LNs (cervical, brachial, axial, brachial) from lethally irradiated recipient (12 Gy) mice receiving allogeneic BALB/c splenocytes were enzymatically digested into a single cell suspension (see Materials and Methods) and stained for flow cytometric analysis. **A.** FRCs were identified as $CD45^{-}$ $podoplanin^{+}CD31^{-}$, LECs as $CD45^{-}podoplanin^{+}CD31^{+}$, and BECs as $CD45^{-}podoplanin^{-}CD31^{+}$. **B.** Macrophages were identified as $F4/80^{+}CD11b^{+}$, cDCs as $CD45^{+}CD11c^{+}MHCII^{hi}$, and pDCs as $CD45^{+}PDCA1^{+}B220^{int}$. LNs were collected at day 1.5 post-transplantation. Data are representative of at least 4 experiments.

or only mildly decreased at early time points for $CD4^+$ T cells (**Fig. 3.10B**). Donor $CD4^+$ and $CD8^+$ T cells normally upregulated the early activation markers CD44 (**Fig. 3.10C,D**) and CD69 (not shown). CD25 was decreased in $Tg^{Ccl19\text{-}cre^+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ hosts (**Fig. 3.10C,D**), consistent with reports that Notch can regulate *Il2ra* expression in T cells [83, 85, 142]. Altogether, Dll1/4 loss in FRCs maintained T cell recruitment and initial activation, consistent with preservation of classical FRC functions but selective loss of Dll1/4-mediated instructive signals to incoming T cells.

Irradiation rapidly alters the microanatomy of SLOs

To assess the impact of allo-BMT on SLO organization with respect to $Ccl19^+$ FRCs, we studied $Tg^{Ccl19\text{-}cre^+};ROSA26^{eYFP}$ mice. Allo-BMT reduced LN cellularity due to loss of radiosensitive hematopoietic cells, while absolute numbers of ROSA26-$eYFP^+$ cells were preserved (**Fig. 3.11A**). Flow cytometric analysis and immunofluorescence microscopy revealed markedly increased relative frequencies of ROSA26-$eYFP^+$ stromal cells in LN and spleen after allo-BMT (**Fig. 3.11A-B**, **Fig. 3.12A**), concomitant with depletion of radiosensitive lymphocytes and thickening of the subcapsular macrophage layer (**Fig. 3.11C**, **Fig. 3.12B**). ROSA26-$eYFP^+$ fibroblastic cells from allo-BMT mice were located within both the T zone and B follicles, as delineated by the stromal markers podoplanin and CD35, respectively. T zone $Ccl19^+$ fibroblastic cells strongly upregulated podoplanin and CD157/BP3 expression, while stromal cells in B follicles upregulated CD35, CD157/BP3, and MAdCAM-1 (**Fig. 3.11D**, **Fig. 3.12C**). Thus, allo-BMT rapidly altered the spatial organization of lymphoid organ-resident cells, exposing donor T cells to a dense network of highly activated stromal cells.

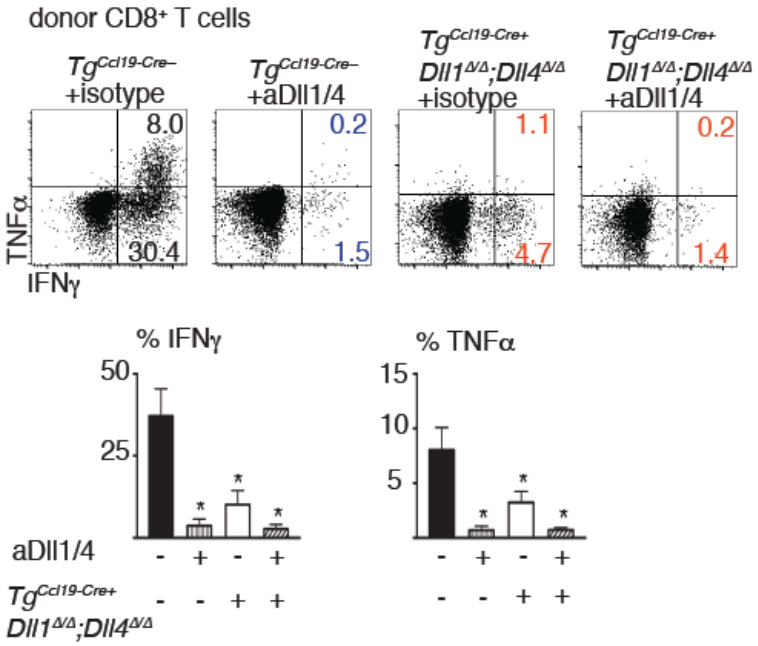

Figure 3.7. Impact of stromal cell-specific inactivation of *Dll1/4* Notch ligand genes on proinflammatory cytokine production by CD8$^+$ donor-derived T cells
Detection of intracellular cytokines in donor CD8$^+$ T cells after anti-CD3/anti-CD28 restimulation (day 6 post-transplantation, flow cytometry) (n = 5 mice/group). Control $Tg^{Ccl19\text{-}cre–}$ recipient mice treated with isotype control antibodies were compared to $Tg^{Ccl19\text{-}cre–}$ mice receiving anti-Dll1/4 antibodies vs. $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice treated with isotype control or anti-Dll1/4 antibodies.

Defined fibroblastic niches deliver Notch ligands to donor alloreactive T cells

We next investigated the distribution of Notch ligands in SLOs after allo-BMT, focusing on Dll4 since it is the dominant Notch ligand driving GVHD [72]. We examined cell-surface Dll4 within five distinct populations of CD45$^-$ LN stromal cells, as defined by expression of CD31, podoplanin, CD157/BP3 and CD21/35 (**Fig. 3.13A**). Comparison of control $Tg^{Ccl19\text{-}cre–}$ and $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice identified cellular subsets expressing functionally relevant Notch ligands after allo-BMT. Interestingly, Dll4 expression was not uniformly distributed among all *Ccl19*$^+$ stromal cells, but observed in a fraction of podoplanin$^+$CD31$^-$ FRCs marked by high levels of CD157/BP3 and in podoplanin$^+$CD31$^-$CD21/35^{hi} cells (consistent with follicular dendritic cells). LECs expressed only slightly decreased Dll4 in $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice, in keeping with low *Ccl19-Cre* activity in LECs (**Fig. 3.5A**). *Ccl19-Cre*$^+$ podoplanin$^+$CD31$^-$

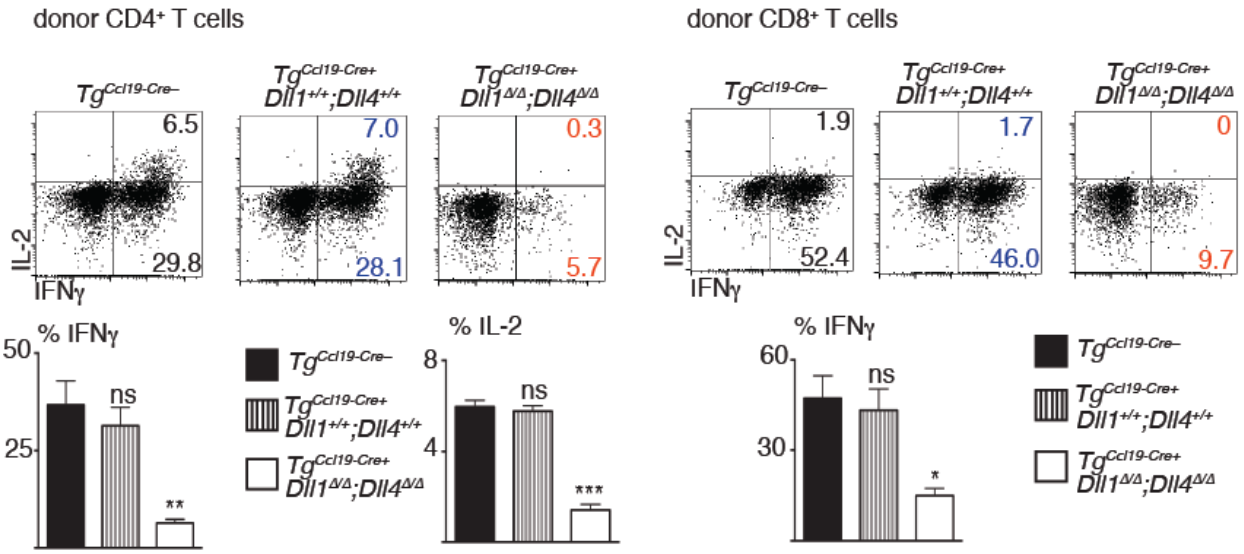

Figure 3.8. *Ccl19-Cre* expression by itself has no impact on T cell alloreactivity after allogeneic bone marrow transplantation
$10x10^6$ TCD BM + $20x10^6$ allogeneic BALB/c splenocytes were transplanted into lethally irradiated (12 Gy) B6 control $Tg^{Ccl19\text{-}cre-}$, $Tg^{Ccl19\text{-}cre+};Dll1^{+/+};Dll4^{+/+}$ or $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice. Some $Tg^{Ccl19\text{-}cre-}$ mice received i.p injections of isotype control or anti-Dll1/4 on days 0, 3. Detection of intracellular cytokines in donor $CD4^+$ T cells and $CD8^+$ T cells after anti-CD3/anti-CD28 stimulation at day 6 post-transplantation (flow cytometry) (n = 5 mice/group). *P<0.05, **P<0.01. Data are representative of 2 experiments, with error bars representing SD.

$CD157^{low}$ FRCs had no detectable Dll4, while BECs expressed Dll4 both in control and $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice (**Fig. 3.13A**). Immunofluorescence microscopy of spleens from $Tg^{Ccl19\text{-}cre+};Dll1^{+/+};Dll4^{+/+};ROSA26^{eYFP}$ and $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta};ROSA26^{eYFP}$ allo-BMT mice also demonstrated that only a subset of *Ccl19-Cre* lineage-traced $eYFP^+$ cells expressed high levels of Dll4 protein. Loss of Dll4 expression was observed in both ROSA26-$eYFP^+CD35^+$ (**Fig. 3.13C**) and ROSA26-$eYFP^+CD157^+$ cells (**Fig. 3.13D**) in $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta};ROSA26^{eYFP}$ mice. To assess if alloantigen-specific T cells first localize in SLOs next to Dll4-expressing stromal cells, we tracked alloantigen-specific $CD4^+$ T cells during their early window of Notch sensitivity. We detected proliferating donor T cells close to both Dll4-expressing $CD157^+$ and $CD35^+$ fibroblasts (**Fig. 3.13E-F**). Altogether, our data suggest that defined fibroblastic subsets

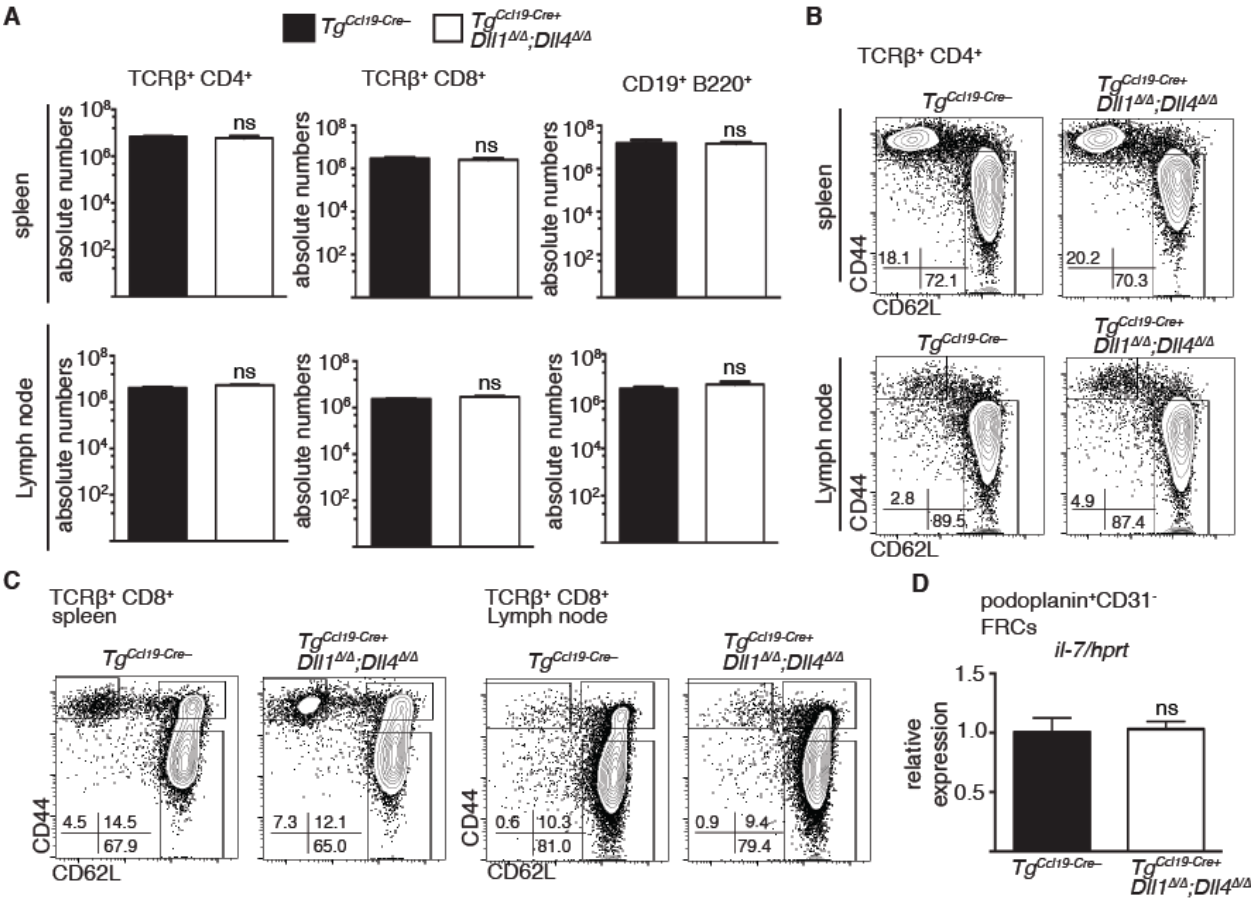

Figure 3.9. *Ccl19-Cre*-mediated *Dll1 and Dll4* inactivation preserves lymphocyte numbers and distribution in SLOs at steady state.
A. Absolute numbers of $CD4^+$ T cells, $CD8^+$ T cells and B cells in spleen and LNs of B6 control $Tg^{Ccl19\text{-}cre-}$ or $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice at steady state (n = 5 mice/group). **B-C.** CD62L and CD44 expression in $CD4^+$ T cells **(B)** and $CD8^+$ cells **(C)** from spleen and LNs of $Tg^{Ccl19\text{-}cre-}$ or $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice at steady state. **D.** Abundance of *Il7* transcripts (qRT-PCR) in sort-purified podoplanin$^+$CD31$^-$ FRCs from $Tg^{Ccl19\text{-}cre-}$ or $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice (n = 5 mice/group). Data are representative of at least 3 experiments, with error bars representing SD.

form specialized niches that provide Dll4 Notch ligands to incoming T cells and promote their pathogenic properties at the onset of GVHD.

Discussion

Preclinical models identified donor and host hematopoietic APCs as critical cellular partners of donor T cells at the onset of GVHD [128, 143]. Early interactions between donor T cells and

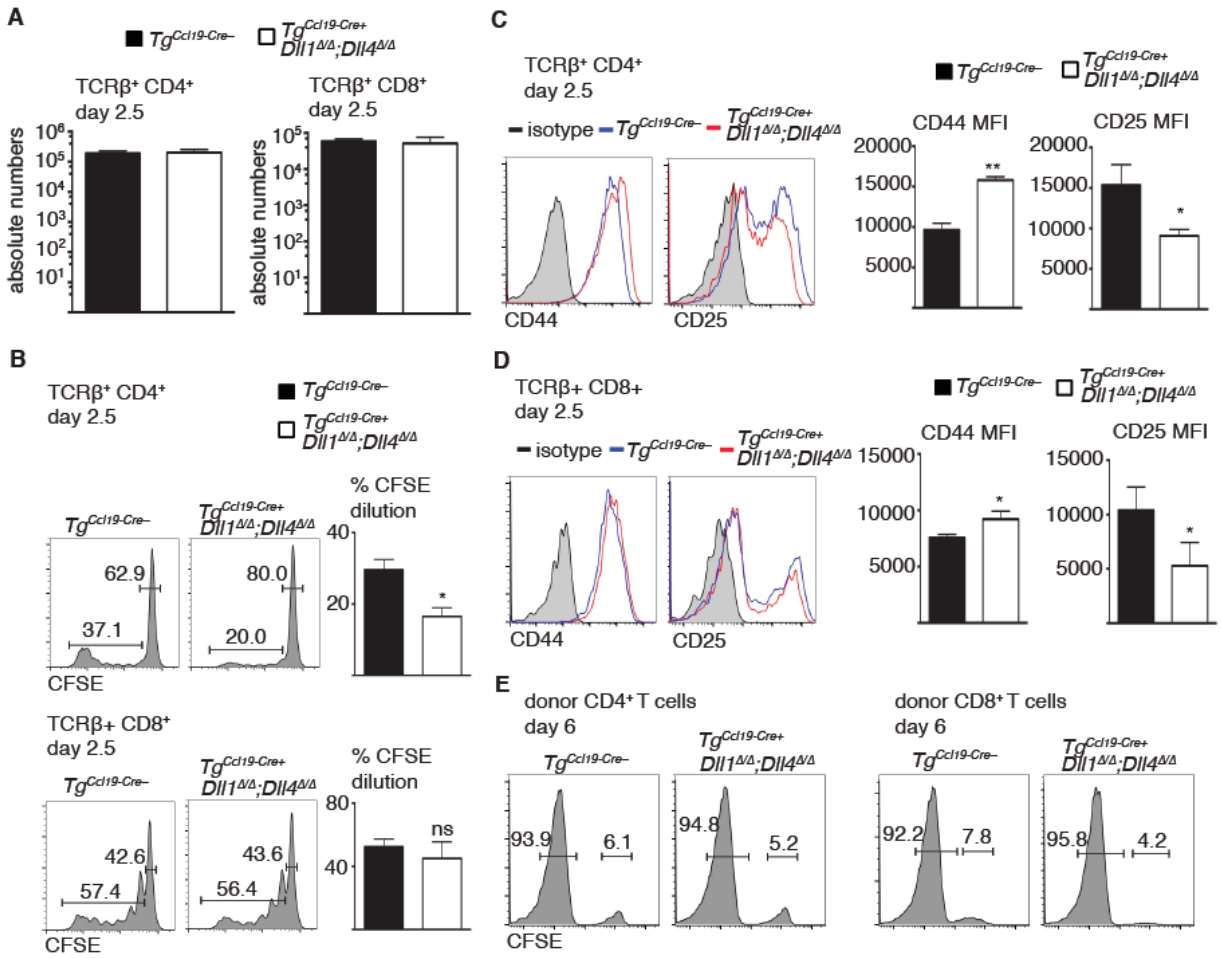

Figure 3.10. *Ccl19-Cre*-mediated *Dll1* and *Dll4* inactivation does not impair T cell recruitment and proliferation in SLOs post-irradiation.

A-D. Absolute numbers **(A)**, proliferation (CFSE dilution)**, (B)** and expression of activation markers **(C-D)** by donor-derived $CD4^+$ and $CD8^+$ T cells after transplantation into lethally irradiated (12 Gy) control $Tg^{Ccl19\text{-}cre-}$ or $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ B6 recipients. Donor cells were isolated at day 2.5 post-transplantation (n = 5 mice/group). **E.** Proliferation (CFSE dilution) of donor $CD4^+$ and $CD8^+$ T cells at day 6. *P<0.05, **P<0.01. Data are representative of at least 3 experiments, with error bars showing SD.

APCs, in concert with inflammatory signals from the microenvironment, are thought to provide essential instructive signals to induce T cell alloreactivity. Our findings revise this prevailing model of GVHD pathogenesis, as they identify LN and spleen-resident $Ccl19^+$ FRCs as key initiators of alloimmune T cell pathogenicity through induction of Notch signals in donor-derived T cells. Contrary to past assumptions, donor and host hematopoietic APCs were not responsible for delivery of Notch signals to donor T cells, as genetic inactivation of Delta-like

Notch ligands within donor and host hematopoietic APCs failed to ameliorate GVHD. In contrast, selective inactivation of Notch ligands within *Ccl19*$^{+}$ FRCs resulted in long-term protection from GVHD. Thus, specialized fibroblastic niches are uniquely important in the context of alloimmunity.

Immunofluorescence microscopy and flow cytometric analysis of SLOs post-allotransplantation demonstrated that Delta-like Notch ligand expression was not uniformly distributed throughout all FRCs. Instead, we observed focal niches of Notch ligand expression within *Ccl19-Cre*$^{+}$ cells marked by the stromal surface markers CD157 and CD21/35. Concomitant expression of chemokines and Delta-like Notch ligands in resident non-hematopoietic cells within defined SLO niches bears similarities to the co-regulated expression of chemokines and Dll4 ligands in cortical thymic epithelial cells. In the thymus, Foxn1 controls expression of CCL21/25 and Dll4, which is critical to attract lymphoid progenitors and induce T lineage development [133]. We speculate that SLOs rely on a similar organizational module to attract immune cells to defined niches with specialized immunological functions in which they are exposed to Notch ligands and other regulatory signals. This is consistent with the role of FRCs as a source of Notch ligands for marginal zone B cells, Esam^{hi} DCs and T follicular helper cells [123]. During alloimmune responses, direct interaction of T cells with these defined stromal niches may deliver important molecular cues in a temporally and spatially controlled fashion. An in-depth characterization of the cellular and humoral signals delivered within these defined niches should provide additional critical insight into the molecular pathogenesis of GVHD. Furthermore, upstream signals and transcription factors controlling the expression of Delta-like Notch ligands and chemokines in FRC niches remain to be discovered.

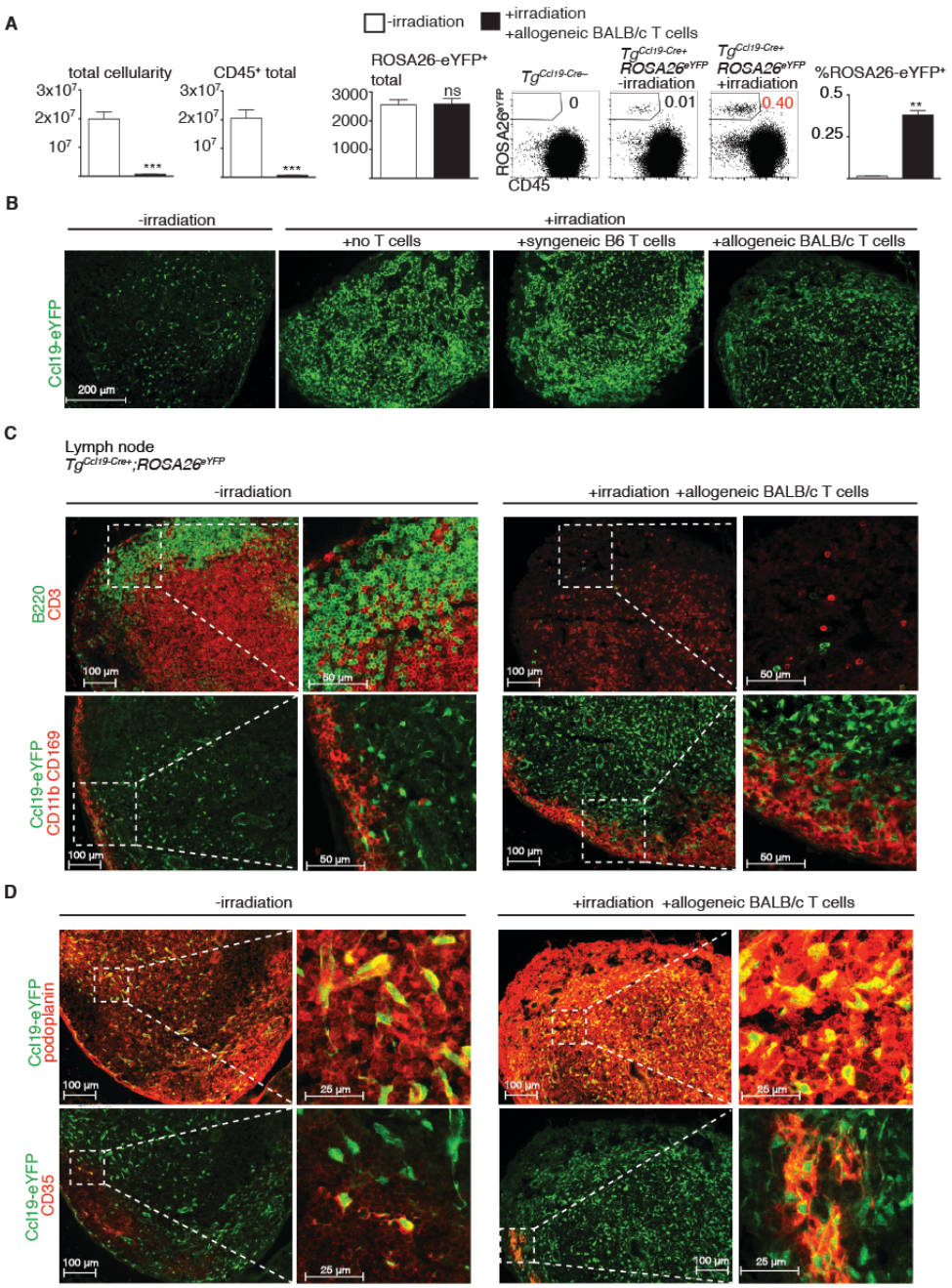

Figure 3.11. Irradiation increases the relative density and activation of *Ccl19*$^+$ stromal cells
A. Total cellularity, absolute numbers of CD45$^+$ cells, and absolute numbers and frequencies of eYFP$^+$ cells in LNs of unirradiated or lethally irradiated (12 Gy) *Tg*$^{Ccl19\text{-}cre^+}$*;ROSA26*^{eYFP} reporter mice receiving allogeneic BALB/c splenocytes (n = 6 mice/group). **B.** Immunofluorescence microscopy of LN cryosections from *Tg*$^{Ccl19\text{-}cre^+}$*;ROSA26*^{eYFP} mice stained for GFP. Cryosections were prepared from unirradiated or lethally irradiated (12 Gy) mice receiving no T cells, syngeneic B6 CD4$^+$ T cells, or allogeneic BALB/c CD4$^+$ T cells. **C-D.** Immunofluorescence microscopy of LN cryosections from *Tg*$^{Ccl19\text{-}cre^+}$*;ROSA26*^{eYFP} mice stained for B220 and CD3 (**C, top panel**), GFP, CD11b, and CD169 (**C, bottom panel**), GFP and podoplanin/gp38 (**D, top panel**) or GFP and CD35 (**D, bottom panel**). Cryosections were prepared from unirradiated or lethally irradiated (12 Gy) mice receiving allogeneic BALB/c CD4$^+$ T cells at day 1.5 post-transplantation. Data are representative of 2 experiments.

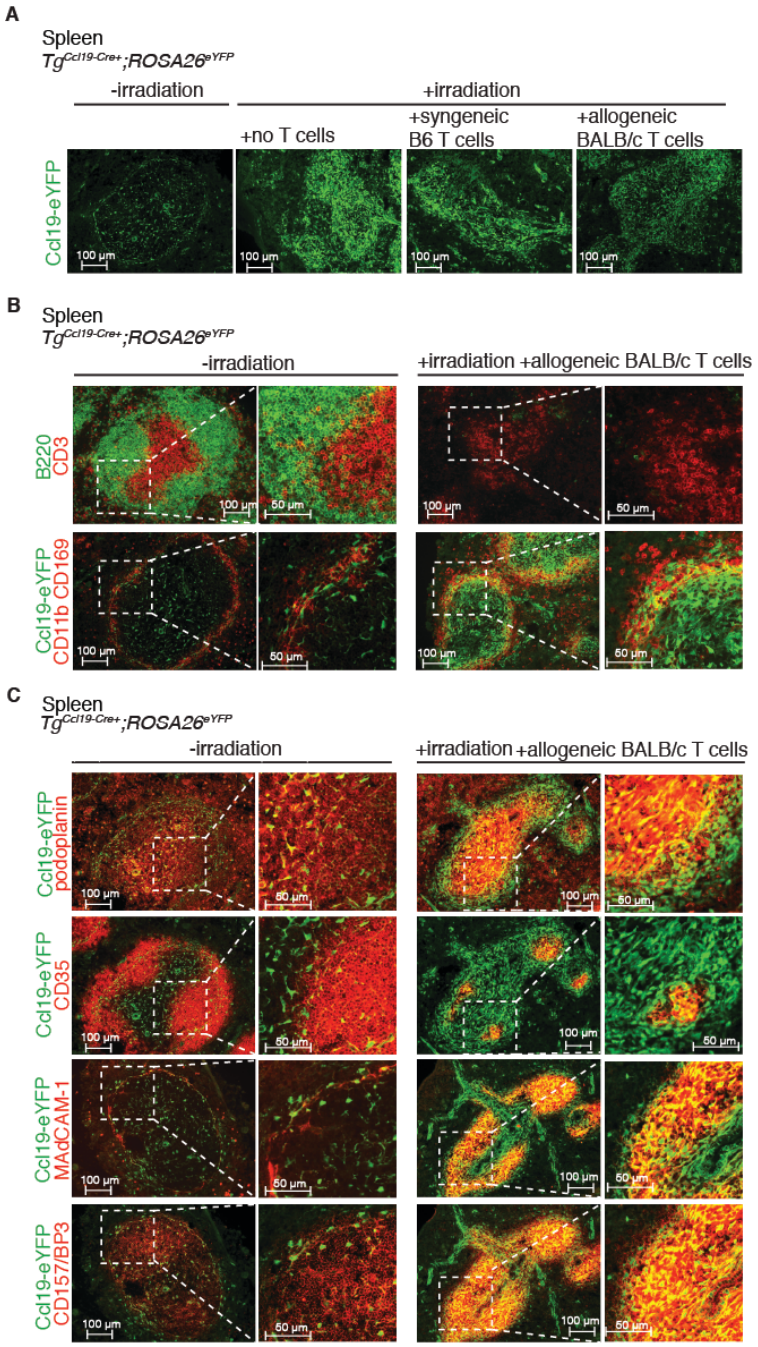

Figure 3.12. Impact of allogeneic bone marrow transplantation on spleen architecture.
A-C. Immunofluorescence microscopy of spleen cryosections from $Tg^{Ccl19\text{-}cre^{+}};ROSA26^{eYFP}$ reporter mice stained for GFP only (**A**), B220 and CD3 (**B, top panel**), GFP, CD11b, and CD169 (**B, bottom panel**), GFP and podoplanin/gp38 (**C, first panel**), GFP and CD35 (**C, second panel**), GFP and MAdCAM1 (**C, third panel**), or GFP and CD157/BP3 (**C, fourth panel**). Cryosections were prepared from unirradiated or lethally irradiated (12 Gy) mice receiving allogeneic BALB/c $CD4^{+}$ T cells at day 1.5 post-transplantation. The high intensity of CD35 staining in the absence of irradiation is due to expression of CD21/35 by B cells. After irradiation and depletion of radiation-sensitive B cells, CD35 staining highlighted stromal cells in the B cell follicles consistent with follicular dendritic cells. Data are representative of 2 experiments.

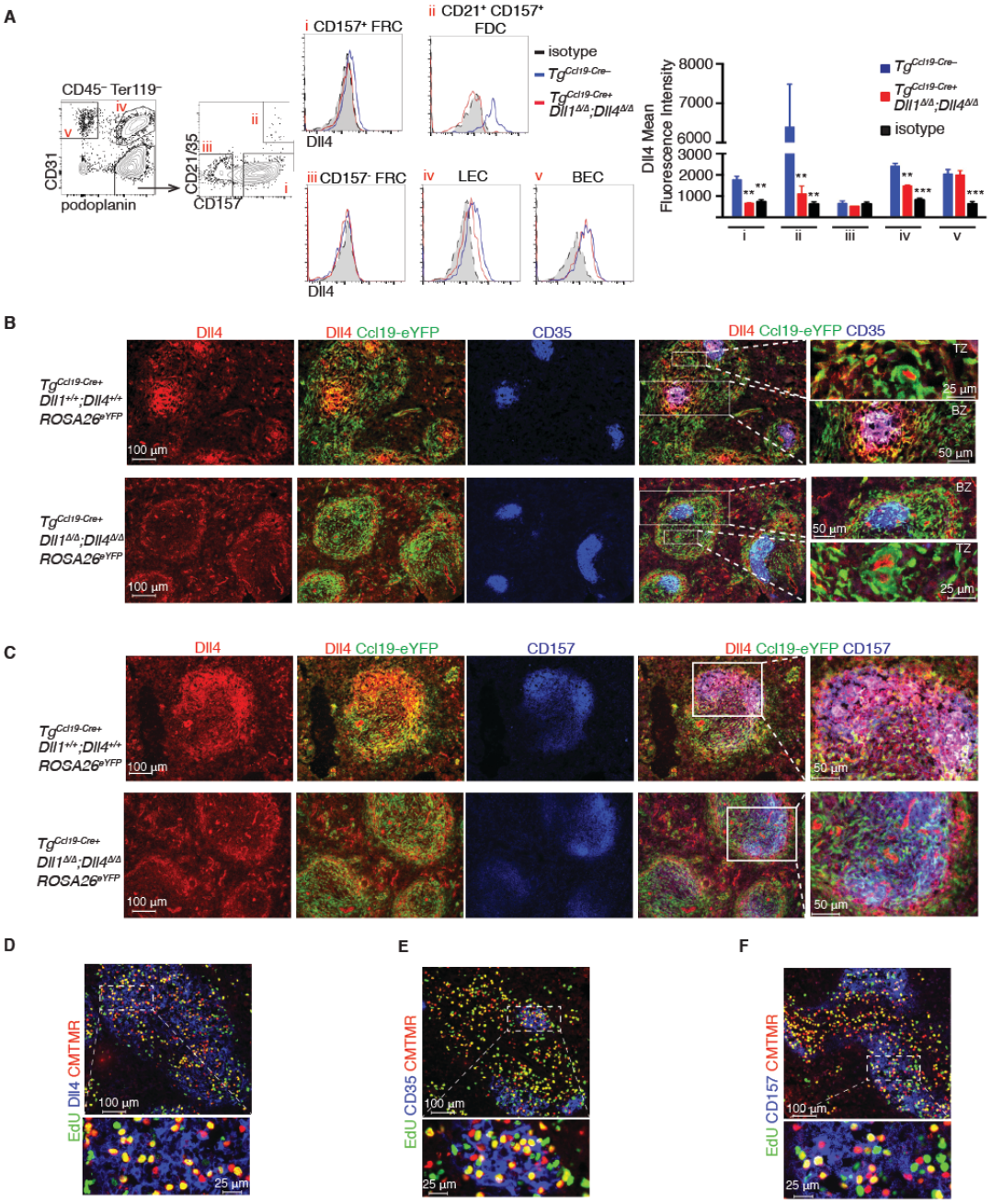

Figure 3.13. Fibroblastic niches express Delta-like1/4 Notch ligands and localize next to alloreactive T cells.

A. Dll4 expression in LN-resident non-hematopoietic cells from control $Tg^{Ccl19\text{-}cre–}$ and $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice transplanted with allogeneic BALB/c splenocytes. **B-C.** Immunofluorescence microscopy of spleen cryosections from $Tg^{Ccl19\ cre+};Dll1^{+/+};Dll4^{+/+};ROSA26^{eYFP}$ or $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta};ROSA26^{eYFP}$ mice stained for GFP, CD35 and Dll4 **(B)** or GFP, CD157, and Dll4 **(C)**. **D-F.** Immunofluorescence microscopy of spleen cryosections from lethally irradiated (8.5 Gy) BALB/c mice transplanted with CMTMR-labeled alloantigen-specific $CD4^+$ 4C T cell receptor transgenic cells and pulsed with EdU 12 hours prior to organ collection. Cryosections were stained with Alexa Fluor 488 picolyl azide for EdU, along with anti-Dll4 **(D)**, anti-CD157 **(E)** or anti-CD35 **(F)**. Organs were collected at day 1.5 post-transplantation. **P<0.01, ***P<0.001. Data are representative of at least 2 experiments, with error bars indicating SD.

FRCs support steady-state immune homeostasis through the secretion of IL-7 and CCL19 [106]. *In vitro*, FRCs enhance naïve $CD4^+$ or $CD8^+$ T cell survival and viability in an IL-7/CCL19-dependent manner, while *Ccl19* loss *in vivo* results in decreased total numbers of $CD4^+$ and $CD8^+$ T cells. Importantly, genetic *Dll1/4* inactivation of Notch ligands with *Ccl19-Cre* did not negatively impact the ability of FRCs to maintain naïve T cell homeostasis, as the total number of immune cells within LN and spleen and the distribution of naïve/memory cells at steady-state were unaffected. Consistent with this notion, *Il7* transcripts were unaltered in sort-purified FRCs from $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice, showing that Dll1/4 loss did not globally disrupt FRC function.

FRCs can regulate immunity through multiple mechanisms. FRCs form a scaffold to which DCs can adhere and present antigens to naïve T cells, thus using the FRC network as a 'road system' for their migration [92]. This random migration is enhanced by the chemokines CCL19 and CCL21, constitutively expressed by FRCs. Physical removal of FRCs after SLO development prevents the efficient activation and proliferation of antigen-specific T cells [139]. In contrast, in our studies, genetic *Dll1/4* inactivation with *Ccl19-Cre* preserved T cell homing, as equivalent numbers of donor $CD4^+$ and $CD8^+$ T cells were isolated in SLOs. Furthermore, donor T cells upregulated the early activation markers CD69 and CD44 upon transfer into $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice, suggesting their preserved ability to encounter and respond initially to alloantigens. Finally, donor T cells demonstrated no obvious impairment in their ability to proliferate in response to antigen. Thus, our observations differ from the broader dysfunction of SLOs observed upon physical elimination of $Ccl19^+$ FRCs [139]. Instead, our targeted genetic approach suggests that FRC subsets regulate specific aspects of immune cell biology through dedicated signaling pathways (e.g. Notch), consistent with the delivery of

unique activation codes to immune cell subsets. Moreover, the post-transplant environment may enhance the delivery of Notch signals to incoming T cells though FRC activation after irradiation and loss of radiation-sensitive host lymphocytes.

Our *in vivo* results contrast with past work identifying a role for stromal cells as negative regulators of immune responses [113-115], as the dominant role of these cells after allo-BMT in our study was to promote rather than to restrain alloimmune reactivity. In previous studies, the addition of *ex vivo* isolated FRCs to T cell/DC cocultures suppressed T cell proliferation in a dose-dependent fashion. Suppression was mediated by IFNγ-dependent upregulation of NOS2 and COX1/2-dependent metabolites within FRCs. While *nos2* inactivation resulted in enhanced T cell proliferation *in vivo,* the lack of specificity of the genetic targeting strategy made it difficult to assess whether FRCs were in fact responsible for enforcing this suppressive mechanism. Furthermore, the expression of Notch ligands on FRCs was not examined in the *in vivo* or *in vitro* studies. In our *in vivo* studies, irradiation of allotransplant recipients upregulated expression of several surface markers on stromal cells, changed their morphology, and resulted in profound remodeling of SLO microanatomy within the first few days after transplantation. Thus, it is possible that the highly inflammatory environment that ensues post-irradiation, in combination with the differences in the nature and availability of antigen, can account for the extreme proinflammatory functions of FRCs after allo-BMT. It is also possible that alloreactive T cells gain unique access to fibroblastic niches from which T cells are typically excluded, such as follicular dendritic cells or follicular FRCs residing in the B cell zone. Alternatively, our observations may reveal a previously unrecognized proinflammatory function of stromal cells mediated by Notch signaling that also operates in other contexts.

In terms of temporal requirement for Notch signaling, systemic delivery of Dll1/4 blocking antibodies at the time of transplantation had profound effects on T cell cytokine production, Treg expansion, and overall T cell alloreactivity, but inhibition delayed by only two days failed to efficiently block GVHD. This narrow window of therapeutic sensitivity is significant in terms of translational applications. Indeed, short-term Dll1/4 inhibition during this critical time emerges as an attractive therapeutic strategy to prevent GVHD without exposing recipients to the risks of long-term Dll1/4 blockade. Mechanistically, this narrow window of therapeutic sensitivity might reflect the temporal delivery of a physical pulse of Notch signaling in defined microanatomical niches after allo-BMT. This physical pulse may result from transient exposure to cellular sources of Notch ligands that are typically inaccessible to alloreactive T cells, or from prolonged initial contacts with FRC subsets that upregulate Dll1/4. Alternatively, our findings could reflect a unique window of T cell sensitivity to Notch signaling during early stages of priming and activation.

Altogether, our study reveals for the first time the existence of specialized subsets of host nonhematopoietic fibroblastic cells delivering Notch signals to donor T cells at early stages after allo-BMT to program their pathogenicity. Donor and host hematopoietic APCs were dispensable sources of Notch ligands, thus challenging the widely accepted hypothesis that motile APCs simultaneously provide both antigen and Notch ligands to prime T cells. These findings illustrate the importance of exploring Notch signaling *in vivo* using loss-of-function approaches, as relevant sources of Notch ligands may be missing from established *in vitro* experimental systems. Our work also highlights the utility of precisely targeting immunomodulatory pathways in stromal cells, as we uncovered a previously unrecognized pathogenic role for FRCs independent of their functions in structural integrity and immune homeostasis. In GVHD,

allograft rejection and other immune disorders, our findings pave the way towards selective inhibition of niche-derived signals that drive deleterious immune responses, without interfering with other essential immunological functions of the lymphoid environment.

Chapter 4

Molecular effects of Notch signaling on alloreactive CD4$^+$ T conventional cells during *in vivo* priming

Abstract

Graft-versus-host disease (GVHD) is the most serious complication of allogeneic bone marrow transplantation (allo-BMT). We recently identified that Notch signaling during the first 48 hours after allo-BMT drives proinflammatory cytokine production in conventional CD4$^+$ T cells (Tconv) and inhibits the expansion of CD4$^+$ FoxP3$^+$ regulatory T cells (Tregs). Inhibition of Notch signals during this 48-hour window results in long-term clinical protection from GVHD. However, it is unclear whether the clinical consequences of Notch inhibition are dependent on its effects on Tconv, Tregs, or both. Furthermore, limited tools are available for examining the molecular events that occur in alloantigen-specific T cells during this early window of Notch activity. In this study, we identified a Tconv-intrinsic role for Notch signaling in mediating acute GVHD. We established a new model of acute GVHD with a clonal population of alloantigen-specific CD4$^+$ Tconv cells that enabled us to examine the molecular impact of Notch signaling on alloreactive T cell priming. During T cell priming, Notch-deprived T cells exhibited no defects in the early steps of activation, preserved their ability to produce IL-2, proliferated normally, and induced the transcription of *tbx21, rorc,* and *gata3*. In contrast, Notch inhibition prevented the acquisition of IFNγ and IL-17 production, diminished mTORC1 and

ERK1/2 activity, and impaired the transcription of a subset of Myc-dependent target genes. Thus, unlike standard global immunosuppression, Notch inhibition in alloantigen-specific Tconv $CD4^+$ cells resulted in the dissociation of proliferation from effector function acquisition. These findings provide a mechanistic explanation for how Notch inhibition modulates T cell alloreactivity differently than standard global immunosuppressants.

Introduction

Notch signaling is an evolutionarily conserved signaling pathway that has important effects during development and homeostasis of the immune system, and during active immune responses [144]. Physical interactions between Notch1-4 receptors and Delta-like1/3/4 (Dll1/3/4) or Jagged1/2 Notch ligands trigger sequential ADAM10 and γ-secretase complex-mediated proteolytic cleavage of Notch, releasing the intracellular domain to regulate transcription of target genes. Notch has emerged as an essential regulator of T cell alloreactivity in mouse models of graft-versus-host disease and allograft rejection [71-73, 75-78]. We previously demonstrated that genetic blockade of Notch signaling within donor $CD4^+$ and $CD8^+$ T cells resulted in long-term protection from GVHD mortality [71, 73]. Pharmacologic inhibition of Dll1/4 Notch ligands with systemic neutralizing antibodies also achieved the same effect [72]. Mechanistically, Notch-deprived $CD4^+$ and $CD8^+$ T cells at day 5 post-transplantation exhibited profoundly defective IFNγ and IL-2 production, blunted Ras/MAPK signaling, and increased transcript levels of negative regulators of T cell signaling [73]. Importantly, these effects appeared to occur independently of the master transcription factors (TFs) T-bet and Eomesodermin (Eomes), as Notch inhibition preserved the expression of both. However, as we recently identified that nearly all essential Notch signals are delivered to

alloreactive T cells during the first 2 days post-transplantation (**Chapter 3**), it is unclear whether these observed defects are direct consequences of Notch inhibition, or whether they are reflective of secondary effects and/or compensatory networks. Therefore, we sought to assess the impact of Notch signaling on molecular events that occur during alloreactive T cell priming, which coincides with when essential Notch signals are delivered.

Studies in T cell acute lymphoblastic leukemia (T-ALL) cell lines, >50% of which harbor Notch gain-of-function mutations, have provided key insights into the potential molecular mechanisms that operate downstream of Notch [24]. Chromatin immunoprecipitation (ChIP) and γ-secretase inhibitor (GSI) washout studies revealed a comprehensive list of direct transcriptional targets of Notch [25, 26]. Two functionally important direct targets, *myc* and *hes1,* have been extensively studied. Overexpression of Myc is sufficient to rescue some GSI-treated T-ALL cell lines from undergoing G0/G1 cell cycle arrest [145, 146]. Notch directly regulates *myc* expression by forming dimerized transcriptional complexes on a long-range enhancer that is >1 Mb 3' distal to the gene body [147, 148]. The basic-helix-loop-helix gene *Hes1* encodes a transcriptional repressor that is important for neuronal stem cell maintenance, T cell development, and T-ALL maintenance and survival [149-151]. During T cell development and in T-ALL, Hes1 was shown to directly represses *pten* expression by binding to its promoter region, in addition to other putative functions. Hes1-mediated inhibition of *pten* transcription increases AKT activity, resulting in increases in cell survival and growth [152, 153]. Therefore, the Notch1-Hes1-PTEN axis can promote AKT signaling.

While RNA-Seq and ChIP-Seq studies have not been performed in Notch-dependent mature $CD4^+$ T cell responses, several findings have indicated that it behaves in a highly context-dependent manner [67, 70, 73, 86]. Recently, two major studies proposed two different (but not

mutually exclusive) models for how Notch operates. In the first model, Notch acts as an unbiased amplifier of T helper differentiation by simultaneously binding to multiple T helper lineage fate transcription factor [55] and cytokine loci [69]. Prior work potentially supported this model, as they identified direct binding of Notch to *foxp3, gata3, rorc, tbx21, il4, ifng,* and *il9* [50, 69, 82, 154, 155]. In the second model, Notch promotes antigen sensitivity in a CD28-dependent fashion [70]. Concomitant exposure of antigen-specific T cells to Dll4 signals enhanced T cell proliferation, upregulation of activation markers, and IL-2 production. As both studies primarily utilized *in vitro* approaches with artificial levels of Notch ligand and antigen, it is unclear whether these mechanisms would operate *in vivo* in the context of T cell alloimmunity.

A recent study demonstrated that genetic inactivation of Notch signaling specifically in FoxP3$^+$ regulatory T cells (Tregs) was sufficient to confer long-term protection from acute GVHD [78]. However, the importance of Notch within FoxP3$^-$ T conventional cells (Tconv) was not assessed. Given that genetic inhibition of Notch signaling in mature CD4$^+$ and CD8$^+$ T cells exerts effects on both Tconv and Tregs, we first assessed the relative importance of Tconv and Tregs in mediating the protective effects of Notch inhibition in a polyclonal model of MHC-mismatched GVHD. Next, we established a novel model of GVHD with a clonal population of donor alloantigen-specific CD4$^+$ T cells. This model allowed us to dissect the molecular effects of Notch within alloantigen-specific cells during *in vivo* T cell priming. We assessed the impact of Dll1/4 inhibition on early T cell activation, signal transduction, and acquisition of cytokine production. Furthermore, we performed RNA-Seq analysis on Notch-deprived alloreactive T cells during priming. We identified an important role for Notch signaling within Tconv cells. Notch inhibition within Tconv cells preserved early T cell activation, early T cell expansion, IL-2 production, and the transcriptional induction of *tbx21*, *gata3*, and *rorc*. In contrast, Notch-

deprived T cells failed to induce the transcription of several proinflammatory cytokines, displayed reduced mTORC1 and MAPK activity, and exhibited features of diminished Myc function despite preserved *myc* transcription. Collectively, these data suggest that Notch inhibition results in the dissociation of proliferation from effector function acquisition.

Results

Notch signaling in CD4$^+$ T conventional cells is an important mediator of acute GVHD

We previously reported an essential role for Notch signaling in mature CD4$^+$ T cells during acute GVHD [71-73]. Conditional expression of the pan-Notch inhibitor dominant negative Mastermind-like (DNMAML) in mature T cells inhibits proinflammatory cytokine production by FoxP3$^-$ CD4$^+$ conventional T cells (Tconv) and expands pre-existing FoxP3$^+$ natural regulatory T cells (nTregs). Whether long-term protection from GVHD mortality is dependent on the effects of Notch inhibition on Tconv, nTregs, or both Tconv and nTregs is unclear. To address this question, we used B6 *FoxP3-IRES-RFP* [156] or B6 *FoxP3-IRES-RFP*;*Tg$^{cd4\text{-}cre}$*;*ROSA$^{DNMAML/+}$* (FIR-DNMAML) mice as a source of donor CD4$^+$ Tconv and nTregs. WT RFP$^-$ Tconv, WT RFP$^+$ nTreg, DNMAML$^+$/RFP$^-$ Tconv, and DNMAML$^+$/RFP$^+$ nTreg were sort-purified and mixed together to generate four different Tconv /nTreg donor inoculums: 1) WT Tconv + WT nTreg; 2) WT Tconv + DNMAML nTreg; 3) DNMAML Tconv + WT nTreg; 4) DNMAML Tconv + DNMAML nTreg (**Fig. 4.1A**). We took this approach because we had previously observed that depletion of nTregs from DNMAML donors prior to transplantation completely prevented the expansion of Tregs [73]. Thus, Notch inhibition expands pre-existing nTregs, rather than converting Tconv to induced Tregs [73]. Each inoculum was adoptively transferred with T cell-depleted (TCD) bone marrow (BM) into lethally irradiated MHC-mismatched

BALB/c recipients. As expected, a majority of recipients of WT Tconv + WT nTreg (56%) succumbed to GVHD mortality by day 45 post-transplantation. Similarly, recipients of WT Tconv + DNMAML nTreg (80%) also succumbed to GVHD mortality (**Figure 4.1B**). There was no statistically significant difference in survival rate between WT Tconv + WT nTreg and WT Tconv + DNMAML nTreg recipient groups, suggesting that DNMAML nTreg by themselves were not sufficient to confer long-term protection from GVHD mortality. In contrast, 70% of recipients of DNMAML Tconv + WT nTreg, and 89% of recipients of DNMAML Tconv + DNMAML nTreg survived by day 45 post-transplantation (**Fig. 4.1B**). There was no statistically significant difference in survival rate between DNMAML Tconv + WT nTreg and DNMAML Tconv + DNMAML nTreg recipient groups, suggesting that Notch inhibition in Tconv was sufficient to confer long-term protection from GVHD mortality. Thus, in contrast to the recent finding that genetic inactivation of Notch within Tregs is sufficient to protect from GVHD, these data highlight an essential role for Notch in $CD4^+$ Tconv cells in mediating acute GVHD.

Establishment of a MHC-mismatched GVHD mouse model with a clonal population of donor $CD4^+$ T cells

Sort purification and mixing of Tconv with Tregs from WT or DNMAML mice allowed us to identify a functionally important role for Tconv-intrinsic Notch signaling during GVHD. As we had previously determined that essential Notch signals are delivered during the first 48 hours after allo-BMT (**Chapter 3**), we next sought to assess the impact of Notch on alloantigen-specific Tconv cells during this early window of activity. However, this was not achievable in the B6 anti-BALB/c model of GVHD, as neither cell proliferation nor the early activation

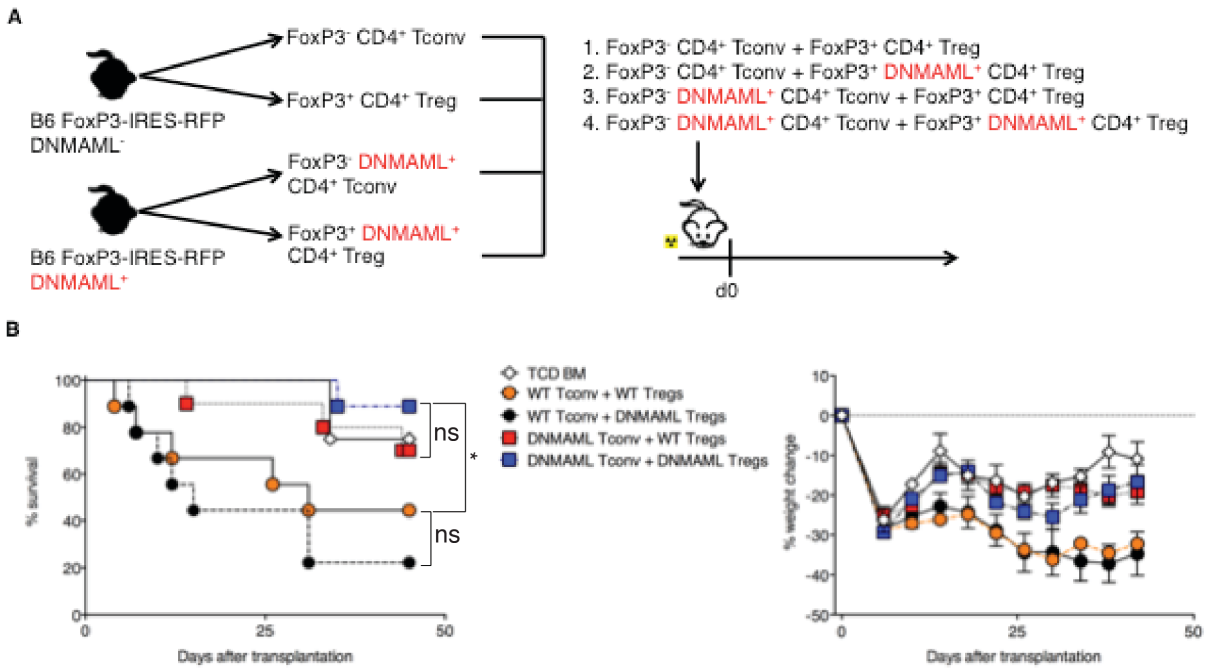

Figure 4.1. Conventional T cell-intrinsic Notch signaling is important for mediating GVHD after MHC-mismatched allogeneic bone marrow transplantation

A. Experimental design for assessing the importance of conventional T cells (Tconv) and regulatory T cells (Tregs) in mediating the protective effects of Notch inhibition after allogeneic bone marrow transplantation. Tconv and Tregs were sort purified from $CD4^+$-enriched cells from B6 *FoxP3-IRES-RFP* [156] or B6 *FoxP3-IRES-RFP*;$Tg^{cd4\text{-}cre}$;$ROSA^{DNMAML/+}$ (FIR-DNMAML) mice, and mixed at a ratio of 8:1 Tconv:Tregs (500,000:62,500) to generate four different donor cell groups. **B.** Survival and weight changes of lethally irradiated (8 Gy) BALB/c mice transplanted with the four experimental groups described in **A** (n = 8-10 per experimental group). *P<0.05. Data are representative of 2 experiments.

markers CD69 and CD44 were sufficient to distinguish alloantigen-specific T cells from bystander cells (data not shown). Therefore, we established a new mouse model of MHC-mismatched GVHD that utilized 4C x $Rag1^{-/-}$ TCR transgenic mice as a source of donor $CD4^+$ T cells. 4C cells are a clonal population of $V\beta13^+$ $CD4^+$ T cells that react against $I\text{-}A^d$ MHC class II through direct antigen recognition [157]. Utilizing 4C x $Rag1^{-/-}$ donor mice conferred two major benefits that were unattainable with our first experimental approach. First, it enabled us to isolate and study a pure, clonal population of alloreactive $CD4^+$ T cells that were not contaminated with confounding bystander cells that were not alloantigen-specific. Second, it

allowed us to assess the importance of Notch in Tconv in the complete absence of Tregs, as $Rag1^{-/-}$ mice lack Tregs.

As 4C cells have only been utilized to investigate MHC-mismatched allograft rejection [157], we first investigated whether 4C donor cells could induce GVHD mortality. To this end, we transplanted $>10^5$, $2x10^4$, or $2x10^3$ donor 4C cells with TCD BM into lethally irradiated BALB/c mice and tracked GVHD survival. We included a group of polyclonal B6 donor cells + TCD BM recipients to compare the kinetics of GVHD induction. 4C cells were potent inducers of GVHD mortality in a dose-dependent fashion (**Fig. 4.2A**). Transplantation of $>10^5$ 4C cells resulted in 100% GVHD mortality within 4-5 days, while transplantation of $2x10^4$ 4C cells resulted in 100% GVHD mortality within 9-14 days. $2x10^3$ 4C cells resulted in 50% GVHD mortality, with all lethality happening within 9-14 days. The remaining recipients of $2x10^3$ 4C cells survived long-term, potentially suggesting that 4C cells eventually became exhausted and were unable to cause long-term disease. The rapid kinetics of lethality that was observed with higher 4C donor numbers was reminiscent of hyperacute GVHD. Consistent with a model of hyperacute GVHD, 4C cells caused GVHD lethality much more rapidly than polyclonal B6 donor cells, which induced lethality ~60 days post-transplantation.

Consistent with *in vivo* activation, 4C cells from MHC-mismatched BALB/c recipients displayed greater size and granularity when compared to 4C cells from autologous B6-SJL recipients (**Fig. 4.2B**).

Delta-like1/4-mediated Notch signals within donor $CD4^+$ T cells drive GVHD mortality

We previously reported that in polyclonal models of MHC-mismatched GVHD, $CD4^+$ T cells receive their Notch signals through Dll1/4 ligands [72]. To assess whether 4C $CD4^+$ cells also

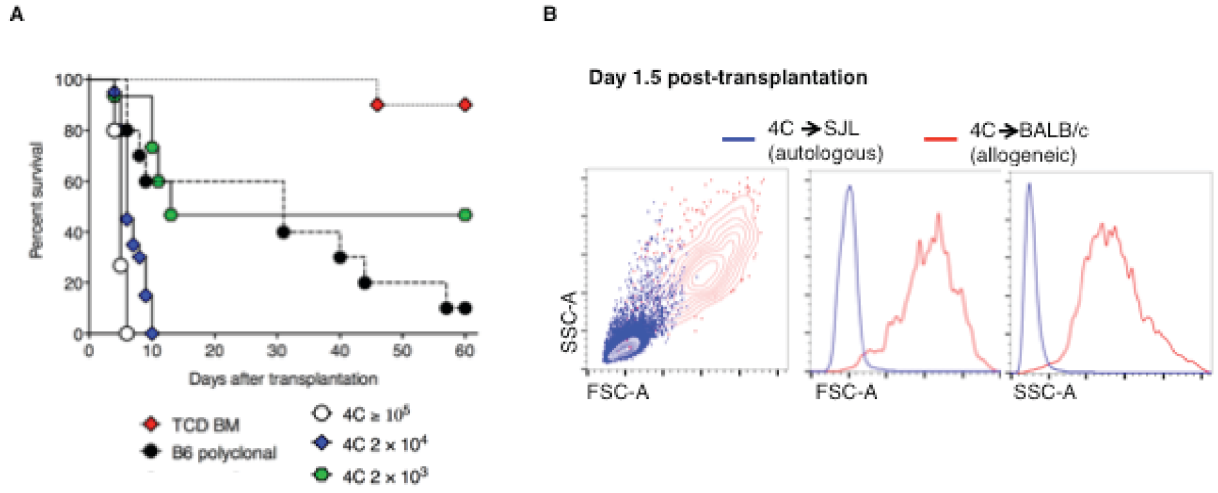

Figure 4.2. CD4$^+$ 4C TCR transgenic donors can induce lethal GVHD after MHC-mismatched allogeneic bone marrow transplantation

A. Survival of lethally irradiated BALB/c mice transplanted with 5x10^6 TCD BM only, 5x10^6 TCD BM + 5x10^6 polyclonal B6 splenocytes, 5x10^6 TCD BM + >10^5 4C Rag1$^{-/-}$ splenocytes, 5x10^6 TCD BM + 4C Rag1$^{-/-}$ splenocytes, or 5x10^6 TCD BM + 2x10^3 4C Rag1$^{-/-}$ splenocytes. **B.** Forward scatter (FSC-A) and side scatter (SSC-A) of 4C cells isolated from lethally irradiated allogeneic BALB/c or syngeneic B6-SJL recipients at day 1.5 post-transplantation. In experiments described in **(A-B)**, BALB/c mice were irradiated at 8 Gy, while B6-SJL mice were irradiated at 12 Gy. Data are representative of at least 3 experiments.

received their Notch signals through Dll1/4 ligands *in vivo*, we transferred 2x10^4 4C cells and TCD BM into lethally irradiated BALB/c mice with or without systemic neutralizing antibodies against Dll1/4. While 100% of isotype control recipients succumbed to GVHD within 10 days, 50% of aDll1/4-treated recipients survived long-term (**Fig. 4.3A**). The same degree of protection (50%) was achieved with 2x10^3 donor 4C cells, as 50% of isotype control recipients succumbed to GVHD, while 25% of aDll1/4-treated recipients survived long-term (data not shown). Consistent with our results from polyclonal donor models of GVHD, systemic aDll1/4 treatment profoundly inhibited IFNγ, IL-2, IL-17, and TNFα cytokine production by *ex vivo* restimulated 4C cells at day 5 post-transplantation (**Fig. 4.3B**). Thus, Notch signals drive 4C alloreactivity *in vivo* in a Dll1/4-dependent manner.

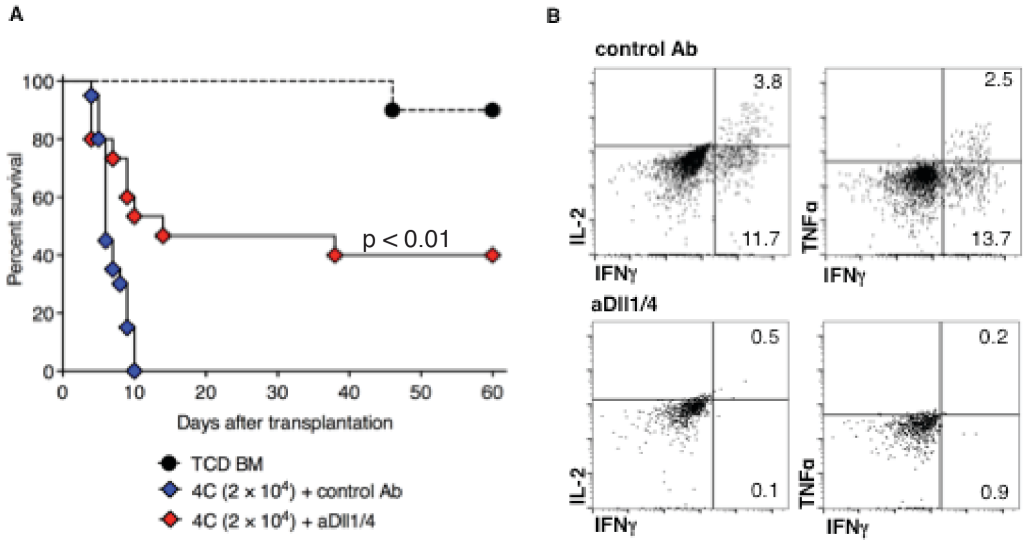

Figure 4.3. Systemic Dll1/4 inhibition protects from CD4$^+$ 4C transgenic donor-induced GVHD mortality after MHC-mismatched bone marrow transplantation
A. Survival of lethally irradiated (8 Gy) BALB/c mice transplanted with 5x10^6 TCD BM only or 5x10^6 TCD BM + 2x10^3 4C Rag1$^{-/-}$ splenocytes. Isotype control or anti-Dll1/4 neutralizing antibodies were injected i.p on day 0 only (n = 10/group). **B.** Intracellular cytokine staining in donor 4C cells after anti-CD3/CD28 restimulation at day 5 post-transplantation (n = 5 mice/group).

Notch inhibition preserves early activation marker expression, but impairs S6 and ERK1/2 phosphorylation

CD4$^+$ T cells undergo T cell priming in three distinct stages within a period of 48 hours [158]. First, CD4$^+$ T cells search and encounter antigen. Upon antigen recognition through the TCR, CD4$^+$ T cells upregulate early activation markers, nutrient sensors, and cytokine receptors. During the second stage, CD4$^+$ T cells acquire the ability to produce cytokines by integrating signals that are received through the TCR and TCR-induced surface markers/receptors [159-161]. Finally, during the third stage, CD4$^+$ T cells proliferate. Given the profound effects of Dll1/4 inhibition on the production of multiple proinflammatory cytokines (**Fig. 4.3B**), we systematically evaluated whether Notch inhibition impacted molecular events that occur during T cell priming.

The *Nur77-GFP* allele has been utilized as a quantitative readout of overall TCR signal strength, both during T cell development and during peripheral T cell responses [162, 163]. Unlike the early activation marker CD69, Nur77-GFP has been reported to be insensitive to inflammatory stimuli such as TLR agonists and type I interferons, thus possibly making it a reliable readout of TCR strength even during highly inflammatory settings such as allo-BMT. However, this has not been formally tested. To assess whether the Nur77-GFP allele could be used to quantify I-A^d alloantigen-dependent signals even after irradiation, we generated 4C x Nur77-GFP x $Rag1^{-/-}$ mice. We compared GFP levels in 4C donor cells that were transplanted into lethally irradiated I-Ad^+ BALB/c recipients or lethally irradiated I-Ad^- autologous B6-SJL recipients. As kinetic analysis of cell division rate with efluor450 dye labeling of donor 4C cells revealed that no cell divisions occurred until after 24 hours post-transplantation (**Fig. 4.4A**), we examined GFP levels at day 1 post-transplantation. Indeed, transfer of 4C x Nur77-GFP x $Rag1^{-/-}$ donor cells into MHC-mismatched BALB/c recipients resulted in higher levels of GFP compared to 4C donor cells that were transferred into autologous B6-SJL recipients (**Fig. 4.4A**), suggesting that the Nur77-GFP could be utilized to quantify TCR signal strength during allo-BMT. Next, we assessed the impact of Dll1/4 inhibition on Nur77-GFP levels, cell size, and the expression of the early activation markers CD69, CD44, and CD25. Dll1/4 inhibition had no effect on Nur77-GFP levels at day 1 post-transplantation (**Fig. 4.4B**). We also observed no changes in cell size at day 1 post-transplantation or expression of the early T cell activation markers CD69 and CD44 at day 1.5 post-transplantation (**Fig. 4.4B**). However, CD25 expression at day 1.5 post-transplantation was reduced, consistent with previous reports that CD25 is a direct target of Notch signaling [35, 83, 85, 142].

Next, we assessed whether Dll1/4 inhibition altered signal transduction during T cell priming. Two major signaling pathways that are collectively activated by the TCR, costimulatory pathways, nutrient sensors, and cytokine receptors are the mTORC1 pathway and the Ras/MAPK pathway. To assess whether Dll1/4 inhibition impacted either of these pathways, we performed phosphoflow cytometry for the mTORC1 target S6 and the MAPK target ERK1/2 at day 1 post-transplantation. To control for specificity of phosphoflow staining, we labeled Thy1.1$^+$ 4C and Thy1.2$^+$ autologous BALB/c with efluor450 cell proliferation dye, and cotransplanted both donor populations into lethally irradiated BALB/c recipients (**Fig. 4.4C**). As expected, pS6 and pERK1/2 levels were higher in alloreactive 4C cells compared to autologous BALB/c cells (**Fig. 4.4C**). Dll1/4 inhibition resulted in a statistically significant decrease in both pS6(S235/S236) and pS6(S240/S244) levels, suggesting an impairment in mTORC1 signaling (**Fig. 4.4D**). Dll1/4 inhibition also resulted in a statistically significant decrease in pERK1/2 levels, suggesting a defect in Ras/MAPK activity. Collectively these data suggested that Notch inhibition impairs key signal transduction events during T cell priming.

Notch inhibition preserves early IL-2 and TNFα production, but impairs IFNγ and IL-17 production

Next, we assessed the impact of Dll1/4 inhibition on proinflammatory cytokine acquisition during the later stages of T cell priming, at day 1.75 post-transplantation. Similar to our observations at day 5 post-transplantation (**Fig. 4.3B**), Dll1/4 inhibition resulted in significant decreases in IFNγ and IL-17 production (**Fig. 4.5A**). In contrast, Dll1/4 inhibition had no

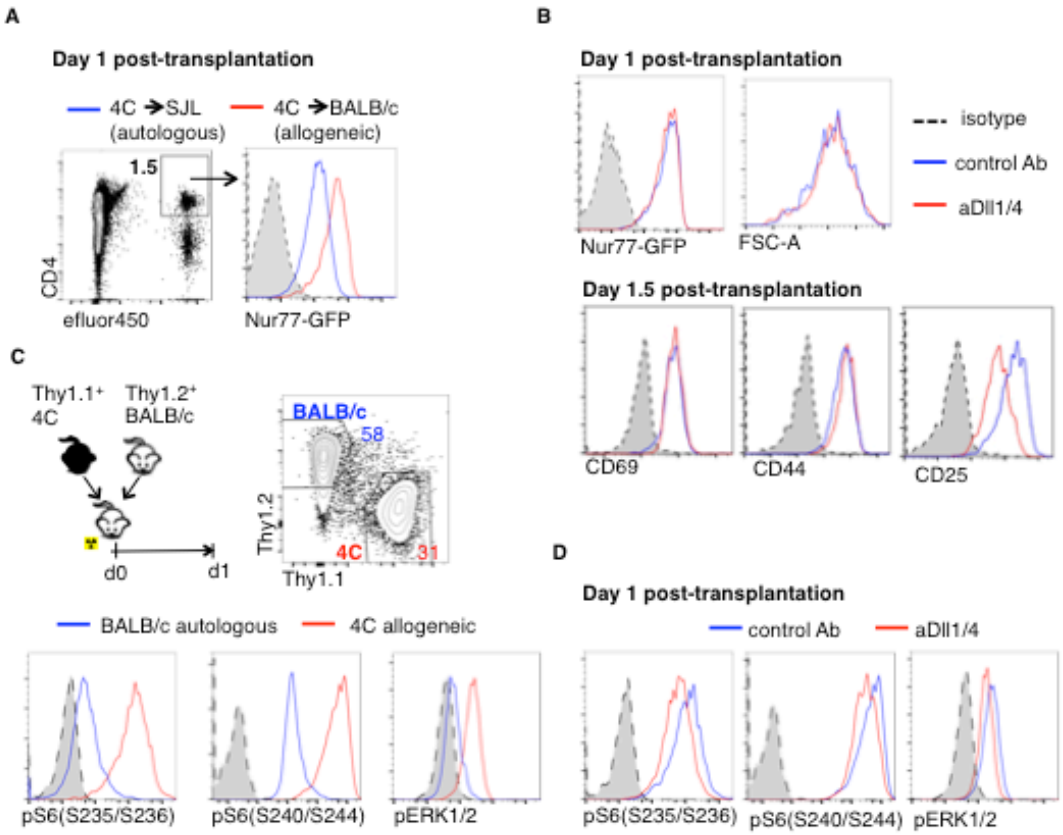

Figure 4.4. Impact of systemic Dll1/4 inhibition on key molecular events that occur during T cell priming

A. Nur77-GFP levels in 4C cells from spleen and LNs of lethally irradiated allogeneic BALB/c or syngeneic B6-SJL recipients at day 1 post-transplantation. **B.** Nur77-GFP levels, FSC-A, and activation marker expression in 4C donors from spleen and LNs of lethally irradiated (8 Gy) BALB/c + isotype control or BALB/c + aDll1/4 recipients. Nur77-GFP and FSC-A was assessed at day 1 post-transplantation, while activation marker expression was assessed at day 1.5 post-transplantation. **C.** Experimental strategy for phosphoflow cytometry analysis. pS6(S235/S236), pS6(S240/S244), and pERK1/2 staining in allogeneic 4C and autologous BALB/c cells from the same lethally irradiated (8 Gy) BALB/c recipient at day 1 post-transplantation. **D.** pS6(S235/S236), pS6(S240/244), and pERK1/2 staining in 4C cells from spleen and LNs of lethally irradiated (8 Gy) BALB/c + isotype control or BALB/c + aDll1/4 recipients at day 1 post-transplantation. In all experiments described in **(A-D)**, isotype control or anti-Dll1/4 neutralizing antibodies were injected i.p. on day 0 only. Data are representative of at least 3 experiments.

impact on IL-2 and TNFα production (**Fig. 4.5A**). Consistent with the initial preservation of IL-2 production, 4C cells from Dll1/4-inhibited BALB/c recipients did not exhibit a defect in the dilution of the cell tracking dye CMTMR (**Fig. 4.5B**). These data suggest that during T cell

priming, Notch-deprived alloreactive $CD4^+$ T cells initially acquire the ability to produce IL-2 and TNFα, while immediately becoming defective in their ability in produce IFNγ and IL-17.

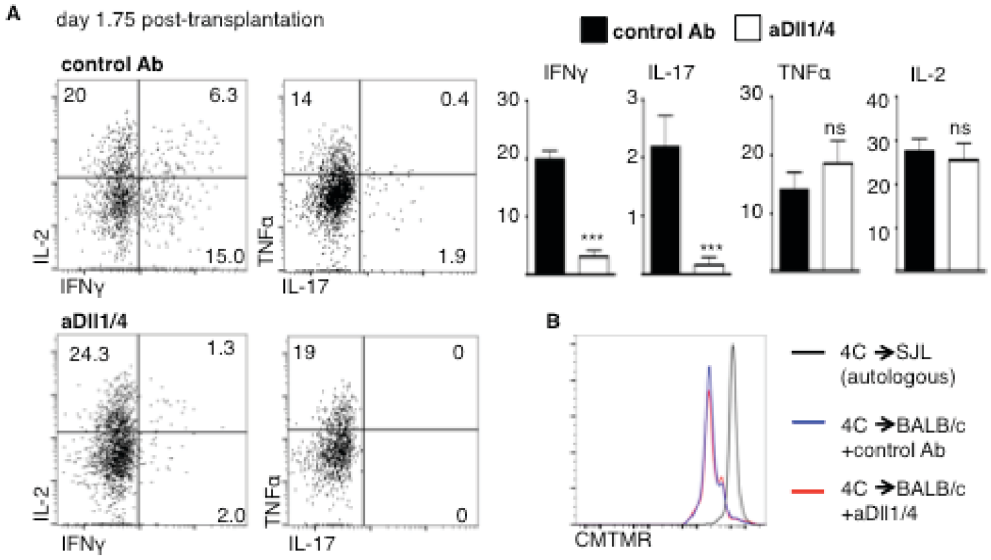

Figure 4.5. Systemic Dll1/4 inhibition preserves the acquisition of IL-2 and TNFα production, but not IFNγ and IL-17 production, during T cell priming
A. Intracellular cytokine staining in donor 4C cells from lethally irradiated (8 Gy) BALB/c + isotype control or BALB/c + aDll1/4 recipients after anti-CD3/CD28 restimulation at day 1.75 post-transplantation (n = 5/group). **B.** Proliferation (CMTMR dilution) by donor 4C cells from lethally irradiated syngeneic B6-SJL, BALB/c + isotype control, or BALB/c + aDll1/4 recipients at day 1.75 post-transplantation. B6-SJL recipients were irradiated at 1200 Gy, while BALB/c recipients were irradiated at 8 Gy. In both panels **A** and **B**, isotype control or anti-Dll1/4 neutralizing antibodies were injected i.p. at day 0 only. *P<0.05. Data are representative of 3 experiments, with error bars showing SD.

Transcriptional profiling of alloreactive T cells

We previously reported that Notch exerts its functional effects on $CD4^+$ and $CD8^+$ T cell alloreactivity through transcriptional regulation of target genes [72, 73]. To define the effects of Notch on the transcriptional landscape of alloreactive $CD4^+$ cells during T cell priming, we performed RNA-Seq analysis on sort-purified $CD4^+$ 4C cells from three different experimental groups at day 1.5 post-transplantation: 1) 4C -> I-Ad^+ BALB/c + isotype control; 2) 4C -> I-Ad^+ BALB/c + aDll1/4; 3) 4C -> I-Ad^- B6-SJL + isotype control. Comparison of group 1 (4C ->

BALB/c + isotype control) to group 3 (4C -> B6-SJL + isotype control) identified genes that were regulated by alloantigen, while comparison of group 1 to group 2 (4C -> BALB/c + aDll1/4) identified genes that were regulated by Notch. Differentially regulated transcripts were identified based on three criteria, which included a sufficient number of alignment reads for statistical analysis, a false discovery rate (q-value) of <0.05, and a relative fold change of >1.5 or <0.67.

In vivo exposure of 4C cells to I-A^d alloantigen differentially regulated their expression of 6212 genes, including the well-established TCR-dependent genes *il2, ifng,* and *cd25* (**Fig. 4.6A**). Systemic Dll1/4 inhibition in MHC-mismatched BALB/c recipients resulted in the differential regulation of 945 genes, among which 526 genes were also upregulated by alloantigen. Among the 945 genes that were differentially regulated by Notch, 619 genes were upregulated, while 326 genes were downregulated. Dll1/4 inhibition downregulated several canonical Notch target genes, including *dtx1, hes, cd25,* and *trib2* (**Fig. 4.6B**). Other previously reported Notch target genes, including *myc, tbx21, gata3, rorc*, *foxp3, hes5, heyL, hey1, hey2,* and *nrarp* were either not downregulated by Dll1/4 inhibition, or expressed below the limit of detection of RNA-Seq analysis (data not shown). Finally, Notch inhibition did not regulate *pten*, thus making it unlikely that the Notch1-PTEN-Hes1 axis is functionally important in $CD4^+$ alloreactive T cells.

Systemic inhibition of Dll1/4-mediated Notch signals broadly impaired the transcription of cytokines during T cell priming, with two notable exceptions. *Ifng, il21, il17a, il17f, il10,* and *il-3* transcripts were all markedly downregulated (**Fig. 4.6C**), while *il2 and tnfa* transcripts were unaltered (**Fig. 4.6D**). These findings were consistent with our intracellular flow cytometry analysis at day 1.75 post-transplantation (**Fig. 4.5A**). The initial preservation of *il2* transcription

was not unique to the 4C model, as we observed the same effects in the BALB/c anti-B6 polyclonal model (**Fig. 4.6E-F**).

Several cytokine receptor transcripts, including *il6st, il12rb2, il23r, il1r1,* and *cd25,* were downregulated by Dll1/4 inhibition (**Fig. 4.6G**). Gene Set Enrichment Analysis (GSEA) was consistent with this result, as we observed an enrichment in downregulated genes that are associated with cytokine signaling pathways (NES = -1.7), MAP kinase signaling pathways (NES = -1.7), and JAK/STAT signaling pathways (NES = -1.9)(**Fig. 4.6H**).

GSEA also revealed that systemic Dll1/4 inhibition results in an enrichment in downregulated genes that are associated with Myc signaling (NES = -1.9) and an enrichment in upregulated genes that are associated with the Treg transcription factor FoxP3 (NES = 2.0)(**Fig. 4.6I**). Both results were surprising, as *myc* transcript levels were unchanged at day 1.5 (**Fig. 4.6J**), while *foxp3* was expressed at nearly undetectable levels in $CD4^+$ 4C cells.

Early Notch inhibition results in aberrant T cell activation

Upon the completion of T cell priming, $CD4^+$ T cells downregulate CD69 while maintaining their expression of CD44 and CD25 [102, 158]. Surprisingly, at day 3 post-transplantation, systemic Dll1/4 inhibition resulted in persistent surface expression of CD69 and decreased expression of CD25 (**Fig. 4.7A**). Consistent with higher levels of CD69 expression, Nur77-GFP levels were also higher (**Fig. 4.7B**). 4C cells from aDll1/4-treated recipient mice were larger, exhibited higher granularity, and expressed higher levels of *c-myc* (**Fig. 4.7C**). Furthermore, despite expressing increased levels of the prosurvival factor Bcl2 (**Fig. 4.7D**), fewer 4C cells were present in spleens from Dll1/4-treated mice (**Fig. 4.7E**). Collectively, these data suggest

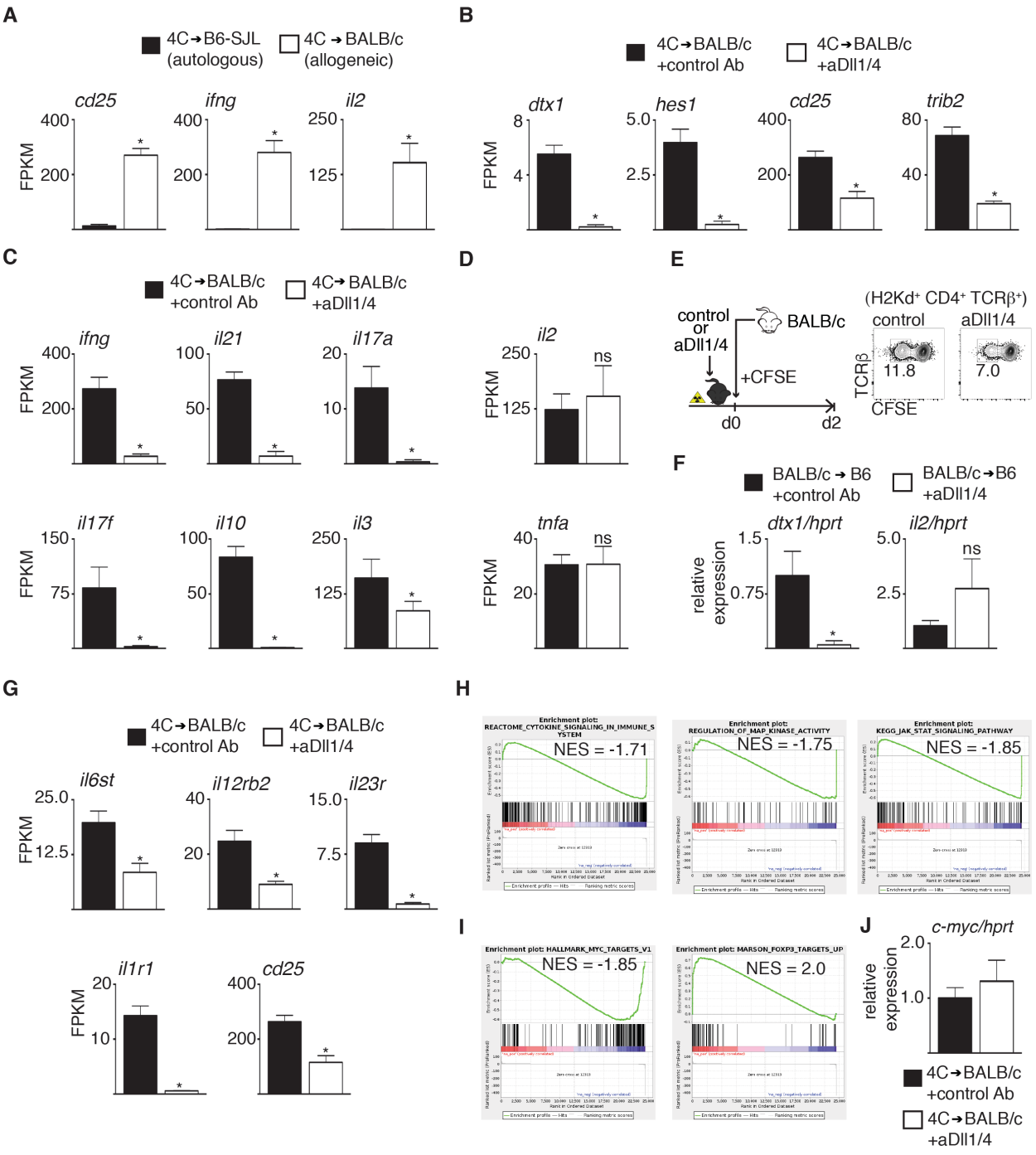

Figure 4.6. RNA-Seq analysis of the Notch transcriptome during alloreactive T cell priming
A. Abundance of *cd25, ifng,* and *il2* transcripts (FPKM) in 4C cells from lethally irradiated syngeneic B6-SJL or allogeneic BALB/c recipients at day 1.5 post-transplantation. **(B-D, G)**. Abundance of *dtx1, hes1, cd25,* and *trib2* transcripts **(B)**, *ifng, il21, il17a, il17f, il10,* and *il3* transcripts **(C)**, *il2* and *tnfa* transcripts **(D)**, *il6st, il12rb2, il23r, ilr1, cd25* transcripts **(G)** in 4C cells from lethally irradiated BALB/c + isotype control or BALB/c + aDll1/4 at day 1.5 post-transplantation. **(E)** Experimental strategy and flow cytometry plots to isolate $CD4^+$ alloreactive T cells at day 2 post-transplantation. Allogeneic BALB/c splenocytes were labeled with CFSE and injected into lethally irradiated (1200 rads) B6 mice. Divided $CFSE^{lo}$ BALB/c cells were sort-purified for gene expression analysis. **(F)** Abundance of *dtx1* and *il2* transcripts (qRT-PCR) in sort-purified $CFSE^{lo}$ $CD4^+$ T cells from lethally irradiated (1200 rads) B6 +

isotype control or B6 + aDll1/4 at d2 post-transplantation (n = 4 mice/group). **(H,I)** Gene set enrichment analysis (GSEA) plots for cytokine signaling, MAP kinase signaling, JAK/STAT signaling (**H**), Myc targets, and FoxP3 targets (**I**). **(J)** Abundance of *myc* transcripts (qRT-PCR) in sort-purified 4C cells from lethally irradiated BALB/c + isotype control or BALB/c + aDll1/4 at day 1.5 post-transplantation. NES, normalized enrichment score. FPKM, fragments per kilobase of transcript per million mapped reads. *P<0.05.

that Dll1/4 inhibition results in the aberrant acquisition of a T cell effector program that exhibits paradoxical features of hyperactivity.

Discussion

In this study, we utilized two different experimental strategies to highlight an important role for $CD4^+$ Tconv-intrinsic Notch signaling in mediating GVHD pathogenicity. First, we sort-purified Tconv and nTregs from B6 FIR or B6 FIR-DNMAML mice to generate "synthetic" donor inoculums of $CD4^+$ $FoxP3^-$ Tconv mixed with $CD4^+$ $FoxP3^+$ nTregs. Notch deprivation within Tconv cells alone was sufficient to protect from GVHD. In contrast, Notch deprivation within Tregs was not sufficient to confer protection from GVHD, as recipients of WT Tconv mixed with Notch-deficient Tregs were susceptible to GVHD. Second, we developed a new model of GVHD with $I\text{-}A^d$-reactive TCR transgenic $CD4^+$ 4C $Rag1^{-/-}$ donors, which completely lack Tregs due to the absence of $I\text{-}A^d$ antigen within the host. While 4C cells were potent inducers of GVHD mortality, they were sensitive to Notch inhibition by neutralizing antibodies against Dll1/4 Notch ligands. Our findings contrast with a recently published report that genetic ablation of Notch signaling specifically within Tregs with *foxp3-Cre* was sufficient to confer long-protection from GVHD mortality [78]. Total splenocytes from *foxp3*^{eGFPCre}*;rbpj*$^{f/f}$ or *foxp3*^{eGFPCre}*;notch1*$^{f/f}$ donors failed to induce mortality in a B6 anti-BALB/c model of GVHD. However, both $CD4^+$ and $CD8^+$ Tconv cells from these mice exhibited a hyporesponsive

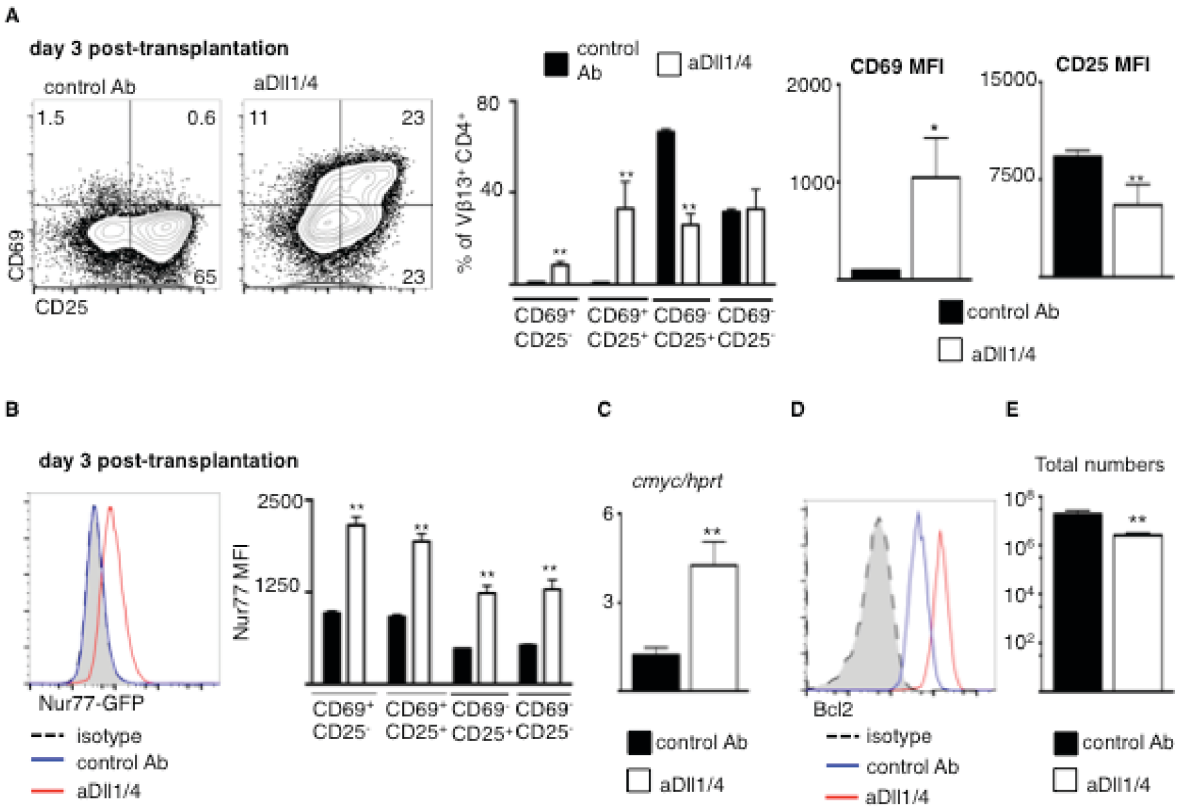

Figure 4.7. Systemic Dll1/4 inhibition results in aberrantly activated alloreactive T cells
CD69 vs. CD25 expression **(A)**, Nur77-GFP levels **(B)**, abundance of *c-myc* transcripts (qRT-PCR) (**C**), intracellular Bcl2 staining (**D**), and absolute numbers (**E**) of 4C cells from spleens of lethally irradiated BALB/c + isotype control or BALB/c + aDll1/4 recipients at day 3 post-transplantation. Isotype control or anti-Dll1/4 neutralizing antibodies were injected i.p. at day 0 only. *P<0.05, **P<0.01. Data are representative of at least 3 experiments, with error bars showing SD.

phenotype on baseline, as they were defective in their ability to produce IFNγ upon PMA/ionomycin restimulation. While the inability to produce IFNγ could have been the result of exuberant suppression of Tconv by Notch-deficient Tregs (which exhibited enhanced suppressive activity), the exact reason for hyporesponsiveness was not explored in detail. Thus, it was possible that the recipients of *foxp3*^{eGFPCre}*;rbpj*$^{f/f}$ or *foxp3*^{eGFPCre}*;notch1*$^{f/f}$ splenocytes were protected from GVHD mortality not because of loss of Notch signaling within Tregs, but because the donor Tconv cells were hyporesponsive. Assessing the ability of purified Tconv

cells from *foxp3*^{eGFPCre}*;rbpj*$^{f/f}$ or *foxp3*^{eGFPCre}*;notch1*$^{f/f}$ donor mice to induce GVHD could directly test this hypothesis.

Analysis of alloreactive 4C cells provided several lines of evidence that inhibition of Notch signaling preserves several aspects of T cell priming. First, Notch-deprived 4C cells displayed no defects in the upregulation of the activation markers CD69 and CD44. Second, transcription and protein synthesis of IL-2, which receives direct inputs from the TCR through the TFs AP-1, and NFAT, was unimpaired by systemic Dll1/4 inhibition. The initial preservation of IL-2 production by $CD4^+$ alloreactive T cells may explain why Notch inhibition in polyclonal models of GVHD results in the expansion of nTregs, which exquisitely depend on IL-2-mediated signals to proliferate and survive [164]. Third, Dll1/4 inhibition did not blunt the initial burst of proliferation by 4C cells. Collectively, these findings conflict with a recent report that Notch enhances CD69 expression, cell size, IL-2 production, and proliferation both *in vitro* and *in vivo* [70]). It is possible that depending on the strength and availability of antigen, Notch exerts different effects.

Both mTORC1 activity and Ras/MAPK activity were impaired in Notch-deprived alloreactive T cells during priming. mTORC1 activation is regulated by several extracellular stimuli, including amino acids, cytokine/growth factor receptors, TCR signaling, and the B7 family of costimulatory molecules [165, 166]. Similarly, Ras/MAPK signaling sits downstream of TCR signaling, cytokine/growth factor receptors, and the B7 family of costimulatory molecules. Therefore, it is unclear as to whether Notch inhibition regulates some or all of the aforementioned pathways to modulate mTORC1 and Ras/MAPK. While Nur77-GFP levels were not altered with Notch inhibition, Nur77-GFP has been reported to mainly read out TCR-mediated Protein Kinase C (PKC) activity [163]. Thus, while diacylglycerol-dependent PKC

activity is most likely intact in Notch-deprived alloreactive T cells, it is entirely possible that Notch modulates specific arms of TCR signaling, such as DAG-dependent Ras/MAPK activation and/or AKT-dependent mTORC1 activation. Alternatively, impairment of mTORC1 and Ras/MAPK could be due to impaired B7-mediated costimulatory signals or diminished cytokine/growth factor signaling. Intact production of IL-2, which receives direct inputs from Ca^{++}-dependent NFAT, costimulation-dependent NF-κB and ERK1/2-dependent AP-1 [167], would argue that both TCR-dependent and B7-mediated ERK1/2 activity is intact.

Our studies represent the first RNA-Seq-based transcriptional analysis of Notch signaling in mature T cells. Dll1/4 inhibition in alloreactive T cells downregulated several canonical Notch target genes, including *dtx1, cd25, trib2,* and *hes1*. Notably, alloreactive 4C cells expressed low levels of *hes1,* while its putative transcriptional target *pten* was unaffected by Notch inhibition (data not shown). Thus, it is unlikely that the previously reported Notch-Hes1-PTEN axis, which plays an important role in both T-ALL and developing thymocytes, operates in alloreactive T cells [152, 153]. Consistent with our previous observations in polyclonal models of GVHD, Notch did not regulate the expression of key TFs that control helper $CD4^+$ lineage fate decisions in mature T cells, including *tbx21, rorc, gata3,* and *foxp3* [71, 73]. On the other hand, a wealth of cytokines (*ifng, il21, il17a, il17f, il10, il3)* and cytokine receptors (*il6st, il12rb2, il23r, il1r1, cd25)* were all downregulated by Dll1/4 inhibition. However, it is unclear whether any of these regulated genes are direct targets of Notch, as the low number of 4C cells at day 1.5 post-transplantation prevented ChIP analysis. Thus, it is difficult to directly compare our findings to the recent report that Notch acts as an unbiased amplifier of T helper cell differentiation [69].

During T cell activation, induction of Myc is essential for the metabolic reprogramming of activated $CD4^+$ and $CD8^+$ T cells [168]. In $CD8^+$ T cells, Myc is asymmetrically partitioned

upon the first division, with Myc^{hi} cells acquiring effector-like properties and Myc^{lo} cells acquiring memory-like properties. Myc^{hi} cells produce higher levels of IFNγ and express high levels of CD25, while Myc^{lo} cells express higher levels of Bcl2 and exhibit lower levels of mTORC1 activity [169, 170]. Intriguingly, Dll1/4 inhibition resulted in the downregulation of Myc-associated target genes, suggesting an overall decrease in Myc function. Unlike what was observed with Notch inhibition in T-ALL cell lines, *myc* transcripts were unaltered by Dll1/4 inhibition, thus making it more likely that Notch promotes a post-translational increase in Myc protein levels and/or function. Notch-inhibited 4C cells exhibited similar features as Myc^{lo} cells, as they expressed higher levels of Bcl2, lower levels of IFNγ and CD25, and exhibited lower mTORC1 activity. Thus, it is tempting to speculate that Notch partially drives $CD4^+$ T cell alloreactivity through positive regulation of Myc activity. If this were indeed the case, this mechanism would be unlike the direct transcriptional regulation of *myc* in Notch gain-of-function T-ALL.

Myc functions by forming obligate heterodimers with MAX [156]. Together, they directly bind to DNA through their basic helix-loop-helix leucine zipper (bHLHZ) domains to regulate transcription. Other bHLHZ domain-containing proteins, such as MAD, antagonize Myc function through recruitment of histone deacetylase complexes. Myc protein levels are posttranslationally regulated through phosphorylation, ubiquitinylation, and acetylation. Specifically, phosphorylation of Myc at Ser-62 by Ras/MAPK, JNK, and CDK1 stabilizes Myc protein levels, while phosphorylation at Thr-58 by GSK3 destabilizes Myc protein levels [171, 172]. If Notch inhibition in alloreactive T cells indeed reduces Myc protein levels, one possible mechanism could be through the reduction of Ras/MAPK-mediated phosphorylation of Myc. We will directly assess Myc protein levels within alloantigen-specific T cells during priming by

crossing 4C mice to GFP-c-Myc reporter mice, which express a GFP-c-Myc fusion protein from the endogenous Myc locus [173]. Importantly, the GFP-c-Myc fusion protein fully retains endogenous Myc functionality, thus serving as a useful tool for quantifying c-Myc protein levels. Alternatively, if Notch inhibition in alloreactive T cells reduces Myc function as opposed to Myc protein levels, one possible mechanism could be through the antagonism of obligate heterodimer formation with MAX, or through enhancement of antagonism by MAD.

In summary, we have identified a $CD4^+$ Tconv-intrinsic role for Notch signaling in alloreactivity and GVHD. Notch-deprived $CD4^+$ Tconv alloreactive T cells did not display overt defects in initial antigen sensitivity, as they exhibited preserved activation marker upregulation, IL-2 production, and initial proliferation. Alloreactive T cells expressed low levels of *hes1*, while Notch inhibition did not alter *pten* expression, making it unlikely that Notch utilizes the Hes1-PTEN-AKT axis to drive alloreactivity. Notch inhibition had no impact on *myc* transcription. In contrast, Notch-deprived $CD4^+$ T cells immediately acquired a defect in IFNγ and IL-17 production, exhibited diminished mTORC1 and Ras/MAPK activity, and failed to upregulate Myc-associated target genes. Collectively, these data suggest that previously reported molecular mechanisms cannot account for the role of Notch in alloreactive T cells, and instead suggest a new set of unique mechanisms through which Notch modulates $CD4^+$ T cell alloreactivity.

Chapter 5

Conclusions and Perspectives

Despite the recent explosion in enthusiasm for CAR T cell therapy for patients with cancer, allogeneic bone marrow transplantation (allo-BMT) remains by far the most commonly used form of adoptive immunotherapy. Although allo-BMT can be highly successful, it is also linked to significant complications, such as GVHD, that limit its success and broad applicability. Thus, there is a need to better understand the pathogenesis of GVHD and to identify new pathways for therapeutic intervention. Notch signaling has emerged as an important regulator of T cell alloreactivity after allo-BMT. In this thesis, I first explored the temporal and spatial regulation of Notch signaling at the onset of GVHD. I found that nonhematopoietic $Ccl19^{+}$ fibroblasts deliver essential Notch signals to alloreactive T cells during the first 48 hours post-transplantation. Next, I explored the molecular impact of Notch on alloreactive Tconv cells during the critical period of Notch activity. Notch inhibition in Tconv cells did not impair early steps of T cell activation, preserving the initial acquisition of IL-2 and maintaining early proliferation. In contrast, Notch-deprived Tconv cells failed to acquire IFNγ and IL-17 production and displayed defects in mTORC1 and Ras/MAPK activity. Transcriptional profiling revealed that Notch inhibition preserved the expression of master T helper lineage transcription factors, but impaired the transcription of several proinflammatory cytokines, cytokine receptors, and Myc-dependent target genes. In this chapter, I will discuss the implications of my results,

provide predictions about the immunobiological functions of Ccl19^{+} fibroblasts and Notch signaling in T cell alloimmunity, as well as discuss future experimental directions.

FRCs in alloimmunity and beyond

Early studies of Notch ligand expression in DCs [124], together with the finding that DCs could determine Th1 or Th2 fates in CD4^{+} T cells through the differential expression of Delta-like or Jagged Notch ligands [51], predicted that DCs would be responsible for the simultaneous presentation of alloantigen and Notch ligands to alloreactive T cells during allo-BMT. However, in our studies, BM chimeras lacking *Dll1* and *Dll4* solely within their hematopoietic tissues remained sensitive to GVHD mortality **(Chapter 3)**, thus ruling out this possibility. Instead, our results revealed a critical role for Ccl19^{+} fibroblasts as a source of Dll1/4 Notch ligands in GVHD and suggest three alternative scenarios for how alloreactive T cells interact with alloantigens and receive Notch signals (**Fig. 5.1**). In the first model, hematopoietic DCs present alloantigens while Ccl19^{+} fibroblasts deliver Notch ligands. In the second possibility, Ccl19^{+} fibroblasts simultaneously provide both alloantigens and Notch ligands. In the third scenario, a nonhematopoietic APC that is not a Ccl19^{+} fibroblast presents alloantigens, while Ccl19^{+} fibroblasts deliver Notch ligands. Of note, all these scenarios profoundly revise our current understanding of GVHD pathogenesis.

The observation that adoptive transfer of host-derived MHC class II-expressing DCs was sufficient to induce disease in GVHD-resistant MHCII$^{-/-}$ mice provided the first suggestion that DCs were responsible for providing alloantigens to CD4^{+} T cells *in vivo* [174, 175]. This concept was consistent with the well-established role for DCs as APCs for CD4^{+} T cells in immune responses outside the context of allo-BMT. Furthermore, BM chimera studies with

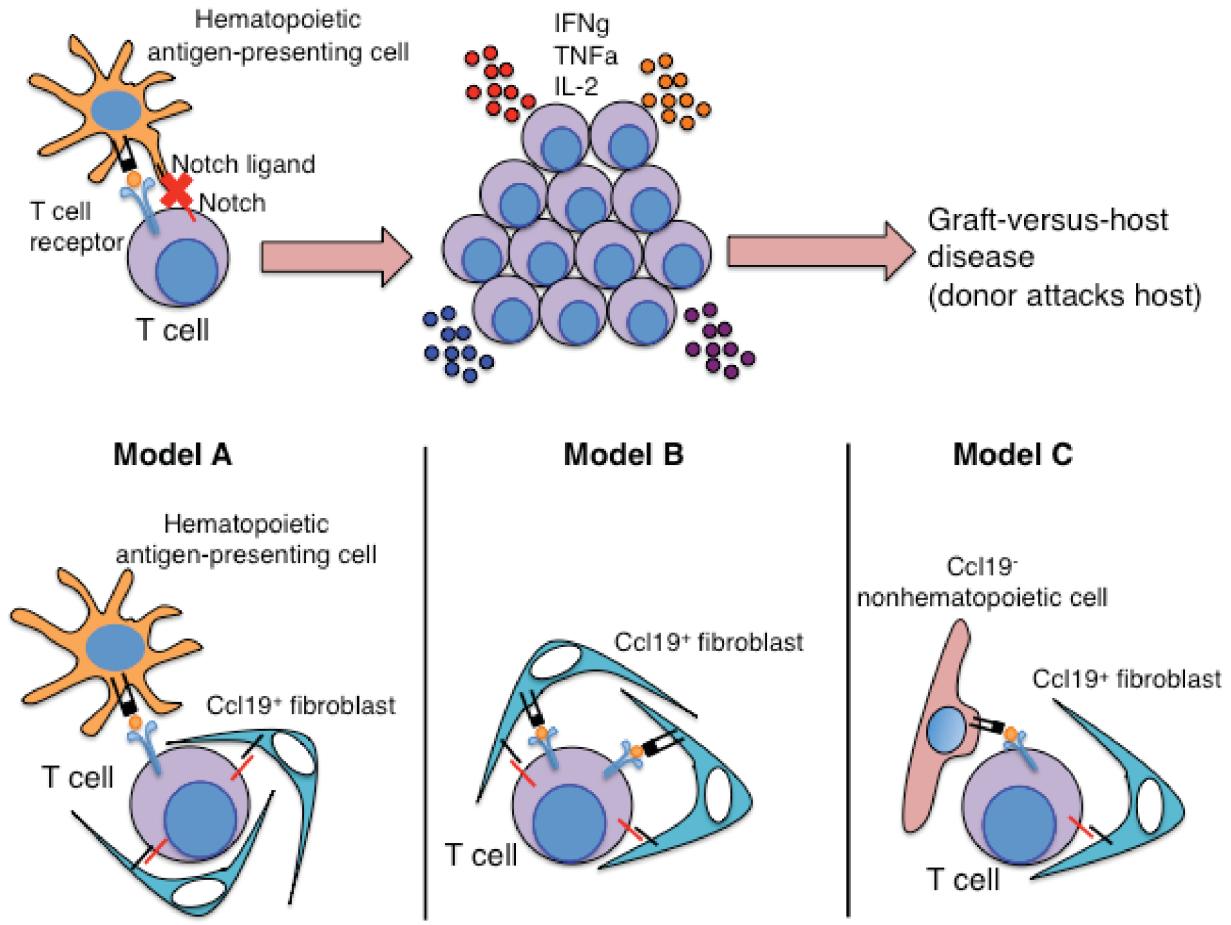

Figure 5.1. Three models for how alloreactive CD4$^+$ T cells receive their alloantigens and Notch ligands during priming

Hematopoietic antigen-presenting cells (APCs) are dispensable sources of Notch ligands (**top panel**). Three possible models for how alloantigen-specific T cells receive their alloantigen and Notch ligands (**bottom panel**). In model A, hematopoietic APCs deliver alloantigens while Ccl19$^+$ fibroblasts provide Notch ligands. In models B and C, hematopoietic APCs are completely dispensable for both alloantigen presentation and delivery of Notch ligands. In model B, Ccl19$^+$ fibroblasts deliver both alloantigens and Notch ligands. In model C, a nonhematopoietic Ccl19$^-$ cell presents alloantigen while Ccl19$^+$ fibroblasts deliver Notch ligands.

MHC class I-deficient β2-microglobulin KO mice had previously demonstrated that host DCs were essential for delivering alloantigen during CD8$^+$-mediated GVHD [12]. However, as the transferred DCs were not irradiated in the add-back studies, it was conceivable that irradiated host DCs would not behave in the same way. Three recent studies suggested that this was most likely the case. Antibody-mediated or genetic depletion of host conventional DCs, plasmacytoid

DCs, and B cells had no impact on GVHD [130, 132]. In an H-Y antigen-dependent model of GVHD, thymectomized BM chimeras that lacked MHC class II within their hematopoietic system remained sensitive to GVHD [131]. Similarly, in a parent-> F1 irradiation-dependent model of GVHD, BM chimeras that lacked MHC class II within donor and host hematopoietic tissues remained sensitive to GVHD lethality, while >60% of BM chimeras that lacked MHC class II within donor hematopoietic and host nonhematopoietic tissues were protected from GVHD lethality [130]. Collectively, these data suggested that nonhematopoietic cells could serve as functional APCs in the absence of functional DCs. Thus, these recent findings would argue against the first model, at least for $CD4^+$ T cell-mediated alloreactivity. Several lines of evidence support the notion that FRCs and or other nonhematopoietic cells can present antigen through MHC class II. Transcriptional profiling revealed that FRCs upregulate both subunits of MHC class II and intracellular antigen presentation components in response to inflammation [107]. Furthermore, FRCs by themselves can drive $CD4^+$ T proliferation in an antigen-dependent manner, either through direct expression of class II or through transfer of class II from other neighboring cells [112, 176, 177]. MHC class II expression is regulated by the master transcription factor class II trans-activator (CIITA), which contains four major promoters regions, pI, pII, pIII, pIV [178-180]. CIITA-pIV is IFNγ-dependent, and is operational in nearly all nonhematopoietic cell types. For example, in an H-Y mismatched model of GVHD, liver endothelial cells and gut epithelial cells that were treated with IFNγ had the capacity to drive H-Y antigen-dependent T cell proliferation *in vitro* [131]. Thus, both the second model, in which $Ccl19^+$ fibroblasts present both alloantigen and Notch ligands, and the third scenario, in which $Ccl19^-$ nonhematopoietic cells present alloantigen while $Ccl19^+$ fibroblasts present Notch ligands, are plausible.

Future studies in the lab will directly test the second model, namely the possibility that Ccl19^{+} fibroblasts present alloantigen. To test whether Ccl19^{+} fibroblasts are important sources of alloantigens, TEa TCR transgenic CD4^{+} donor T cells, which recognize BALB/c-derived I-E^{d} antigen specifically in the context of I-A^{b} MHC class II, will be transplanted with WT TCD B6 BM into irradiated B6 x BALB/c F1 recipients that express both the *Ccl19-cre* transgene and one copy of the floxed *I-A^{b}* allele. In this experimental system, Ccl19^{+} fibroblasts would lack the machinery to present alloantigen to donor TEa cells, as they would lack the expression of I-A^{b} (but would retain expression of BALB/c-derived I-A^{d} and I-E^{d}), while Ccl19^{-} cells would retain expression of MHC class II. This design would circumvent the potential disruptions in immune homeostasis that may be present in B6 *Tg$^{Ccl19\text{-}cre+}$;H2-Ab1$^{\Delta/\Delta}$* mice, as F1 experimental mice could be generated by crossing B6 *Tg$^{Ccl19\text{-}cre+}$;H2-Ab1$^{\Delta/+}$* with BALB/c mice. Alternatively, if B6 *Tg$^{Ccl19\text{-}cre+}$;H2-Ab1$^{\Delta/\Delta}$* do not exhibit baseline defects in immune homeostasis, they could be utilized as recipients of purified BALB/c CD4^{+} T cells and WT B6 TCD BM. Using either model, if genetic inactivation of MHC class II within Ccl19^{+} fibroblasts results in long-term protection from GVHD, it would strongly suggest that Ccl19^{+} fibroblasts are important presenters of alloantigen. If the aforementioned genetic strategies do not lead to long-term protection, we would have to rule out the possibility that Ccl19^{+} fibroblasts acquire MHC class II from neighboring Ccl19^{-} cells before concluding that they are not important for alloantigen presentation.

If genetic studies demonstrate that Ccl19^{+} fibroblasts are indeed responsible for both alloantigen presentation and the delivery of Notch ligands to alloreactive T cells, it is interesting to speculate about the location at which this would occur. It would be entirely conceivable that one population of Ccl19^{+} fibroblast presents alloantigens, while another population provides

Notch ligands. Using flow cytometry and immunofluorescence studies, I demonstrated that CD157$^+$ T zone FRCs and CD21/35$^+$ FDCs both expressed high levels of Dll4 (**Chapter 3**). Given that T cells enter the LN parenchyma through HEV "exit ramps" which are formed by T zone FRCs [92], it would be an obvious location within which alloantigen priming could occur. Preliminary studies with intravital imaging support this possibility (discussed later in "**Molecular mechanisms of Notch in alloimmunity and beyond**"). Alternatively, alloreactive T cells could enter the LN parenchyma and encounter their alloantigens and Notch ligands on FDCs, which can present opsonized antigen, at the center of B cell follicles. While this scenario would be unlikely during a normal immune response due to the strict chemokine and FRC-dependent boundaries that restrict T cell trafficking, it is possible that normal trafficking is disrupted during allo-BMT and that restricted microanatomical areas become accessible to incoming T cells. Supportive of this possibility, I found through static immunofluorescence images that 4C alloreactive T cells could localize within B cell follicles during the first two days post-transplantation (**Chapter 3**). However, it is unclear whether alloreactive cell simply transit through the B follicles to reenter the T cell zone, or whether they persist inside. Finally, in a third scenario, alloantigen presentation by Ccl19$^+$ fibroblasts could occur outside of SLOs, as GVHD can develop in their absence [181] while we and others have observed *Ccl19-Cre* transgene activity in tissues outside of SLOs.

We found that a single dose of Dll1/4 neutralizing antibodies at the time of transplantation was sufficient to confer long-term protection from GVHD. In contrast, delaying administration by two days resulted in a substantial loss of clinical protection, thus identifying an early window of functionally important Notch signaling during allo-BMT **(Chapter 3)**. This narrow temporal window, which coincides with T cell priming, could be explained by a short duration of high

intensity Notch signaling, a short duration of T cell sensitivity to Notch, or both. Two different scenarios could facilitate the short period of high intensity Notch signaling. In the first scenario, prolonged contacts between alloreactive T cells and alloantigen-presenting cells would promote a substantial decline in T cell motility, allowing for the sustained delivery of Notch signals from a focal niche. Upon disengagement from the immune synapse, T cells would once again increase their motility, resulting in decreased exposure to Notch signals. In other words, alloantigen would facilitate the sustained delivery of Notch signals by $Ccl19^{+}$ fibroblasts. In the second scenario, alloreactive T cell would transiently traffic to a unique microenvironment that is rich in Notch ligands (i.e. where FDCs are present). Upon exiting this area, alloreactive T cells would be exposed to lower concentrations of Notch ligands, thus dampening Notch signals.

While our studies prominently highlight $Ccl19^{+}$ fibroblasts as the key cellular sources that deliver Notch signals to alloreactive T cells during GVHD, it remains an open question as to whether our observations are generalizable to other types of T cell-driven responses. It is possible that the highly inflammatory conditions that ensue after total body irradiation, in conjunction with the rapid disappearance of radiosensitive hematopoietic cells, are key factors that determine the relative importance of $Ccl19^{+}$ fibroblasts in delivering Notch ligands to T cells. Recent *in vivo* loss-of-function genetic approaches have generated conflicting results on this matter. In one study, conditional deletion of *Dll4* in host DCs ($Tg^{CD11c\text{-}cre;}\ Dll4^{\Delta/\Delta}$) impaired H-Y antigen responses by adoptively transferred H-Y specific Marilyn TCR transgenic cells [70]. Relative to Marilyn T cells that were transferred into WT male mice, Marilyn T cells that were transferred into male $Tg^{CD11c\text{-}cre;}\ Dll4^{\Delta/\Delta}$ mice were smaller, expressed lower levels of activation markers, and produced less IL-2. In a second study, transfer of OVA-specific OT-II TCR transgenic cells into $Tg^{CD11c\text{-}cre;}\ Dll1^{\Delta/\Delta}$ impaired their survival [84]. In ongoing studies that

were not included in this thesis, I found that Ccl19^{+} fibroblasts were not important cellular sources of Dll4 in a model of myelin oligodendrocyte glycoprotein peptide-induced experimental autoimmune encephalitis. On the other hand, Notch-dependent Tfh differentiation was reported to proceed normally in the absence of Notch ligand expression on hematopoietic cells, and was exquisitely dependent on Dll4 ligands from Ccl19^{+} fibroblasts [123]. Furthermore, in studies that were not included in this thesis, I also found that Ccl19^{+} fibroblasts deliver Dll1/4 ligands to alloreactive T cells in a heterotopic model of cardiac allograft rejection. Thus, it is still unclear whether Ccl19^{+} fibroblasts provide Notch ligands during normal immune responses that occur in the absence of irradiation. Further studies will be required to clarify this question, as it has significant implications about the mechanisms through which Notch signals are delivered to T cells.

Molecular mechanisms of Notch in alloimmunity and beyond

The recent emergence of new prophylactic strategies for GVHD, such as post-transplantation cyclophosphamide, has renewed enthusiasm for understanding early molecular events affecting alloantigen-specific T cells during their initial priming [182-185]. However, the field of experimental GVHD has been limited by the lack of tools for identifying alloantigen-specific T cells at early time points. In parent -> F_1 models of GVHD, cell proliferation and expression of the early activation marker CD69 were utilized to approximate the frequency of alloreactive T cell precursors in polyclonal donor inoculums [186]. However, in irradiation-dependent models of GVHD, both CD69 upregulation and cell proliferation can occur in bystander cells that are undergoing homeostatic proliferation independently of alloantigen (data not shown). Thus, technical limitations prevented molecular analysis of alloantigen-specific T cells in irradiation-dependent models of GVHD. This changed with the development of TCR transgenic cells with

fixed antigen specificity. However, while TCR transgenic cells were employed to assess the long-term impact of clinical interventions and/or specific molecular pathways on GVHD [187, 188], there still remained a wide gap in knowledge about the molecular effects that occur during the early stages of priming. We bridged this gap by establishing a new model of acute GVHD that utilized a clonal population of alloantigen-specific $CD4^+$ T cells. We envision that this model can be used as a broad platform to evaluate the mechanistic impact of nearly any therapeutic intervention, not just Notch inhibition, in alloantigen-specific T cells during priming.

While the dynamics of pathogen-specific T cells have been characterized through intravital imaging, it is unclear whether alloantigen-specific T cells behave in a similar fashion. To gain insights into this question, the Serody group recently performed intravital imaging studies in two polyclonal models of acute GVHD [189]. Allogeneic donor T cells participated in prolonged contacts with DCs within two hours after allo-BMT and exhibited slower instantaneous velocity than syngeneic donor T cells. Furthermore, Tregs reduced the contact time between Tconv cells with DCs. However, there were three major problems with this analysis that could change the interpretation of these results. First, given that alloantigen-specific T cells constitute a low fraction of the donor inoculum, it is most likely that a majority of the observed behavior was attributable to bystander T cells that were not alloantigen-specific. Second, autologous T cells were imaged in separately transplanted mice, suggesting that non-cell autonomous factors or technical variability could have contributed to differences in behavior between allogeneic and autologous cells. Third, a mixture of both $CD4^+$ and $CD8^+$ T cells were infused into the recipient. As $CD8^+$ cells receive alloantigen from host DCs while $CD4^+$ cells may not, their behavior in relation to DCs would most likely be different. Thus, it was clear that imaging studies with alloantigen-specific $CD4^+$ or $CD8^+$ T cells needed to be performed.

In an effort to define the impact of Notch on early alloantigen-specific T cell dynamics in relation to Ccl19^{+} fibroblasts, I collaborated with the Huang laboratory at Case Western Reserve University to perform intravital multiphoton imaging studies. In light of the caveats of the aforementioned Serody study, we established an alternative experimental system utilizing alloantigen-specific CD4^{+} 4C T cells. As we knew that these cells were potent inducers of GVHD lethality (**Chapter 4**), we postulated they would serve as a good tool for intravital imaging analysis. To visualize Ccl19^{+} fibroblasts, we generated F1 B6 x BALB/c mice that expressed both the *Ccl19-cre* transgene and the *ROSA26*^{TdTomato} reporter alleles. The *ROSA26*^{TdTomato} allele was substantially brighter than the *ROSA26*^{eYFP} allele, allowing us to identify Ccl19^{+} fibroblasts with either multiphoton or confocal microscopy. We labeled 4C alloantigen-specific CD4^{+} T cells with the cell tracker dye CMTMR, autologous F1 CD4^{+} T cells with the cell proliferation dye CFSE, transferred both populations into the same irradiated F1 *Tg*$^{Ccl19\text{-}cre+}$*;ROSA26*^{TdTomato} recipients, and immediately began imaging the exposed popliteal LN (**Fig. 5.2**).

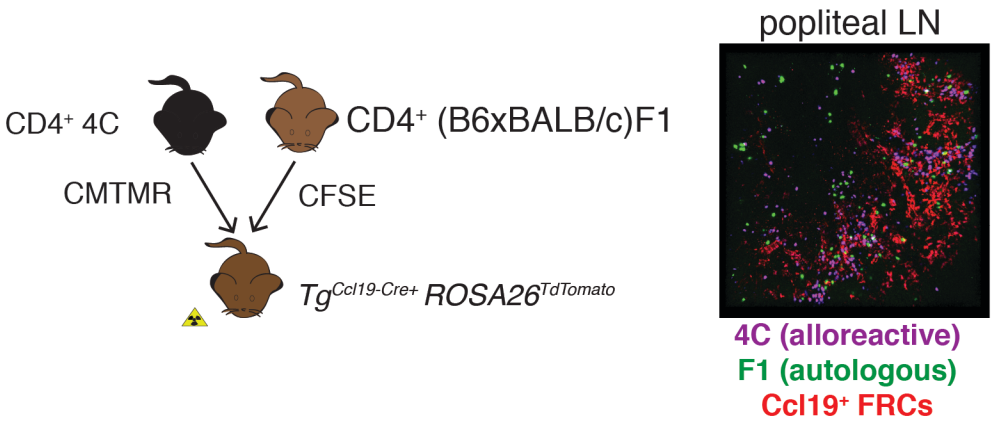

Figure 5.2. Experimental design for intravital imaging of alloantigen-specific CD4^{+} T cells and Ccl19^{+} fibroblasts
Alloantigen-specific 4C^{+} cells were labeled with CMTMR, autologous cells were labeled with CFSE, while recipient mice express both Tg$^{Ccl19\text{-}cre}$ and *ROSA26*^{TdTomato}. Image on the right represents a snapshot of the popliteal LN six hours post-transplantation.

Future studies will involve quantifying the instantaneous velocity and displacement ratios of alloantigen-specific T cells in both irradiation-dependent and irradiation-independent F1 models, characterizing the spatial relationship of alloantigen-specific cells to $Ccl19^+$ fibroblasts and DCs during priming with a special focus on assessing whether alloantigen-specific T cells participate in prolonged contacts with one or both cell types, and defining the features of the microanatomical niches in which alloantigen-specific T cells localize. These data will not only provide insights into the behavior of alloantigen-specific T cells during priming, but also provide clues about their interaction partners. Of course, it will be important to characterize the effects of Notch inhibition on all of the aforementioned parameters. Finally, other more exploratory questions could involve the use of 2C $CD8^+$ transgenic T cells to define $CD8^+$ alloreactivity and the development of strategies to image either the mesenteric LN or the spleen, two major sites to which alloreactive T cells immediately traffic after allo-BMT. Altogether, these findings will allow the field to determine whether alloantigen-specific T cells abide by the classical three-phase model of priming that was initially defined by von Andrian and colleagues [158, 190]. The extraordinarily high abundance of antigen, combined with the inflammatory environment after allo-BMT, could conceivably result in a divergent process of T cell priming.

One tool that would aid in future intravital imaging studies could be the development of a robust Notch reporter that reads out *in vivo* Notch signals in a specific, quantitative and dynamic manner. Currently available reporters, which utilize BAC transgenes that contain tandem RBP-Jκ binding sites upstream of a reporter gene, have failed to achieve one or more of these criteria. Recent work that utilized synthetic Notch receptors with swapped out intracellular transcriptional domains to sense extracellular stimuli could aid in this endeavor [191]. For example, mice that simultaneously express a tetracycline response element promoter upstream of eYFP and a

modified version of Notch1 that replaces the intracellular domain with a tetracycline transactivator could perhaps be useful.

RNA-Seq analysis of the transcriptome of Notch-inhibited alloreactive 4C cells undergoing T cell priming revealed a profound impairment in the transcription of several cytokines, including *ifng, il17a, il17f, il10,* and *il21*. Notably, *il2* and *tnfa* were unchanged with Notch inhibition. Consistent with our previous work, expression of master T helper lineage transcription factors, including *tbx21, gata3, rorc*, *foxp3,* were not regulated by Notch in alloreactive T cells. Thus, it is unlikely that Notch exerts control over T helper lineage fate decisions. Instead, it most likely functions to ensure the appropriate acquisition of effector functions. Notably, Notch inhibition preserved initial proliferation of alloantigen-specific T cells, suggesting a dissociation of proliferation from effector function acquisition. While my studies do not yet provide a clear molecular explanation for how this dissociation occurs, they provide a clear-cut reason for why Notch inhibition does not result in global immunosuppression.

Our mechanistic studies with a clonal population of alloreactive T cells revealed that Notch inhibition also results in the delayed downregulation of CD69 after alloantigen stimulation (**Chapter 4**). This effect was not unique to 4C cells, as we observed the same result when polyclonal BALB/c cells were transferred into lethally irradiated $Tg^{Ccl19\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ mice (data not shown). Given that CD69 expression receives direct inputs from the TCR, these data suggested that Notch-deprived alloreactive T cells could be receiving a longer pulse of TCR signals. As CD69 expression is also regulated by inflammatory stimuli such as Toll-like receptors [162] and type I IFNs [192], it is conceivable that this observation was due to a non-cell autonomous effect. However, assessment of Nur77-GFP levels, which has been reported to be insensitive to inflammatory mediators, showed similar results. While Nur77-GFP levels

rapidly declined to undetectable levels in WT alloreactive cells after priming, Notch-deprived T cells persistently expressed Nur77-GFP. Collectively, these findings supported the possibility that Notch-deprived alloreactive T cells receive a longer duration of TCR signals. Alternatively, it is conceivable that Notch-deprived T cells are globally defective in protein degradation, resulting in delayed turnover. This hypothesis could be tested in pulse-chase experiments with radioactively labeled amino acids.

How do alloreactive T cells quantify the signals they receive during priming? How do alloreactive T cells determine when to disengage from their immune synapse after participating in prolonged contacts with APCs? This process must be tightly controlled, as improper acquisition of signals can result in dysfunctional effector outcomes or ultimately, cell death. One important mechanism that ensures the optimal delivery of signals is the employment of digital and analog elements in classical signal transduction pathways. Analog signals can be converted into digital signals to form signaling thresholds. Upon meeting the threshold for activation, T cells enforce negative feedback loops to turn off signaling. For example, TCR-mediated ERK1/2 activation results in the phosphorylation of the transmembrane scaffold LAT at Thr155 to diminish IP_3-mediated Ca^{++} mobilization [193]. However, classical signal transduction pathways by themselves might not be sufficient for ensuring optimal signal delivery, due to their dependence on signal amplification. Thus, it is conceivable that alloreactive T cells leverage the unique biochemistry of Notch signaling, which does not rely on signal amplification, to "count" signals in a more precise manner. Alloreactive T cells could more precisely quantify the quality and duration of their interactions with APCs during the prolonged contact phase through the regulation of transcriptional targets downstream of Notch. In the absence of Notch signals, alloantigen-specific T cells would lack the ability to quantify signals in a refined manner,

resulting in miscoordinated release from the synapse. This model would be predicated on the assumption that Notch signals are delivered in parallel to alloantigen signals, which has yet to be demonstrated.

Notch-deprived alloreactive T cells share some common molecular features with exhausted T cells, which develop during situations of persistent antigen exposure such as chronic viral infections and high tumor burden. Exhausted T cells produce diminished amounts of proinflammatory cytokines, express higher levels of inhibitory surface markers, and persistently express the early activation marker, CD69 [194]. Furthermore, while exhausted $CD4^+$ and $CD8^+$ T cells exhibit divergent transcriptional programs, they share the common feature of expressing high levels of the T-box transcription factor, Eomes [195]. Notably, we have also observed that both $CD4^+$ and $CD8^+$ Notch-deprived alloreactive T cells express increased levels of Eomes [73]. A subset of exhausted $CD8^+$ T cells can be rescued with PD-1 blockade. As both $CD4^+$ and $CD8^+$ Notch-deprived T cells exhibit higher levels of PD-1 expression at day 5 post-transplantation, it would be interesting to test whether PD-1 inhibition in Notch-deprived T cells could reverse some molecular features of Notch inhibition. One notable difference between Notch-deprived alloreactive T cells and exhausted T cells is their proliferative capacity, as polyclonal Notch-deprived alloreactive T cells demonstrate minor defects in expansion while exhausted T cells divide at substantially slow rates. Understanding how Notch-deprived alloreactive T cells sustain cell division could provide insights into their unique behavior.

Could Notch act as a costimulatory modulator that requires other ongoing signals to exert its functional effects? This would mean that exposure of mature T cells to isolated Notch signals would be insufficient to alter their function. Instead, Notch would modulate signals from other pathways in a context-dependent manner. Several experimental findings support this concept.

First, in T cell coculture studies with antigen-pulsed artificial APCs that overexpressed different Notch ligands, Notch could only drive cytokine production if polarizing cytokines were included [67]. Second, in T cell coculture studies with antigen-pulsed artificial APCs that overexpressed Dll4, Notch promoted T cell activation and IL-2 production only when the B7 family member CD80 was also expressed on APCs [70]. Third, I observed that naïve T cells already experience Notch signals, as acute inhibition of Dll1/4 ligands with neutralizing antibodies or genetic inactivation of Dll1/4 ligands with *Ccl19-cre* downregulates Notch target genes in naïve T cells. Yet, naïve T cells from Notch-deprived DNMAML mice exhibit no obvious phenotypes on baseline. Thus, the consequences of Notch signaling may become apparent only when T cells engage in an antigen-driven immune response. However, it is important to note that in murine models of GVHD, Notch inhibition achieves superior clinical protection as compared to what is achieved with inhibition of individual costimulatory pathways. For example, both CD28 KO and ICOS KO donor T cells are delayed in their induction of GVHD, but ultimately cause GVHD lethality [187, 196]. Thus, it is likely that Notch plays a more complicated role than simply acting like the classical B7 and TNF family of costimulatory molecules.

Several interesting questions about Notch signaling in mature T cells require further exploration. Are the molecular effects of Notch in T cells reversible? If not, could this explain the narrow window of sensitivity to Notch inhibition that we observed during allo-BMT? Epigenetic studies of mature T cells have revealed the highly plastic nature of $CD4^+$ T helper subsets, arguing in support of reversibility [55]. Are the functional outcomes of persistent exposure to Notch different than that of transient exposure to Notch? MZBs certainly require persistent Notch signaling for maintenance, while T-ALL cell lines require persistent Notch signals for their survival. Furthermore, *in vitro* studies with GSI inhibitors demonstrated that

persistent Notch signaling is necessary for maintaining for T helper programs [69]. Do mature T cells experience different functional outcomes when exposed to differing levels of Notch signal strength?

Therapeutic potential of Notch in T cell alloimmunity

The finding that a single injection of Dll1/4 neutralizing antibodies conferred long-term protection from acute GVHD highlights the prophylactic potential of Notch inhibition for allo-BMT patients. Importantly, a single dose of Dll1/4 antibodies not only inhibited proinflammatory cytokine production by Tconv, but also expanded Tregs. As the intestine, thymus, and vasculature are exquisitely dependent on Dll1/4 signals for their maintenance, regeneration, and/or function, the efficacy of a single dose is highly encouraging. However, it is unclear whether Notch inhibition would serve as an effective form of therapy for patients who already manifest with acute GVHD symptoms. In fact, it is possible that Notch inhibition in these patients could exacerbate their intestinal problems, due to on target effects.

While delayed Dll1/4 inhibition by two days did not achieve the same level of clinical protection as Notch inhibition at the time of transplantation, ~20% of the allo-BMT recipients were protected long-term. In a model of cardiac allograft rejection, systemic Notch inhibition for the first 2 weeks after transplantation reduced the development of germinal center and plasma B cells, which may depend on Notch signals that extend beyond the first two days [74]. Thus, it is unclear whether the narrow therapeutic window for Notch inhibition is unique to acute GVHD or generalizable to other Notch-dependent T cell responses. A deeper understanding of the basic mechanisms that regulate Notch function could shed light on this question. Exploring the therapeutic potential of Dll1/4 inhibition in other alloimmune and autoimmune contexts will continue to serve as an exciting avenue with high translational potential.

The identification of fibroblastic niches that deliver Dll1/4 ligands to incoming alloreactive T cells opens up the possibility of targeting the niches themselves for therapeutic purposes. One approach would be to target the molecular inputs that regulate Dll1/4 ligand expression within fibroblastic niches. While Notch ligands are expressed within many tissues, it is plausible that different cell types employ distinct regulatory mechanisms to control Dll4 expression. For example, it was recently reported that sex steroids selectively regulate Dll4 expression within thymic epithelial cells but not endothelial cells [197]. Thus, understanding how Notch ligand expression is enforced within $Ccl19^{+}$ fibroblastic niches could lead to selective therapies that have few side effects.

Within the thymus, the transcription factor Foxn1 coregulates the expression of the Notch ligand *dll4* and T cell progenitor recruitment chemokines *ccl25, cxcl12,* and *scf* [133]. It is conceivable that SLOs also coregulate the expression of Notch ligands and the chemokines CCL19/CCL21 in an analogous fashion, allowing for FRCs to efficiently deliver both recruitment signals and Notch-dependent priming signals to alloreactive T cells. While microarray studies of $gp38^{+}$ $CD31^{-}$ FRCs suggest that they express low levels of *foxn1*, it is likely that only a small subset of FRCs that expresses Dll4 utilizes this regulatory network. Alternatively, FRCs might utilize an alternative transcription factor, such as one that defines mesenchymal cells, to enforce a similar transcriptional network. Comparing the transcriptomes of $Ccl19^{+}$ $CD157^{+}$ FRCs, which are enriched for Dll4 expression, to $Ccl19^{+}$ $CD157^{-}$ FRCs could provide insight into this question.

Chapter 6

Materials and Methods

Mice. BALB/c (H-2^d), C57BL/6 (H-2^b, CD45.2) [B6] and B6-CD45.1 (H-2^b, CD45.1) mice were bred at the University of Michigan. $Tg^{Mx1\text{-}cre}$, $Dll1^{f/f}$, $Dll4^{f/f}$, *Foxp3-IRES-RFP*, *Nur77-GFP*, and 4C $Rag1^{-/-}$ T cell receptor transgenic mice were previously described [34, 117, 157, 162, 198]. $Tg^{Mx1\text{-}cre}$;$Dll1^{f/f}$;$Dll4^{f/f}$ mice were kindly provided by Freddy Radtke. 4C $Rag1^{-/-}$ mice were kindly provided by Todd Brennan. *Nur77-GFP* mice were kindly provided by Carey Lumeng. $Tg^{Mx1\text{-}cre}$ allows for interferon-inducible activation of Cre expression, e.g. via systemic administration of poly(I:C). 4C $Rag1^{-/-}$ mice have a monoclonal population of $CD4^+$ T cells specific for the I-A^d MHC class II alloantigens, which enables tracking of alloantigen-specific T cells *in vivo*. 4C $Rag1^{-/-}$ mice were crossed to *Nur77-GFP* mice and backcrossed onto the $Rag1^{-/-}$ background. $Tg^{Mx1\text{-}cre}$;$Dll1^{f/f}$;$Dll4^{f/f}$ mice were maintained on the B6 background and backcrossed to the BALB/c background for >5 generations. *Ccl19-Cre* BAC transgenic mice ($Tg^{Ccl19\text{-}cre}$) express Cre recombinase in FRCs under the control of a 90 kb regulatory sequence upstream of the *Ccl19* transcriptional start site [123, 126, 139, 140]. $Tg^{Ccl19\text{-}cre}$ mice were kindly provided by Burkhard Ludewig and Freddy Radtke. $Tg^{Ccl19\text{-}cre}$ mice were crossed to B6 $Dll4^{f/f}$;$Dll1^{f/f}$;$ROSA26^{eYFP}$ mice (expressing Cre-inducible eYFP under control of the *ROSA26* promoter).

Induction and assessment of GVHD. 6-10 week old BALB/c or 8-12 week old B6 recipients underwent allo-BMT as previously described [71-73]. Both females and males were used as recipients, and were distributed equally among experimental groups. BALB/c mice received 8.5-9 Gy (^{137}Cs source) 4 hours prior to allo-BMT, while B6 mice received two doses of 6 Gy (^{137}Cs) separated by 3 hours. T cell-depleted bone marrow (TCD BM) was prepared with anti-Thy1.2 antibodies and complement (Cedar Lane Laboratories) [72], resulting in >95% depletion of Thy1.2$^+$ cells. BALB/c recipients received 5-10x10^6 TCD BM +/- 5x10^6 donor splenocytes, while B6 recipients received 10-15x10^6 TCD BM +/- 20x10^6 splenocytes. Clinical GVHD score and weight changes were monitored at least weekly, as described [71-73].

Antibody-mediated inhibition of Delta-like Notch ligands. Humanized IgG1 neutralizing antibodies against Dll1, Dll4 and isotype control were described previously [72, 199, 200]. Nearly unlimited quantities of Dll1, Dll4, and isotype control antibodies were kindly provided by Christian Siebel and Minhong Yan at Genentech. All antibodies were injected i.p. (5 mg/kg twice weekly). The potency and specificity of each antibody batch was verified by assessing their capacity to achieve *in vivo* depletion of Dll1-dependent marginal zone B cells or Dll4-dependent thymocytes [72].

Generation of BM hematopoietic chimeras and GVHD induction. B6-CD45.2 $Tg^{Mx1\text{-}cre+};Dll1^{f/f};Dll4^{f/f}$ donor mice or control littermates received 5 i.p. injections of 50 μg poly(I:C) (Amersham) every other day. Two weeks after the last poly(I:C) dose, 6-10 week old female B6-CD45.1 recipients received 9 Gy of irradiation and were reconstituted with syngeneic BM from poly(I:C)-induced $Tg^{Mx1\text{-}cre+};Dll1^{\Delta/\Delta};Dll4^{\Delta/\Delta}$ or control littermates. Donor chimerism was quantified 12 weeks later by determining the relative frequency of CD45.2$^+$ cells within mature cell populations in blood and spleen. Efficiency of Cre-mediated *Dll1* and *Dll4* recombination

was assessed by PCR in sort-purified blood myeloid cells using the following primers: Dll1-fwd: CACACCTCCTACTTACCTGA; Dll1-rev: GAGAGTACTGGATGGAGCAAG; Dll1-loxas: GGCGCTCAAAGGATATGGGA; Dll4-fwd: GTGCTGGGACTGTAGCCACT; Dll4-rev: TGTTAGGGATGTCGCTCTCC; Dll4-revdel: CTCGTCTGTTCGCCAAATCTTAC.

BM chimeras were rested at least 12 weeks to allow for establishment of steady-state hematopoiesis before GVHD induction. BM chimeras were irradiated with two doses of 5.5 Gy each (^{137}Cs) separated by 3 hours, and received $8x10^6$ TCD BM +/- $30x10^6$ splenocytes from BALB/c donors (or syngeneic B6 donors as negative control). Clinical GVHD score and weight changes were monitored at least weekly [71-73].

Lymph node stromal cell isolation. Stromal cells were isolated from LNs using a modified version of a previously described protocol [106, 123]. Briefly, peripheral LNs (cervical, axial, brachial, inguinal) from unirradiated or irradiated mice were coarsely chopped with a scalpel and incubated at 37°C for 15 minutes in digestion solution containing DMEM, 2% FBS, 1.2 mM $CaCl_2$, penicillin/streptomycin, 1.0 mg/mL collagenase IV (Invitrogen) and 40 μg/mL DNAse I (Roche). Samples were pipetted gently and reincubated at 37°C until all solid material appeared to be dissolved. Suspensions were filtered through a 70 μM strainer and analyzed by flow cytometry.

Flow cytometry. The following antibodies were from BioLegend: anti-CD4, CD8α, CD19, CD11c, CD11b, F4/80, PDCA1, B220, MHCII, CD44, CD62L, podoplanin/gp38, CD31, Ter119, CD157, CD44, CD25, CD21/35, CD45, TCRβ, H-2K^b, H-2K^d, IFNγ, TNFα and IL-2. Anti-FoxP3 antibodies were from eBioscience. For assessment of T cell cytokine production, donor splenocytes were stimulated with plate-bound anti-CD3 (2.5 μg/mL, clone 145-2C11) and anti-

CD28 (2.5 μg/mL, clone 37.51) for 2-3 hours prior to addition of monensin (Golgistop, BD Biosciences). Samples were fixed and stained for intracellular proteins per manufacturer's instructions. Flow cytometric analysis was performed on a 3-laser Fortessa or 4-laser FACS AriaII/III (Becton Dickinson). Dead cells were excluded with DAPI (Sigma Aldrich) or Zombie Aqua fixable viability dye (Biolegend).

Immunofluorescence microscopy. LN and spleens were fixed in 1% PFA for 2 hours, sunk in 30% sucrose overnight, and embedded/frozen in Tissue-Tek OCT in an ethanol/dry ice bath. Staining of 6-8 μm thin cryosections was performed for 60' or overnight at room temperature using the following antibodies: monoclonal anti-CD3ε (145-2C11), B220 (RA3-6B2), CD35 (8C12), podoplanin/gp38 (8.1.1), MAdCAM-1 (Meca-89) and polyclonal goat anti-Dll4 (AF1389; R&D systems). Secondary antibodies were: donkey anti–rat biotin, donkey anti–rat Cy3, donkey anti–rabbit Cy3, donkey anti–sheep APC (Jackson ImmunoResearch), streptavidin Alexa Fluor 488, donkey anti–rabbit Alexa Fluor 647 (both Invitrogen). For Dll4 labeling, primary antibodies were detected using a biotinylated donkey anti–goat IgG followed by HRP-coupled streptavidin (Jackson ImmunoResearch) and tyramide Signal Amplification (Invitrogen) according to the manufacturer's instructions, but using a borate buffer (0.1 M in PBS, pH 8.5) for tyramide dilution. eYFP was detected using a rabbit anti-GFP antibody followed by Alexa Fluor 488–conjugated donkey anti–rabbit IgG (both Invitrogen). Images were acquired on an AxioImager.Z1 microscope with an AxioCam, and were processed in Adobe Photoshop. Specifically, image contrast was adjusted using the Levels tool, while image sharpness was improved with the Unsharpen Mask command. Tissue sections were stained with three different fluorochromes, but processed images were often simplified into two color images with ImageJ's "split channels" and "merge channels" functions.

EdU incorporation and detection. Mice were injected i.p with 2.5 mg/mL EdU 12 hours prior to tissue collection. LN and spleen were fixed in 1% PFA for 2 hours, sunk in 30% sucrose overnight, and embedded and frozen in OCT in an ethanol/dry ice bath. EdU was detected with Click-iT EdU Alexa Fluor 488 imaging kit (Molecular Probes), per manufacturer's instructions.

Quantitative real-time PCR. Donor T cells or host stromal cells were sort-purified directly into TRIzol (Invitrogen). Total RNA was extracted with phenol/chloroform and purified with RNeasy Micro kit (Qiagen). cDNA was generated with SuperScript II (Invitrogen), and subjected to quantitative PCR with either Taqman or SYBR Green PCR Master Mixes on Mastercycler ep realplex (Applied Biosystems). Gene expression analysis was performed using the following primers: *dtx1* (Mm00492297_m1, Applied Biosystems); *hes1* (Mm01342805_m1, Applied Systems); Hprt-fwd: CTCCTCAGACCGCTTTTTGC, Hprt-rev: TAACCTGGTTCATCATCGCTAATC; Il7-fwd: GTGCCACATTAAAGACAAAGAAG; Il7-rev: GTTCATTATTCGGGCAATTACTATC. Relative gene expression was determined via the ΔΔCT method, with normalization to *hprt*.

Isolation of 4C alloreactive T cells. 4C cells were isolated from spleen and LN using an enzymatic digestion protocol. Spleen and LNs were finely chopped with a scalpel and incubated at 37°C for 20 minutes in 2 mL digestion solution containing RPMI, 0.75 mg/mL collagenase IV (Invitrogen), 0.2 mg/mL collagenase D (Roche), and 40 μg/mL DNAse I (Roche). Samples were then vigorously aspirated and expirated with a 1 mL pipette to disrupt the organ capsule, and undissolved organ fragments was allowed to settle for 2 min. Dissolved material was then transferred into 10 mL of FACS buffer (PBS + 4% FBS + 2 mM EDTA), while undissolved fragments were resuspended in 2 mL of new digestion solution. Samples were pipetted continuously for 10 min, after which undissolved organ fragments were allowed to settle for 2

min. Dissolved material was added to the same 10 mL of FACS buffer, while undissolved fragments were once again resuspended in 2 mL digestion solution. Cells were pipetted vigorously for another 10 min, until all solid material appeared to be dissolved. Suspensions were filtered through a 70 μM strainer and utilized for downstream applications.

Phosphoflow. Samples were stained with surface markers for 15 min on ice, washed twice with FACS buffer, and fixed in 4% PFA for 30 min on ice. Samples were pelleted and permeabilized with ice-cold 90% methanol for 30 min on ice. Samples were then washed twice in FACS buffer, and samples were stained overnight with 100 uL intracellular antibody cocktail mix. Samples were acquired the next day.

RNA-Seq analysis. 10,000 4C cells were sort-purified directly into TRIzol. Total RNA was extracted with phenol/chloroform and purified with RNAEasy PLUS Micro kit (Qiagen) to remove contaminating DNA. Total RNA was depleted of ribosomal RNA with Ribogone columns (Clontech). RNA-Seq libraries were prepared with the SMARTer Ultra-Low input stranded kit and sequenced using the Illumina Hi-Seq platform. For bioinformatics analysis, Clontech SMARTer adapters were trimmed, and raw reads were aligned to the UCSC mm10 reference genome using TopHat and Bowtie. Cufflinks/CuffDiff were utilized for differential expression analysis.

Statistical analysis. Sample size for *in vivo* mouse experiments was determined empirically based on prior experience, and used to calculate power with the 'pwr' statistical package in R. After assessing for normality of data with the Shapiro-Wilk test, variances of different treatment groups were compared with a two-tailed F-test. Means of two different treatment groups were compared with a two-tailed unpaired Student's t test only if they had statistically similar

variances. Significance was calculated and noted as *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$. Survival curves were compared using Log-rank (Mantel-Cox) test. Bar graphs were generated with GraphPad Prism.

Ethics statement. All experiments were performed according to NIH guidelines and approved by the University of Michigan's Committee on Use and Care of Animals.

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