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## Early Notch Signals Induce a Pathogenic Molecular Signature during Priming of Alloantigen-Specific Conventional CD4<sup>+</sup> T Cells in Graft-versus-Host Disease

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# Early Notch Signals Induce a Pathogenic Molecular Signature during Priming of Alloantigen-Specific Conventional CD4<sup>+</sup> T Cells in Graft-versus-Host Disease

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Graft-versus-host disease (GVHD) is the most serious complication of allogeneic hematopoietic cell transplantation. Notch signals delivered during the first 48 h after transplantation drive proinflammatory cytokine production in conventional T cells (Tconv) and inhibit the expansion of regulatory T cells (Tregs). Short-term Notch inhibition induces long-term GVHD protection. However, it remains unknown whether Notch blockade blunts GVHD through its effects on Tconv, Tregs, or both and what early Notch-regulated molecular events occur in alloantigen-specific T cells. To address these questions, we engineered T cell grafts to achieve selective Notch blockade in Tconv versus Tregs and evaluated their capacity to trigger GVHD in mice. Notch blockade in Tconv was essential for GVHD protection as GVHD severity was similar in the recipients of wild-type Tconv combined with Notch-deprived versus wild-type Tregs. To identify the impact of Notch signaling on the earliest steps of T cell activation in vivo, we established a new acute GVHD model mediated by clonal alloantigen-specific 4C CD4<sup>+</sup> Tconv. Notch-deprived 4C T cells had preserved early steps of activation, IL-2 production, proliferation, and Th cell polarization. In contrast, Notch inhibition dampened IFN- $\gamma$  and IL-17 production, diminished mTORC1 and ERK1/2 activation, and impaired transcription of a subset of Myc-regulated genes. The distinct Notch-regulated signature had minimal overlap with known Notch targets in T cell leukemia and developing T cells, highlighting the specific impact of Notch signaling in mature T cells. Our findings uncover a unique molecular program associated with the pathogenic effects of Notch in T cells at the earliest stages of GVHD. *The Journal of Immunology*, 2019, 203: 557–568.

Notch signaling is an evolutionarily conserved signaling pathway with multiple roles in immune cell development and function (1). Notch has emerged as an essential regulator of T cell alloreactivity in mouse models of graft-versus-host disease (GVHD) and allograft rejection (2–11). We previously demonstrated that genetic blockade of Notch signaling within donor CD4<sup>+</sup> and CD8<sup>+</sup> T cells and therapeutic neutralization of the Notch Delta-like ligand (Dll) 1 and Dll4 results in long-term protection from GVHD morbidity and mortality after allogeneic hematopoietic cell transplantation (allo-HCT) while largely

preserving graft-versus-leukemia (GVL) activity (2, 4, 6). We identified host fibroblastic stromal cells in secondary lymphoid organs as the critical source of Dlls that drive pathogenic Notch activity in donor T cells within 48 h posttransplantation (10). GVHD inhibition via Notch blockade was associated with decreased IFN- $\gamma$  and IL-17 production as well as expansion of pre-existing Foxp3<sup>+</sup> regulatory T cells (Tregs). At peak expansion, Notch-deprived CD4<sup>+</sup> and CD8<sup>+</sup> T cells exhibited blunted Ras/MAPK signaling and upregulated several negative regulators of T cell signaling while preserving the expression of

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The online version of this article contains supplemental material.

Abbreviations used in this article: allo-HCT, allogeneic hematopoietic cell transplantation; BM, bone marrow; CMTMR, 5-(and-6)-((4-chloromethyl)benzoyl)amino) tetramethylrhodamine; Dll, Delta-like ligand; DNAML, dominant negative mastermind-like 1; GO, Gene Ontology; GVHD, graft-versus-host disease; IRES, internal ribosome entry site; RNA-Seq, RNA sequencing; T-ALL, T cell acute lymphoblastic leukemia; TCD, T cell-depleted; Tconv, conventional T cell; Treg, regulatory T cell; WT, wild-type.

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the master transcription factors T-bet and Eomesodermin (2, 4, 6). In addition, the selective genetic inactivation of Notch signaling in Tregs was recently reported as sufficient to confer long-term protection from acute GVHD (9), although the existence of secondary functional changes in conventional T cells (Tconv) could not be ruled out. Therefore, further work is needed to define whether Notch signaling functions primarily to promote Tconv pathogenicity, destabilize Treg suppressive potential, or impact both populations to aggravate GVHD. Understanding the downstream molecular consequences of Notch signaling in T cells will provide new insights into its effects at the earliest stages of alloreactivity.

Studies in T cell acute lymphoblastic leukemia (T-ALL), >50% of which harbor Notch gain-of-function mutations, have provided key insights into the molecular mechanisms that operate downstream of Notch in this context (12). Chromatin immunoprecipitation and  $\gamma$ -secretase inhibitor washout studies revealed a range of direct transcriptional targets of Notch in T-ALL, many of which are associated with distal enhancers (13–16). However, it remains unclear if Notch regulates the same targets in mature T cells because systematic studies have not been performed in Ag-reactive T cell subsets, which rely on a very different context-specific enhancer landscape (4, 17–19). Cleaved intracellular Notch has been proposed to function either as an amplifier of Th cell differentiation by binding to Th lineage fate master transcription factor and cytokine loci (20) or by enhancing Ag sensitivity in a B7/CD28-dependent fashion via professional hematopoietic APCs (19, 21–24). Although Notch blockade failed to impact the expression of master transcription factors driving individual effector T cell lineages during GVHD (2, 4, 11), the contribution of other individual mechanisms to the role of Notch in T cell alloreactivity remains unknown (2, 4, 11).

The earliest posttransplant time window represents a critical period of alloreactive T cell priming and Notch activity that defines subsequent GVHD. Thus, we investigated the impact of Notch signaling on cellular and molecular events in alloreactive T cells during this time to gain insight into the molecular impact of Notch on alloimmunity. As Notch inhibition in mature CD4<sup>+</sup> and CD8<sup>+</sup> T cells exerts effects on both Tconv and Tregs, we first established the relative importance of Tconv and Tregs in mediating the protective effects of Notch inhibition in a polyclonal model of MHC-mismatched GVHD. After identifying Notch signaling in Tconv as essential for GVHD pathophysiology, we dissected alloreactive Tconv responses during the immediate posttransplant period. As the majority of donor polyclonal T cells are nonalloreactive bystander cells that do not contribute to GVHD, we established an MHC-mismatched model of acute GVHD driven by a defined clonal population of alloantigen-specific CD4<sup>+</sup> T cells. This strategy allowed us to define the cellular and molecular effects of Notch within alloantigen-specific cells during *in vivo* T cell priming. Notch inhibition within Tconv preserved early T cell activation, expansion, and IL-2 production as well as the induction of *tbx21*, *gata3*, and *rorc* transcription. In contrast, Notch-deprived T cells had markedly decreased transcripts for multiple proinflammatory cytokines, including IFN- $\gamma$ , IL-17A/F, and IL-21, showed reduced mTORC1 and MAPK activity, and exhibited features of diminished Myc function. In this relatively narrow transcriptional signature, we identified targets with a broad potential impact on T cell alloimmunity that were regulated by Notch blockade. Our study highlights the molecular signature that underlies the impact of Notch signaling on the acquisition of pathogenic CD4<sup>+</sup> T cell effector functions during GVHD.

## Materials and Methods

### Mice

BALB/c (H-2<sup>d</sup>, CD45.2<sup>+</sup>), BALB/c-CD45.1 (H-2<sup>d</sup>, CD45.1<sup>+</sup>), C57BL/6 (B6, H-2<sup>b</sup>, CD45.2<sup>+</sup>), and C57BL/6.Ptprca (B6-SJL, H-2<sup>b</sup>, CD45.1<sup>+</sup>) mice were bred at the University of Michigan or the University of Pennsylvania. *ROSA26<sup>DNMAML</sup>* mice (dominant negative mastermind-like 1 [DNMAML]; H-2<sup>b</sup>, CD45.2<sup>+</sup>) containing a Cre-inducible cassette encoding the DNMAML-GFP pan-Notch inhibitor under the *ROSA26* promoter were crossed with Foxp3-internal ribosome entry site (IRES)-RFP reporter mice [Foxp3-IRES-mRFP, strain 008374; The Jackson Laboratory (25)]. Because no effect of Cre expression was observed in alloreactive T cells (data not shown), both *Cd4-Cre<sup>+</sup>* or *Cd4-Cre<sup>-</sup>* controls were used. 4C *Rag1*<sup>-/-</sup> TCR transgenic mice on B6 background (H-2<sup>b</sup>), reactive to I-A<sup>d</sup>, were previously described (26). Transgenic mice expressing a GFP reporter under the control of Nur77 (*Nr4a1*) promoter sequences (Nur77-GFP, H-2<sup>b</sup>, strain 016617; The Jackson Laboratory) were described before (27). All littermates (experimental and control animals) were cohoused until experimental use. Animals were 8–16 wk old at the time of use. All protocols were approved by the University of Pennsylvania's Office of Regulatory Affairs and the University of Michigan's Committee on Use and Care of Animals.

### Bone marrow transplantation, systemic Ab-mediated Notch inhibition, and GVHD assessment

BALB/c recipients were irradiated with 8–8.5 Gy (<sup>137</sup>Cs source) and B6-SJL recipients with 12 Gy (in two split doses separated by 3 h). T cell-depleted (TCD) bone marrow (BM) was prepared as described (6) and 5–10 × 10<sup>6</sup> TCD BM was injected alone or with T cells in doses outlined in the appropriate figure legends. To allow sufficient cell retrieval, we transplanted 5 × 10<sup>6</sup> 4C T cells for all experiments in which downstream T cell analyses were done prior to 48 h posttransplant. In select experiments, T cells were labeled with CFSE or CellTracker Orange 5-(and-6)-((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR) Dye (Invitrogen). Humanized IgG1 mAbs specific for Dll1 or Dll4 (28, 29) were injected i.p. (5 mg/kg) on day 0 pretransplant. An anti-HSV gD human IgG1 Ab was used as an isotype control. The quality of each mAb batch was tested *in vivo* by assessing the inhibition of Dll4-dependent T cell development and Dll1-dependent marginal zone B cell development (30–33). Systemic clinical GVHD was graded twice a week using an established system (34), and survival was monitored daily.

### Flow cytometry

Single cell suspensions from lymphoid organs (spleen, cutaneous, and mesenteric lymph nodes) were prepared using a modified version of a previously described protocol (35, 36). Briefly, organs were coarsely chopped with a scalpel and incubated at 37°C for 20 min in digestion solution containing RPMI 1640, 2% FBS, 1.0 mg/ml collagenase IV (Invitrogen), and 40  $\mu$ g/ml DNase I (Roche), followed by 10 min of sample pipetting at 37°C to disrupt the tissue. This process was repeated up to two times until tissue dissolution, followed by passage through a cell strainer. Detection of cytokine production in alloreactive T cells harvested within the first 48 h after allo-HCT was done following a 4 h incubation with brefeldin A (BD Biosciences) only. Alloreactive T cells harvested beyond the first 48 h posttransplant were restimulated for 6 h in 96-well round-bottom plates precoated with 2.5  $\mu$ g/ml anti-CD3 $\epsilon$ /anti-CD28 (BioLegend), with brefeldin A added during the last 4 h, before subsequent staining. Surface staining was performed following staining with Zombie Aqua Fixable Viability dye (BioLegend). Intracellular staining was done following the manufacturer's protocol (eBioscience). Phospho-flow analysis was done as described (37). Abs, clones, and manufacturers are described in the Supplemental Table I. Analysis was performed on an LSRFortessa (BD Biosciences). Sorting was performed with FACSARIA III (BD Biosciences). Data were analyzed using FlowJo (TreeStar).

### RNA sequencing and bioinformatics analysis

4C T cells were sort purified to >95% purity. Total RNA was isolated using TRIzol (Invitrogen) and RNeasy Micro Kit (QIAGEN). Libraries were prepared with Clontech SMARTer Kit, and sequencing was performed using Illumina Hi-Seq platform. The quality of the raw reads was assessed using FastQC (version 0.10.1). The first three bases from the 5' end from all reads were trimmed to remove Clontech SMARTer adapter, and the quality of all raw reads was then assessed using FastQC (version 0.10.1). FASTQ files were aligned to genome build GRCh38 using the STAR aligner (version 2.5.2b) and the Ensembl annotation (release 87) as a splicing guide. Gene counts were obtained using Subread featureCounts

(version 1.5.1) using the same Ensembl annotation, counting second strand alignments, not counting multi mapping alignments, and assigning reads to features with the largest overlap. Genes with <10 counts across all samples were discarded. Differentially expressed genes were identified using DESeq2 (version 1.14.1). All three conditions were normalized together, and results were obtained by applying contrasts between any two conditions. Significant genes were filtered for an adjusted  $p$  value <0.01 and an absolute log<sub>2</sub> fold change >0.585 (1.5 $\times$ ). Heat maps were prepared using the regularized log<sub>2</sub> values obtained from DESeq2 and the pheatmap function in R. Gene Set Enrichment Analysis (GSEA) was performed by first transforming the mouse identifiers into human homologs using annotation from Ensembl BioMart and using the PreRanked function of GSEA with the log<sub>2</sub> fold changes reported from DESeq2. RNA sequencing (RNA-Seq) data (GSE126518) have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>).

### Statistics

GraphPad Prism 7.03 was used for statistical analysis. Values are presented as mean  $\pm$  SEM. Statistical differences were calculated using one-way ANOVA with Tukey correction for multiple comparisons, Student  $t$  test, or log-rank test, as indicated for specific experiments. Only statistically significant differences with a  $p$  value <0.05 are highlighted.

## Results

### Active Notch signaling in CD4<sup>+</sup> Tconv is necessary for acute GVHD development

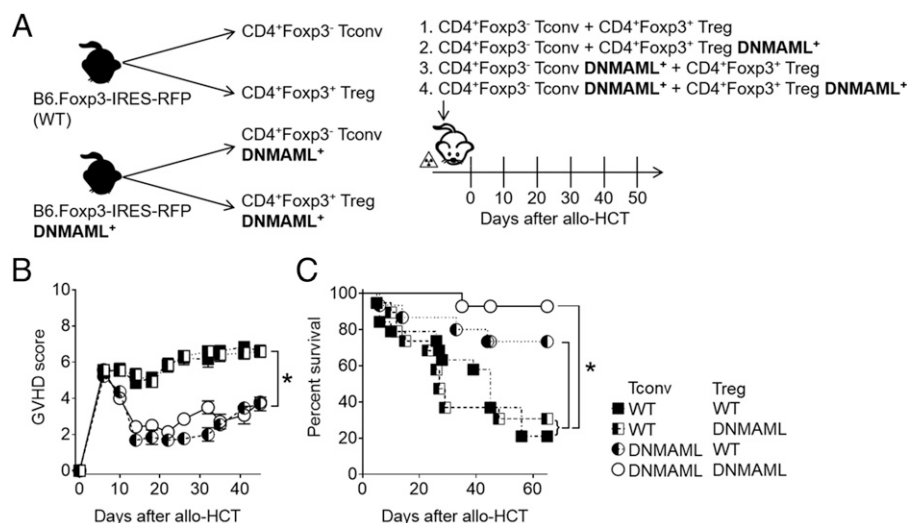
Using complementary genetic and biochemical approaches, we previously reported an essential role for Notch signaling in all mature CD4<sup>+</sup> T cells during acute GVHD in mouse allo-HCT models (2, 4, 6). A recent study suggested that Notch inhibition specifically in Tregs was sufficient to improve acute GVHD outcomes after MHC-mismatched transplantation in mice (9). Thus, the respective importance of Notch signaling in Foxp3<sup>+</sup> Tconv versus Foxp3<sup>+</sup> Tregs during acute GVHD remains unclear. To address this question, we relied on a B6 *Foxp3-IRES-mRFP* reporter strain (25) and crossed it with B6-DNMAML mice (expressing GFP and lacking all canonical Notch signals in mature T cells). This strategy allowed us to engineer T cell inocula for transplantation into MHC-mismatched irradiated BALB/c mice, in which Notch would be selectively inhibited in Tconv, Treg, or both (Fig. 1A). We pursued this approach as we previously observed that the

expansion of Tregs following Notch blockade was driven by pre-existing Tregs rather than Tconv conversion to induced Tregs (4). Each inoculum was infused with TCD BM into lethally irradiated BALB/c recipients, followed by serial monitoring for acute GVHD severity and survival. Recipients of wild-type (WT) Tconv developed severe and lethal acute GVHD, regardless of Notch blockade in the Treg compartment (Fig. 1B, 1C). Pan-Notch blockade via DNAML expression in Tconv was highly protective against acute GVHD as measured by clinical scores and survival, if Notch was active in coinfused Tregs (38). Thus, our data highlight an essential role for Notch signaling in CD4<sup>+</sup> Tconv to mediate acute GVHD.

### Establishment of an MHC-mismatched GVHD mouse model with a monoclonal population of donor alloantigen-specific CD4<sup>+</sup> T cells

We recently reported that key Notch signals are delivered to T cells during the first 48 h after allo-HCT (10). This narrow window provides an ideal opportunity to investigate cellular and molecular mechanisms of Notch signaling that shape pathogenic alloresponses in T cells. However, only a minor fraction of donor Tconv recognizes alloantigen and contributes to acute GVHD pathogenesis. Within this earliest posttransplant window, these rare cells remain difficult to track in standard models of acute GVHD. To bypass this pitfall and enable direct analyses of alloantigen-specific GVHD-causing cells, we established a new mouse model of MHC-mismatched GVHD with T cells from 4C $\times$ *Rag1*<sup>-/-</sup> TCR transgenic mice. 4C CD4<sup>+</sup> T cells are a clonal population that react against I-A<sup>d</sup> MHC class II through direct Ag recognition (26) and can be tracked easily by flow cytometry using TCR V $\beta$ 13 and/or CD90.1-directed Abs. Use of 4C T cells on *Rag1*<sup>-/-</sup> background allowed us to focus specifically on Tconv as 4C $\times$ *Rag1*<sup>-/-</sup> mice lack Tregs.

Although 4C T cells have been studied in mouse models of skin transplantation (26), their capacity to cause GVHD has not been examined previously. We first studied GVHD phenotypes arising from the transplantation of varying doses of 4C T cells, using polyclonal WT B6 T cells as a point of comparison. Lethally irradiated BALB/c mice transplanted with WT TCD BM



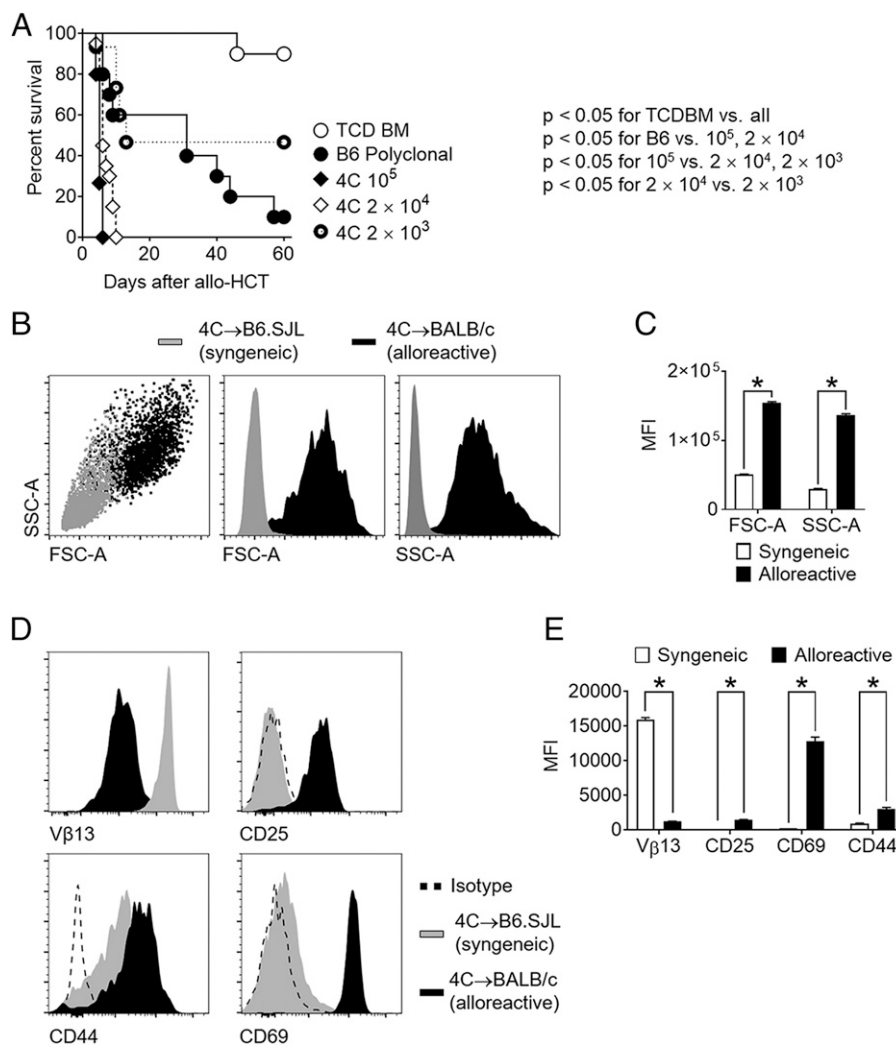
**FIGURE 1.** Tconv-intrinsic Notch signaling is essential for GVHD induction after MHC-mismatched allogeneic BM transplantation. (A) Experimental design to assess the importance of Tconv and Tregs in mediating the protective effects of Notch inhibition after allogeneic BM transplantation. Tconv and Tregs were sort purified from CD4<sup>+</sup>-enriched cells from B6 *Foxp3-IRES-mRFP* or B6 *Foxp3-IRES-mRFP*; *Tg<sup>cd4-cre</sup>; ROSA<sup>DNAML/+</sup>* (FIR-DNMAML) mice and mixed at a ratio of 8:1 Tconv:Tregs (by analogy to the physiological ratio found in WT donors; 500,000:62,500) to generate four different donor cell groups. (B) Clinical GVHD score and (C) overall survival of lethally irradiated (8 Gy) BALB/c mice transplanted with the four experimental inocula described in (A). Data are representative of two experiments and at least 15 mice per group. \* $p$  < 0.05, Student  $t$  test (B), log-rank test (C).



**FIGURE 2.** Alloantigen-driven 4C T cells induce lethal GVHD after MHC-mismatched allogeneic BM transplantation.

(A) Survival of lethally irradiated BALB/c mice transplanted with  $5 \times 10^6$  TCD BM only or TCD BM supplemented with  $5 \times 10^6$  polyclonal B6 splenocytes,  $10^5$  4C T cells,  $2 \times 10^4$  4C T cells, or  $2 \times 10^3$  4C T cells.

(B–E) Cellular hallmarks of the alloantigen-specific response in the 4C→BALB/c model of allo-HCT. Lethally irradiated recipients (B6.SJL, syngeneic; BALB/c-CD45.1, allogeneic) received TCD BM and  $5 \times 10^6$  4C T cells. Then, 1.5 d after allo-HCT, secondary lymphoid organs were processed, and lymphocytes were isolated for analysis. (B and C) Forward scatter (FSC-A) and side scatter (SSC-A) showed a dramatic increase in T cell size and granularity, consistent with acute activation. (D and E) The alloantigen-specific response led to robust 4C T cell activation. Histogram plots and cumulative data showed increased expression of the activation markers CD25, CD44, and CD69 and a decrease in Vβ13 TCR chain surface density. Data are representative of at least two experiments and at least 10 mice per group. \* $p < 0.05$ , log-rank test (A), Student  $t$  test (C and E).

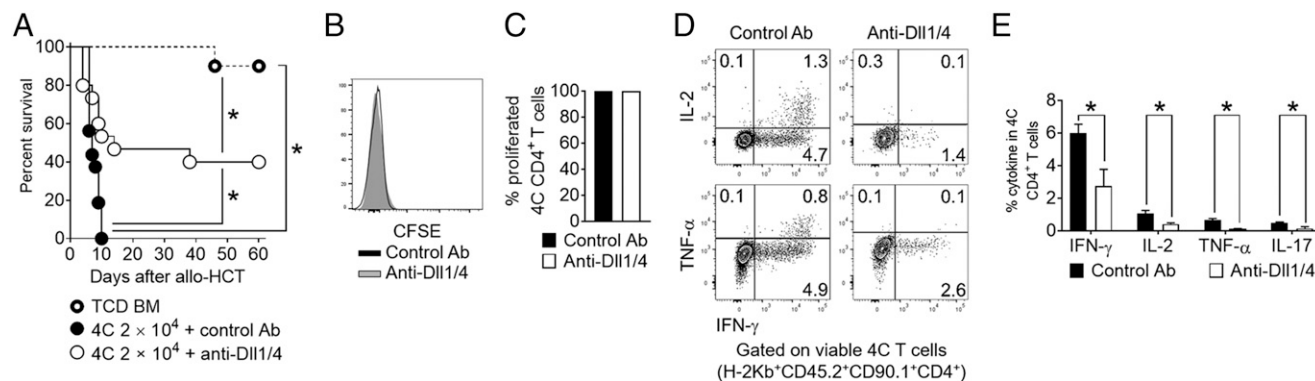


and  $5 \times 10^6$  B6 splenocytes developed severe GVHD resulting in the lethality of almost all recipients by day 60 after allo-HCT (Fig. 2A). Transplantation of as few as  $2 \times 10^4$  4C T cells induced universally lethal acute GVHD. Dosage reduction to  $2 \times 10^3$  4C T cells allowed partial recipient survival with evidence of acute GVHD. Thus, our new model was ideal to further investigate cellular changes in 4C T cells within the 48-h window during which critical Notch signals are delivered (10). To document the allo-specificity of T cell activation in this model, 4C T cells were transplanted into irradiated B6.SJL (syngeneic) or BALB/c (allogeneic) hosts (Supplemental Fig. 1A, 1B). Alloreactive 4C T cells showed hallmarks of Ag-driven T cell activation as compared with changes seen in 4C T cells undergoing homeostatic proliferation in syngeneic B6.SJL recipients. Alloreactive 4C T cells displayed greater size and granularity (Fig. 2B, 2C). Decreased density of cell surface Vβ13<sup>+</sup> TCR was consistent with downregulated surface TCR upon 4C T cell activation (Fig. 2D, 2E). The allospecific T cell response was associated with significant upregulation of the early T cell activation markers CD69 and CD25, as seen both in their cell surface density (Fig. 2D, 2E) and in the high proportion of 4C T cells expressing them (Supplemental Fig. 1B). In contrast, CD44 was induced to a larger extent in syngeneic recipients, indicating higher sensitivity of this marker to lymphopenic states independent of alloantigen encounter. Similar results were observed when 4C and BALB/c T cells were cotransferred to lethally irradiated BALB/c recipients and retrieved 36 h after allo-HCT (Supplemental Fig. 1C–F). Thus, 4C T cells are capable of causing

severe acute GVHD driven by alloantigen-specific T cell activation and effector differentiation.

#### *Intrinsic Delta-like1/4-mediated Notch signals in donor CD4<sup>+</sup> T cells drive GVHD mortality*

We previously reported in multiple mouse models of minor and major histocompatibility Ag-mismatched allo-HCT that Notch signaling is regulated by inputs from Dll1 and Dll4 ligands and established Dll1/4 targeting as a promising preclinical therapeutic strategy of GVHD prevention (6, 10, 11). In these studies, although the penetrance, severity, and kinetics of disease varied, Notch inhibition and Dll1/4 blockade resulted in profound protection from GVHD mortality/morbidity while largely preserving graft-versus-leukemia activity. To assess if the same Notch ligands are necessary for 4C T cell pathogenicity, lethally irradiated BALB/c recipients were treated with anti-Dll1/4 or isotype control Abs and transplanted with TCD BM plus  $2 \times 10^4$  4C T cells (a uniformly lethal dose in the presence of Notch signaling). Whereas 100% of isotype control recipients succumbed to GVHD within 10 d, anti-Dll1/4-treated recipients showed GVHD protection, leading to long-term survival in ~40% of the recipients (Fig. 3A). Thus, the degree of protection was similar to that afforded by a 10-fold reduction in T cell dose in this model (Fig. 2). Similar to our results with polyclonal T cells in models of acute GVHD (6, 10), anti-Dll1/4 therapy had no effect on initial 4C T cell proliferation, as shown at day 5 posttransplant (Fig. 3B, 3C). At this time point, Dll1/4 blockade inhibited the production of proinflammatory



**FIGURE 3.** Notch blockade mitigates acute GVHD driven by 4C CD4<sup>+</sup> Tconv. Lethally irradiated BALB/c mice were transplanted with  $5 \times 10^6$  TCD BM only or TCD BM supplemented with  $2 \times 10^3$  4C T cells. 4C T cell recipients were treated with isotype control (black-filled circles) or anti-Dll1 plus anti-Dll4 (white-filled circles) Abs (5 mg/kg i.p. on day 0). **(A)** The percentage of surviving transplanted animals are plotted as a function of time.  $*p < 0.05$ , log-rank test. **(B–E)** Lethally irradiated BALB/c mice transplanted with 4C T cells and treated with isotype control or anti-Dll1/4 Abs were sacrificed 5 d after allo-HCT, and spleens were retrieved for immunophenotypic analysis. **(B and C)** Notch blockade did not impair 4C Tconv proliferation as assessed through the dilution of CFSE. **(D and E)** Anti-Dll1/4 treatment led to impaired cytokine production in 4C Tconv. Representative flow cytometry plots **(D)** and cumulative data **(E)** showing the percentage of donor-derived 4C T cells expressing individual intracellular cytokines after anti-CD3/CD28 restimulation.  $*p < 0.05$ , Student *t* test.

cytokines important for GVHD pathogenesis in spleen, mesenteric lymph nodes, and peripheral blood (IFN- $\gamma$ , IL-2, IL-17, and TNF- $\alpha$ ; Fig. 3D, 3E, Supplemental Fig. 1G, 1H). Thus, Notch signals drive 4C T cell alloreactivity in vivo in a Dll1/4-dependent manner.

#### *Notch inhibition preserves early alloreactive T cell activation and TCR signaling strength but impairs S6 and ERK1/2 phosphorylation*

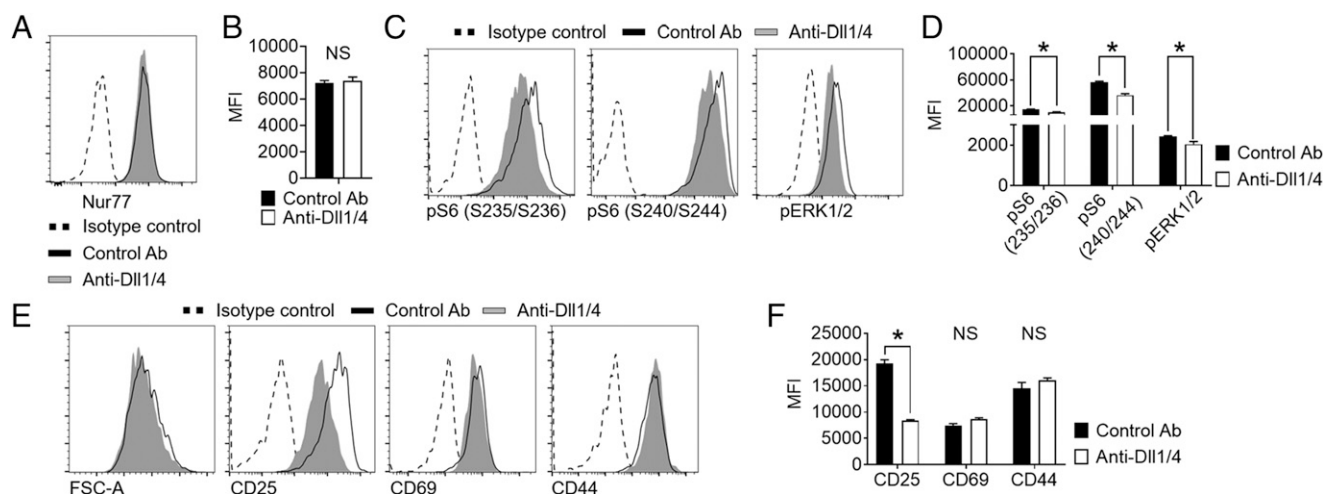
The earliest stages of CD4<sup>+</sup> T cell priming proceed in distinct phases. Following Ag encounter, TCR signaling drives the upregulation of early activation markers, nutrient sensors, and cytokine receptors followed by the proliferation and cytokine production (39–42). The potential importance of Notch signaling in regulating each of these steps has been reported in other contexts (2, 6, 19, 20, 43–46). However, dominant cellular and molecular mechanisms that operate upon in vivo Notch inhibition during alloresponses remain unclear.

T cell fate during Ag-specific responses depends upon TCR signal strength, with stronger TCR inputs leading to enhanced effector differentiation and reactivity (47). To investigate if Notch blockade negatively regulates TCR signal strength, leading to impaired effector fitness of alloreactive 4C T cells, we first evaluated intracellular expression of Nur77. Past work using a Nur77 (*Nr4a1*)-GFP allele showed that Nur77 expression is induced rapidly upon TCR activation. Unlike the early activation marker CD69, Nur77 is insensitive to inflammatory stimuli such as IL-2, TLR agonists, or type I IFNs that are abundant during early allo-HCT, thus directly reflecting TCR signal strength (27, 48). Notch blockade had no impact on TCR signaling strength in 4C cells as measured through Nur77 expression at 24 h, assessed by intracellular Nur77 staining (Fig. 4A, 4B). Similar results were obtained upon assessing GFP expression in 4C T cells transplanted from 4C×Nur77-GFP donors (data not shown). We then assessed whether Dll1/4 inhibition altered specific aspects of signal transduction during early T cell activation. Two major signaling pathways that are collectively activated by the TCR, costimulatory pathways, nutrient sensors, and cytokine receptors are the mTORC1 and the Ras/MAPK pathways. To quantify the impact of Notch blockade on these pathways, we performed phospho-flow cytometry for the mTORC1 target S6 and the MAPK targets ERK1/2 24 h posttransplantation. We coinjected both alloreactive

4C donors and syngeneic BALB/c donors into lethally irradiated BALB/c recipients and compared phosphorylation levels within the same sample, allowing us to control for non-cell-autonomous effects and technical variability. Both pathways showed significantly higher activity in alloreactive 4C T cells than in coinjected syngeneic BALB/c T cells (Supplemental Fig. 2A, 2B). Dll1/4 inhibition resulted in a significant decrease in both pS6 (S235/S236) and pS6 (S240/S244) levels, suggesting a cell-autonomous impairment in mTORC1 signaling as well as defective Ras/MAPK activity, as seen through decreased pERK1/2 levels (Fig. 4C, 4D). In contrast to the negative effect on pS6 and pERK1/2 levels, Notch blockade had a limited impact on the expression of activation markers in alloreactive 4C T cells. Aside from decreased levels of CD25 [a known direct target of Notch signaling (44, 49–51)], CD69 and CD44 as well as cell size, all hallmarks of T cell activation were unaffected by anti-Dll1/4 administration (Fig. 4E, 4F). Collectively, these data suggested that Notch inhibition impairs select key signal transduction events during T cell priming but not TCR signal strength nor overall T cell activation.

#### *Notch blockade preserves early IL-2 and TNF- $\alpha$ production*

Notch blockade did not impact the early proliferation of 4C T cells, despite profoundly inhibiting the production of multiple cytokines on day 5 posttransplant (Fig. 3B, 3E), similar to our observations in polyclonal systems (2, 6, 10, 11). To better understand this finding, we assessed the effects of Notch blockade on proliferation and cytokine production during T cell priming at earlier time points. In 4C T cells retrieved 42 h posttransplant from recipients treated with anti-Dll1/4, we observed a minimal decrease in the proportion of cells that underwent initial division (Fig. 5A, 5B). Simultaneously, Notch-blocked 4C T cells showed no impairment in the production of IL-2 or TNF- $\alpha$  (Fig. 5C, 5D). In contrast, IFN- $\gamma$  and IL-17A production was already markedly reduced at this early time point. These data suggest that during T cell priming, early Notch inputs are essential for inducing IFN- $\gamma$  and IL-17A production. In contrast, early Notch signals are dispensable for the induction of IL-2 and TNF- $\alpha$  production but necessary for sustaining IL-2 and TNF- $\alpha$  secretion at later time points. Thus, Notch-deprived alloreactive CD4<sup>+</sup> T cells initially maintained the production of IL-2, although it subsequently decreased by day 5, while immediately losing the



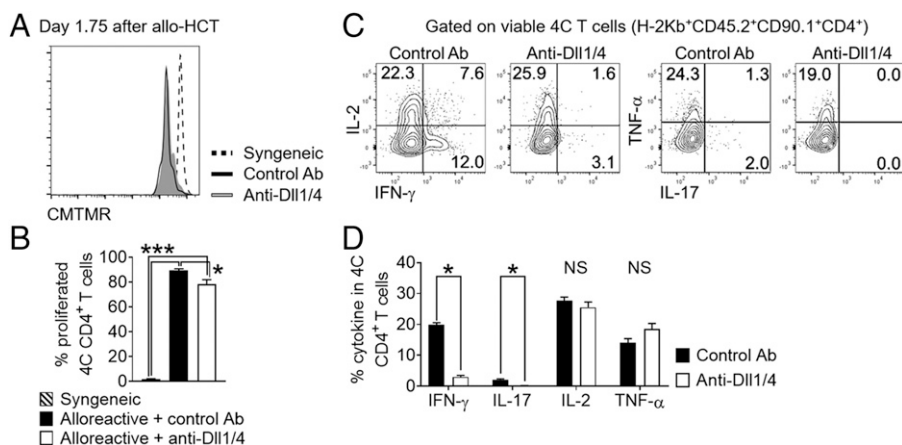
**FIGURE 4.** Impact of systemic Notch blockade on key cellular events in T cells during their in vivo priming by alloantigens. (A–F) Lethally irradiated BALB/c mice were transplanted with TCD BM supplemented with  $5 \times 10^6$  4C T cells and treated with isotype control or anti-Dll1/4 Abs on day 0. Animals were sacrificed 24 h after allo-HCT, and secondary lymphoid organs were retrieved for analysis. (A and B) Representative histogram plots (A) and cumulative data of mean fluorescence intensity (B) demonstrate that Notch blockade did not impact overall TCR signaling strength, as seen with the indistinguishable Nur77 levels in 4C T cells isolated from mice treated with isotype control or anti-Dll1/4 mAb. (C and D) Notch blockade impaired mTORC1 and Ras/MAPK signaling in alloreactive 4C T cells. Histogram plots (C) and cumulative data of mean fluorescence intensity (D) showing the abundance of phosphorylated S6 (S235/S236 and S240/S244 residues) and ERK1/2 in alloreactive 4C T cells. Data are representative of at least three experiments. \* $p < 0.05$ , Student  $t$  test. (E and F) Notch blockade had limited impact on the surface markers of alloreactive 4C T cell activation during in vivo Ag-mediated priming. Histogram plots (E) and cumulative data of mean fluorescence intensity (F) showing flow cytometric analysis of early T cell activation markers CD25 (a direct Notch target), CD69, and CD44. Data are representative of at least three experiments and 10 mice per group. \* $p < 0.05$ , Student  $t$  test.

ability to produce other inflammatory cytokines with a central role in GVHD.

#### *Notch blockade impacts a limited number of genes regulated by the alloresponse*

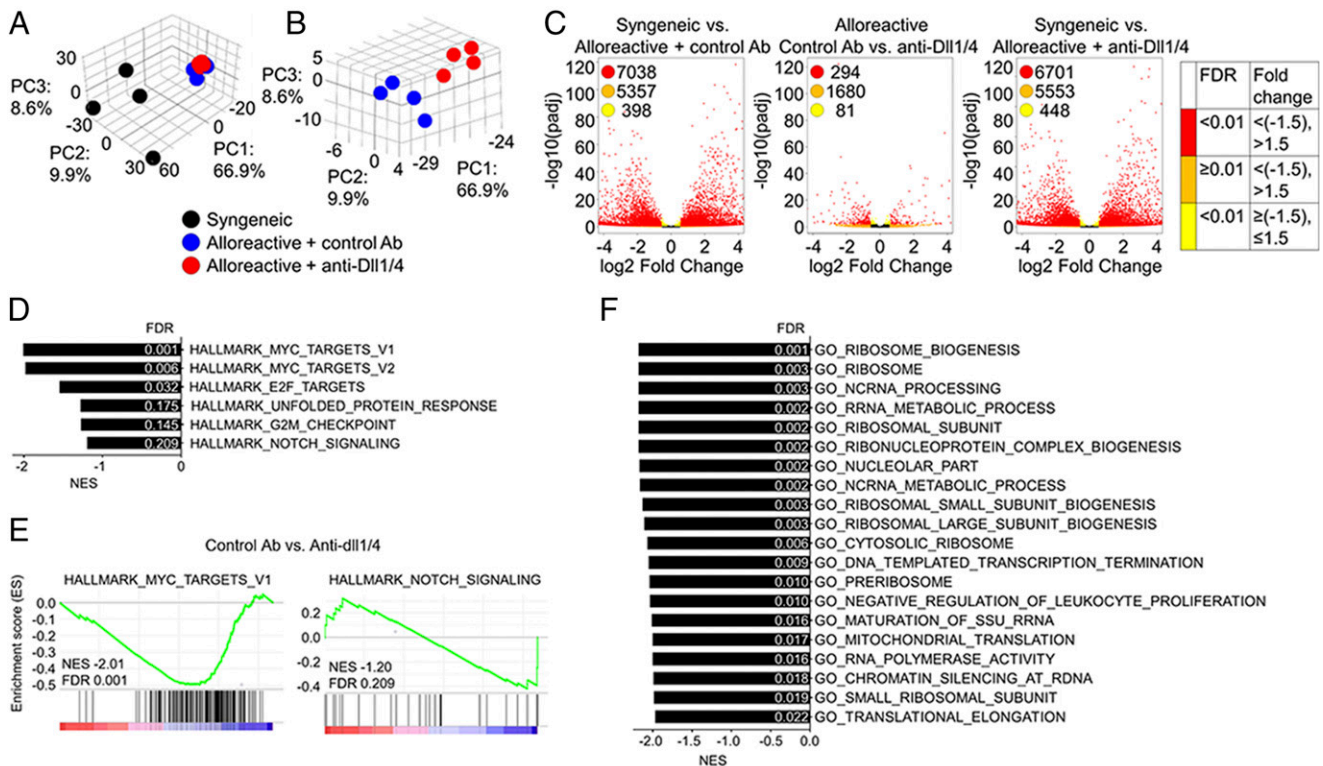
Given that all of the effects of Notch blockade are mimicked by the expression of DNMA1L, an inhibitor of the Notch transcriptional activation complex (2, 4), we hypothesized that transcriptional changes arising after early Notch blockade have mechanistic relevance in GVHD. To probe the effects of Notch on the 4C T cell transcriptome during T cell priming in an unbiased fashion, we performed RNA-Seq analysis. To account for noncognate gene

expression changes driven by the inflammatory milieu and the lymphopenic environment, we sort purified 4C T cells from the following experimental groups of irradiated recipient mice: 1) 4C→B6.SJL (syngeneic control group); 2) 4C→BALB/c treated with control Abs; and 3) 4C→BALB/c treated with anti-Dll1/4. 4C T cells were retrieved 36 h after allo-HCT, followed by RNA isolation and the generation of stranded amplified cDNA library before sequencing and analysis. Principal component analysis highlighted a distinct alloantigen-driven signature (Fig. 6A) with limited variance caused by Dll1/4 Notch ligand inhibition (Fig. 6B). Consistently, we identified a robust differential gene expression between syngeneic and both alloreactive 4C T cell



**FIGURE 5.** Systemic Notch blockade preserves selected early effector 4C T cell functions after allo-HCT. B6-SJL (syngeneic) or BALB/c (allogeneic) mice were irradiated and transplanted with TCD BM supplemented with  $5 \times 10^6$  4C T cells. BALB/c recipients were treated with isotype control or anti-Dll1/4 mAb on day 0. Mice were sacrificed 42 h after allo-HCT, and secondary lymphoid organs were retrieved for analysis. (A and B) CMTMR dilution by donor 4C cells showed robust initial alloreactive T cell proliferation minimally affected by Notch blockade. (A) Representative histogram plots and (B) cumulative data with four animals analyzed from one of three experiments and a total of 10 animals per group. (C and D) Intracellular cytokine staining in donor 4C T cells showed no impact of Notch blockade on IL-2 and TNF-α production but markedly decreased IFN-γ and IL-17. Representative plots (C) and cumulative data (D) from one of two experiments. \* $p < 0.05$ , \*\*\* $p < 0.001$ , Student  $t$  test (B), ANOVA (D).





**FIGURE 6.** Notch signaling has a unique but limited transcriptional footprint during the priming of alloreactive T cells. **(A)**  $5 \times 10^6$  CMTMR-labeled 4C T cells were transplanted into lethally irradiated BALB/c (allogeneic) or B6-SJL (syngeneic) recipient. Allogeneic recipients received anti-Dll1/4 or isotype control Ab on day 0. Forty-two hours after transplantation, 4C T cells were sort purified for RNA isolation, library generation, and RNA-Seq. **(A)** and **(B)** Principal component analysis of biological samples was used in this study. The percentage of variance captured by each of the three principal components demonstrated alloantigen regulation of T cell response (A) and more limited changes of the alloreactive T cell transcriptome as a result of Notch blockade (B). **(C)** Volcano plots displaying differentially expressed genes in 4C T cells within the three different comparison groups (syngeneic versus alloreactive plus control Ab; syngeneic versus alloreactive plus anti-Dll1/4 Ab; alloreactive control versus anti-Dll1/4 Ab). Red color-coded dots represent genes with significant differential expression at predefined 1.5-fold change. **(D–F)** GSEA analysis of RNA-Seq data showing individual gene sets and select enrichment plots from Hallmark (D and E) and gene sets from GO (F) collections significantly regulated by Notch blockade in 4C T cells.

groups. Based on predefined cutoffs (gene expression fold change [ $>1.5\times$ ,  $<-1.5\times$ ] and false discovery rate [ $<0.01$ ]), Notch blockade in alloreactive T cells differentially regulated the expression of 294 out of a total 20,034 identified genes, whereas ~7000 genes were seen as differentially regulated between both alloreactive data sets and syngeneic 4C T cells (Fig. 6C). To uncover the regulation of distinct pathways by Notch blockade (thus highlighting mechanistically relevant pathways in GVHD that are regulated by Notch), we performed GSEA for Hallmark and Gene Ontology (GO) gene sets from the Molecular Signatures Database. Whereas *myc* mRNA was only mildly reduced (~20%) by anti-Dll1/4 administration, Notch blockade consistently downregulated the expression of multiple Myc targets (Fig. 6D, 6E). Normalized enrichment scores for the HALLMARK\_NOTCH\_SIGNALING gene set did not reflect a major impact of Dll1/4 blockade on genes previously published as regulated by Notch in other contexts (Fig. 6D, 6E). In the GO data set, Notch blockade also broadly regulated ribosome biology-associated genes (Fig. 6F), suggesting a potential impact on ribosome homeostasis and translation.

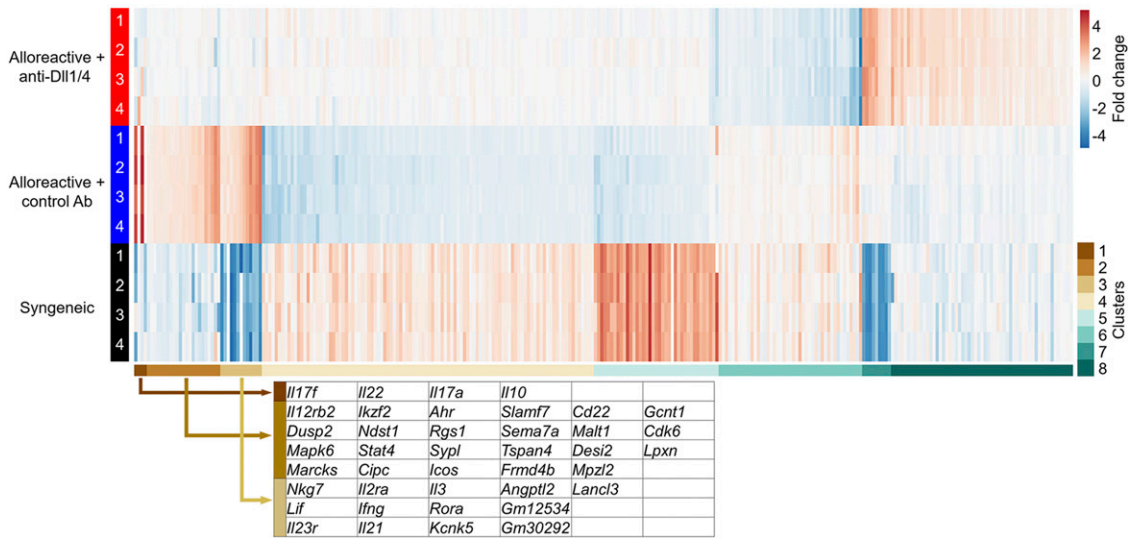
To provide further insight into genes regulated by Notch blockade and identify groups of genes with similar expression patterns, we performed k-means clustering (Fig. 7, Supplemental Table II). Interestingly, clustering analysis revealed a grouping of genes downregulated by Notch and with emerging roles in GVHD (such as *Il17* and *Il10*-family cytokines in cluster 1 or *Ahr* and *Icos* in cluster 2). Cluster 4 contained genes upregulated by Notch blockade, hence not direct targets, including *Cblb*, a negative

regulator of T cell activation that we reported previously as up-regulated in allogeneic T cells upon Notch blockade (4). Interestingly, this cluster also contained *Bach2*, another repressor of T cell function. *Bach2* is particularly interesting given that its effects on regulatory and effector T cell function mimic our observations with effective Notch blockade in GVHD (52–55). Overall, k-means analyses suggest that Notch blockade synergistically regulated multiple alloantigen-induced pathogenic pathways.

#### The transcriptional landscape regulated by Notch in mature T cells differs from T-ALL

Our prior work and results presented in this study identified a broad regulation of cytokine production in alloantigen-specific T cells, with no effects on IL-2 and TNF- $\alpha$  production within the T cell priming phase. To better characterize the impact of Notch signaling on early cytokine polarization in alloreactive 4C T cells, we extracted data on cytokine expression from our RNA-Seq dataset. Notch blockade downregulated the transcription of genes encoding cytokines with mechanistic relevance in GVHD (e.g., *Il17f*, *Il21*, and *Il22*). However, other cytokines with direct pathogenic importance in GVHD, including *Il2*, *Tnfa*, and *Il27*, remained unaffected by Dll1/4 blockade (Fig. 8A). Because the multifaceted role of Notch signaling in mature T cells was also ascribed to its ability to regulate multiple Th transcription factor loci (56), we analyzed the impact of Notch blockade on the expression of transcription factors with known functions in T cell effector differentiation. Notch signaling did not significantly affect prototypic



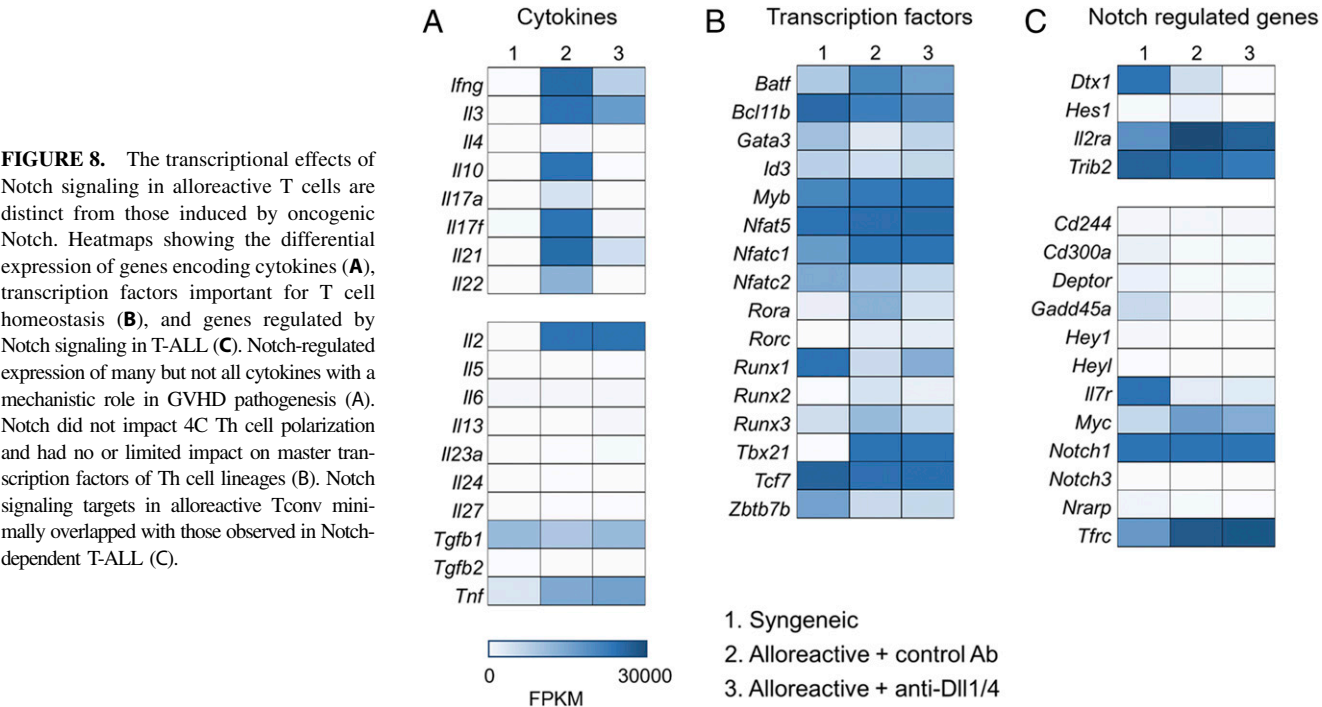


**FIGURE 7.** k-means clustering analysis identifies Notch regulation of multiple GVHD-associated genes in alloreactive 4C T cells. k-means clustering of 294 genes differentially regulated by Notch blockade in alloreactive 4C T cells during T cell priming period. Individual members of clusters 1–3 are highlighted and reveal several genes with a known mechanistic relevance in GVHD (*Il17f*, *Il21*, *Il2ra*, *Ahr*, *Icos*). Primary data from these clusters and all other clusters are presented in Supplemental Table II.

Th transcription factor expression (*Tbx21*, *Gata3*, *Rorc*; Fig. 8B), indicating that cytokine production defects were not a result of skewed Th differentiation but rather driven by alternative mechanisms. In addition, Notch signaling blockade showed limited to no impact on the expression of other transcription factors with a role in T cell homeostasis (*Batf*, *Bcl11b*, *Runx1*, *Runx3*; Fig. 8B), suggesting that Notch-induced changes in alloreactive T cells were independent of Th polarization.

Notably, our dataset in the GSEA analysis did not highly correlate with published data (57–61) on Notch-regulated pathways. Moreover, prototypic Notch targets uncovered during studies of oncogenic Notch signaling in T-ALL have thus far failed to provide an obvious mechanistic explanation for decreased GVHD upon Notch inhibition in mature T cells. To investigate the

regulation of individual putative Notch targets, we compared Notch targets identified in published T-ALL datasets with our RNA-Seq data. Dll1/4 inhibition downregulated the expression of only a few well-known Notch target genes, including *Dtx1*, *Hes1*, *Il2ra* (*Cd25*), and *Trib2* (Fig. 8C). In concordance with GSEA results, the Notch blockade signature had a limited overlap with previously published data on Notch targets in T-ALL (14). Other Notch target gene transcripts, such as *Tbx21*, *Gata3*, *Rorc*, *HeyL*, *Hey1*, and *Nrarp* were either not downregulated by Dll1/4 inhibition or present below the limit of detection of RNA-Seq analysis (Fig. 8B, 8C). Finally, Notch inhibition did not affect the abundance of *Pten* mRNA. Collectively, the genetic signature of active Notch in the T cell alloresponse was starkly different from that induced by oncogenic Notch. Instead, Notch signaling in alloreactive



**FIGURE 8.** The transcriptional effects of Notch signaling in alloreactive T cells are distinct from those induced by oncogenic Notch. Heatmaps showing the differential expression of genes encoding cytokines (A), transcription factors important for T cell homeostasis (B), and genes regulated by Notch signaling in T-ALL (C). Notch-regulated expression of many but not all cytokines with a mechanistic role in GVHD pathogenesis (A). Notch did not impact 4C Th cell polarization and had no or limited impact on master transcription factors of Th cell lineages (B). Notch signaling targets in alloreactive Tconv minimally overlapped with those observed in Notch-dependent T-ALL (C).

Tconv induced distinct molecular pathways with converging roles in pathogenic alloreactivity.

## Discussion

We previously demonstrated the central role of Notch signaling in mature T cells to drive pathogenic alloresponses in multiple major and minor histocompatibility Ag-mismatched mouse models of acute and chronic GVHD (2, 4, 6, 10). However, prior to the current study, the key cellular compartment (effector T cell versus Treg) through which the beneficial effects of Notch inhibition was mediated was unclear. In this study, we engineered mixed T cell grafts to uncover the essential function of active Notch in conventional rather than Tregs to mediate GVHD pathogenicity. Furthermore, we identified unique cellular effects and a molecular landscape associated with Notch inhibition in alloantigen-specific Tconv at very early stages after transplantation.

We and others have reported an increased expansion of donor-derived Tregs after allo-HCT and during GVHD protection upon genetic or biochemical Notch inhibition (2, 4, 6, 10, 11). To specifically investigate the role of Notch signaling in Tregs in this context, Charbonnier et al. (9) used a *Foxp3-Cre* transgene to inactivate essential components of the Notch pathway (including *Pofut1*, *Rbpj*, and *Notch1*) in Tregs only. Using this approach, they reported protection from GVHD after allogeneic transplantation of total splenocytes from mice with Notch-deficient Tregs, suggesting that Notch inhibition in Tregs was sufficient to protect from GVHD. However, this interpretation did not take into account the fact that chronic Notch inhibition in Tregs appeared to impact the composition and function of the Tconv compartment (Tconv hyporesponsiveness, decreased cytokine production, and increased prevalence of memory T cells) and that engineering of the graft would be necessary to formally restrict Notch inhibition to the Treg compartment. In our study, graft engineering offered an experimental advantage and enabled us to document directly the central pathogenic role of Notch in Tconv but not Tregs during GVHD.

In view of these findings and to better understand Notch regulation in Tconv, we developed a novel Tconv-driven MHC-mismatched model of acute GVHD, mediated by I-A<sup>d</sup>-reactive TCR transgenic CD4<sup>+</sup> 4C *Rag1*<sup>-/-</sup> donor T cells, which lack Tregs because of the absence of I-A<sup>d</sup> Ag during T cell development in the thymus and because of the absence of endogenous *Rag1* expression. 4C T cells were potent inducers of GVHD mortality yet remained sensitive to Notch inhibition by neutralizing Abs against Dll1/4 Notch ligands.

Thus, this model was ideal to focus our analyses on pathogenic T cells, avoid pitfalls of bulk T cell analyses at early time points, and define the cellular and molecular events underlying Notch-driven alloresponses in the absence of any potential changes in the TCR repertoire. Focusing on the previously identified early window of critical Notch activity in GVHD (10), we described relatively limited effects of Notch signaling on 4C T cells during their priming and early activation. Our analysis provided several lines of evidence that Notch inhibition preserves key 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 transcription factors AP-1 and NFAT, was unimpaired by systemic Dll1/4 inhibition at early time points while decreasing later. How the lack of sustained IL-2 production influences 4C T cell (or alloreactive CD4<sup>+</sup> T cell, in general) proliferation remains unclear. It is possible that in the context of strong TCR stimulation such as is seen in vivo during alloresponses, IL-2 production is dispensable for continued

proliferation, particularly in the CD4<sup>+</sup> Tconv compartment where TCR-dependent and mitogenic cytokine-independent regulation dominate (62, 63). Even if not important for Tconv proliferative responses early posttransplant, the initial burst of IL-2 in polyclonal GVHD models (10) may help explain the robust expansion of Tregs that we and others have reported upon Notch inhibition because Treg expansion exquisitely depends on IL-2-mediated signals (64). Moreover, Dll1/4 inhibition did not impact transcriptional regulation of Th skewing. Although our findings conflict with a recent report that Notch enhances CD69 expression, cell size, IL-2 production, and proliferation both in vitro and in vivo via a costimulatory-like effect downstream of B7-CD28 signaling (19), it is possible that Notch functions as a costimulatory modulator but with different effects depending on context, Ag strength and availability, and other concomitant signals.

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 (65, 66). Similarly, Ras/MAPK signaling sits downstream of TCR signaling, cytokine/growth factor receptors, and the B7/CD28 family of costimulatory molecules. Given that TCR signaling, T cell costimulation, and cytokine/growth factor signaling simultaneously occur during T cell priming, it is unclear whether Notch inhibition regulates some or all of the aforementioned pathways to modulate mTORC1 and Ras/MAPK. Precise spatiotemporal analysis of phosphorylation signals could be helpful in dissecting contributions from each upstream signal. Although Nur77-GFP levels were not altered with Notch inhibition, Nur77-GFP has been reported to mainly read out TCR-mediated protein kinase C activity proportionately to TCR signal strength (48). Thus, although protein kinase C activity is most likely intact in Notch-deprived alloreactive T cells, it is possible that Notch modulates specific arms of TCR signaling, such as 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 (19) or diminished cytokine/growth factor signaling. Intact early production of IL-2, which receives direct inputs from Ca<sup>++</sup>-dependent NFAT, costimulation-dependent NF-κB, and ERK1/2-dependent AP-1 (67), would argue that both TCR-dependent and B7-mediated ERK1/2 activity are initially intact.

Although global Ras/MAPK and mTORC1 activity were both impaired (but not ablated) in alloreactive T cells during priming, *Il2* transcription and IL-2 protein were initially preserved. These data suggest that the highly inflammatory milieu of allotransplantation conditioning, in conjunction with strong TCR signaling, stimulate alternative upstream signals that are sufficient to drive IL-2 expression, at least at early time points. Given that IL-2 production by Notch-deprived alloreactive T cells was impaired at later time points after allotransplantation (Fig. 3D, 3E), we speculate that dynamic involvement of upstream regulators of *Il2* transcription, with Notch-regulated MAPK/mTORC1 signaling, is dispensable early but essential later for maximal IL-2 production. Our findings should be considered in light of research on the epigenetic regulation of *Il2* transcription that revealed a highly dynamic involvement of proximal regulatory elements and distal enhancers through complex sequential three-dimensional rearrangements of the chromatin architecture (68).

To capture the overall impact of Notch on the molecular landscape of T cell alloreactivity, we relied on an unbiased transcriptional approach in an alloantigen-specific T cell model. Using a monoclonal population of donor cells allowed us to isolate Ag-specific T cells in a precise manner while preserving the profound

impact of Notch inhibition on GVHD mortality/morbidity. Despite differences in disease kinetics and severity as compared with the polyclonal BALB/c→B6 major mismatch and B10.D2→BALB/c minor mismatch models, we observed concordant phenotypic and functional changes as well as regulation of gene expression in both of these models. Thus, our molecular observations have broad relevance to immune pathogenesis in multiple complementary mouse GVHD models. Despite the known limitations of mouse allo-HCT models, and although more research is needed, we speculate that conserved features of Notch as an ancient signaling pathway will apply to human allogeneic transplantation as well.

Our study represents the first RNA-Seq-based transcriptional analysis of Notch effects in pathogenic T cells during GVHD. Despite its profound functional impact on alloantigen-driven responses, Notch had a surprisingly narrow effect on the transcriptional landscape, differentially regulating only 294 of ~7000 genes. Prototypic Notch targets identified in other contexts showed minimal overlap with differentially expressed genes that we identified in alloreactive T cells. In particular, alloreactive 4C cells expressed low levels of *Hes1*, whereas its putative repressed transcriptional target *Pten* was unaffected by Notch inhibition. Thus, the Notch-Hes1-PTEN transcriptional axis might operate in T-ALL and developing thymocytes, as reported (57, 69), but is unlikely to be essential in alloreactive T cells. Several Notch target genes, including *Dtx1*, *Il2ra*, and *Trib2*, were downregulated upon Dll1/4 blockade in alloreactive T cells, although, aside from *Il2ra*, an apparent connection to GVHD pathophysiology is lacking. Consistent with our previous observations in polyclonal models of GVHD, Notch did not regulate the expression of key transcription factors that control Th cell CD4<sup>+</sup> lineage fate decisions in mature T cells, including *Tbx21*, *Rorc*, and *Gata3* (2, 4). In contrast, the expression of multiple cytokines (*Ifng*, *Il17f*, *Il21*, *Il22*) and cytokine receptors (*Il1r1*, *Il23r*) with a reported role in GVHD (70–74) was downregulated early on by Dll1/4 inhibition. Notch blockade also downregulated the expression of *Ahr* and *Icos* (75, 76), identified as therapeutic targets in GVHD. Additional gene expression changes could be related mechanistically to the observed GVHD abrogation. A particularly interesting candidate with a broad impact on adaptive immune responses and upregulated by Notch blockade in our dataset is *Bach2*. Beyond transcriptional regulation, our data suggest early defects in PI3K/Akt/mTOR signaling, normally responsible for BACH2 downregulation (55). BACH2 broadly regulates adaptive immune responses, stabilizing Tregs, promoting memory over effector T cell responses, and promoting tumor immunosuppression (52–55). Although transcriptional disruption is essential to confer the benefits of Notch blockade, the contribution of epigenetic mechanisms to protective benefits against GVHD cannot be ruled out and is also suggested by our identification of multiple epigenetic regulators active in adaptive immunity as impacted by Notch blockade (*Dnmt3a*, *Mbd2*, *Jarid*).

In summary, we have identified a CD4<sup>+</sup> Tconv-intrinsic role for Notch signaling in alloreactivity and GVHD. Notch-deprived alloreactive CD4<sup>+</sup> Tconv did not display overt early defects in overall Ag sensitivity as they exhibited preserved activation marker upregulation, IL-2 production, and initial proliferation. In contrast, Notch-deprived CD4<sup>+</sup> T cells rapidly acquired a defect in IFN-γ and IL-17 production despite preserved *Tbx21* and *Rorc* transcription while exhibiting diminished mTORC1 and Ras/MAPK activity. Finally, our RNA-Seq data defined the Notch-regulated transcriptional landscape in mature alloreactive T cells as very distinct from that observed in T-ALL or developing T cells.

Our study emphasizes the increasingly recognized versatility of Notch signaling in the immune system that extends beyond its developmental roles. Although work presented in this study

focused on CD4<sup>+</sup> T cells, we previously documented many parallels between Notch effects in the CD4<sup>+</sup> and CD8<sup>+</sup> T cell compartments in alloreactivity (4). Collectively, our data suggest that previously reported molecular mechanisms cannot account for the role of Notch in alloreactive T cells and propose a new set of unique mechanisms through which Notch modulates T cell alloreactivity. We provide molecular cues for this functional divergence and, importantly, identify not only known targets with importance in GVHD as regulated by Notch but also others that have a potential impact on GVHD regulation but have not yet been probed in this context. Because Notch blockade affects only selected aspects of alloreactive T cell function without inducing global immunosuppression, we postulate that these targets may identify keys to successful and specific control of GVHD as well as pathologic alloresponses beyond the field of allo-HCT.

## Disclosures

C.W.S. is an employee of Genentech. The other authors have no financial conflicts of interest.

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