The role of Notch signaling in T cell-mediated immune disorders

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Immunology)
in the University of Michigan
2013

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Dedication

To my loving and supportive family, fiancé, and friends. It has been a rollercoaster of a couple of years that could not have been accomplished without all of you by my side. When my dad asks for the *hundredth* time, “when are you going to be done?” I can finally answer, “now!” ;)

Table of Contents

List of Figures

List of Tables

Summary

Notch is a highly conserved cell-to-cell communication pathway that plays an essential role in many biological processes. Notch exerts multiple effects in the hematopoietic system, especially during T cell development. Recent data suggest that Notch also regulates mature T cell differentiation and function. Here, we studied Notch signaling specifically in mature T cells using genetic loss-of-function approaches in two medically relevant T cell-mediated immune disorders: graft-versus-host disease (GVHD) and experimental autoimmune encephalomyelitis (EAE). We found that inhibition of Notch signaling provided long-term protection from lethal GVHD and EAE in mice.

In GVHD, Notch-deprived alloreactive T cells had markedly decreased production of multiple pro-inflammatory cytokines, including IL-17A, and IFNγ, despite preserved expression of the master transcription factors T-bet and Eomesodermin. Notch-inhibited T cells acquired a hyporesponsive phenotype, but maintained *in vivo* expansion and cytotoxic potential. Notch1/2 and Dll1/4 mediated all the effects of Notch signaling in T cells during GVHD. Therapeutic targeting of the Notch ligands Dll1 and Dll4 prevented GVHD even with a short-course of treatment.

In EAE, Notch-deprived T cells had preserved effector differentiation in secondary lymphoid organs but failed to accumulate in the central nervous system (CNS). Parking wild-type T cells with Notch-inhibited T cells allowed Notch-deprived T cells to accumulate in the CNS, although they failed to suppress disease induced by wild-type T cells. Once in the CNS, Notch-deprived T cells produced markedly decreased IL-17A and IFNγ, despite preserved T-bet expression.

Collectively, these data suggest that Notch signaling augments T cell responses in a context-dependent fashion. Inhibition of cytokine production with preserved expression of master transcription factors is reminiscent of costimulation blockade in T cells in mouse models of GVHD and EAE. Notch may regulate T cell responses in a context-dependent manner by acting similarly to a costimulatory receptor. Thus, blockade of Notch signaling could be an attractive therapeutic target in T cell-mediated immune disorders.

Chapter 1

Introduction

1.1 Overview of Notch signaling

Notch signaling was first identified in *Drosophila melanogaster* on the basis of a heritable serrated or "notched" wing phenotype.[1] Subsequently this pathway was named Notch, based on the described wing phenotype. The *Notch* gene was cloned in 1983.[2] Later genetic studies in fruit flies identified multiple other functions for Notch signaling as a result of Notch loss-of-function phenotypes, including neurogenesis, oogenesis, and developmental patterning defects.[3-5] These studies resulted in the identification of two Notch interacting ligands in the fly, Delta and Serrate.[6,7] Duplication events gave rise to four mammalian Notch receptor genes (*Notch1-4*) and five genes encoding mammalian Notch ligands (*Dll1, 3, 4 and Jag1, 2*).[8-11]

In mammals, Notch signaling exerts recurring functions in the development of multiple organ systems, adult tissue homeostasis, and cancer. Physiologically, Notch is required for the development of multiple organs, including kidney, heart, and vasculature, as well as homeostasis of the skin, gut, and other tissues in postnatal life.[12-17] The first involvement for Notch signaling in cancer was the discovery that a t(7, 9) translocation resulted in generation of a constitutively active form of NOTCH1 in rare cases of T cell acute lymphoblastic leukemia (T-ALL).[18,19] The importance of Notch gain-of-function mutations in T-ALL was found to be more extensive with the discovery of point mutations in the heterodimerization and PEST domains that destabilized the Notch receptor and increased the half-life of active Notch.[20] Since then, Notch pathway mutations have been identified in a wide variety of cancers. For instance, gain-of-function mutations were described in mantle cell lymphoma and B cell chronic lymphocytic leukemia, and loss-of-function mutations in cutaneous and lung squamous cell carcinoma and chronic myelomonocytic leukemia.[21-24] Collectively, Notch signaling dynamically regulates multiple cell types in a variety of tissues.

1.2 Notch signaling pathway activation

Notch receptors are present on the cell surface as heterodimeric transmembrane proteins after proteolytic cleavage by a furin-like convertase at the "S1" site during transport through the Golgi apparatus (**Figure 1.1A**).[25,26] Once present on the cell surface, Notch receptors (Notch 1-4) interact with Notch ligands of the Delta-like (Dll1, 3, 4) or Jagged (Jag1-2) families to initiate Notch signaling **(Figure 1.1A).**

The expression of Notch receptors and ligands is highly regulated in the hematopoietic system, with interactions between specific Notch ligands and receptors being favored. For example, Notch1 in developing thymocytes interacts with Dll4 expressed by the thymic epithelium, while Notch2 directs marginal zone B cell development through its interaction with Dll1.[27-30] All the mechanisms accounting for preferential Notch ligand-receptor interactions are not fully understood. However, specific Notch receptors acquire enhanced avidity for specific Notch ligands, at least partially, through post-translational modifications mediated by Fringe family glycosyltransferases.[31,32] Modification of Notch receptors by Fringe proteins results in differential ligand binding affinity and receptor activation.[32] This modification of ligand/receptor specificity by Fringe proteins has been elegantly described in the case of T cell development as well as in the regulation of endothelial proliferation, during which Dll4 preferentially interacts with Fringe-modified Notch1.[33-35]

Upon receptor-ligand binding, the negative regulatory region of Notch receptors is displaced allowing matrix metalloproteases of the ADAM family to gain access to the "S2" cleavage site (**Figure 1.1A).**[36] In developing thymocytes and marginal zone B cells, two well-described Notch-dependent populations, the "S2" cleavage is mediated by ADAM10.[37] The "S2" cleavage renders the remaining Notch receptor unstable, leading to its intramembrane proteolysis by the γ-secretase complex, a rate-limiting step in Notch signaling activation.[38] Although the γ-secretase complex can be pharmacologically inhibited to study Notch signaling, the γ-secretase complex is not specific to Notch signaling.[39] Cleavage at the "S3" site by the γ-secretase complex results in the release of the intracellular domain of Notch (ICN) (**Figure 1.1A).**[38] ICN acts as the effector of Notch signaling and translocates into the nucleus to interact with a core transcriptional activation complex.

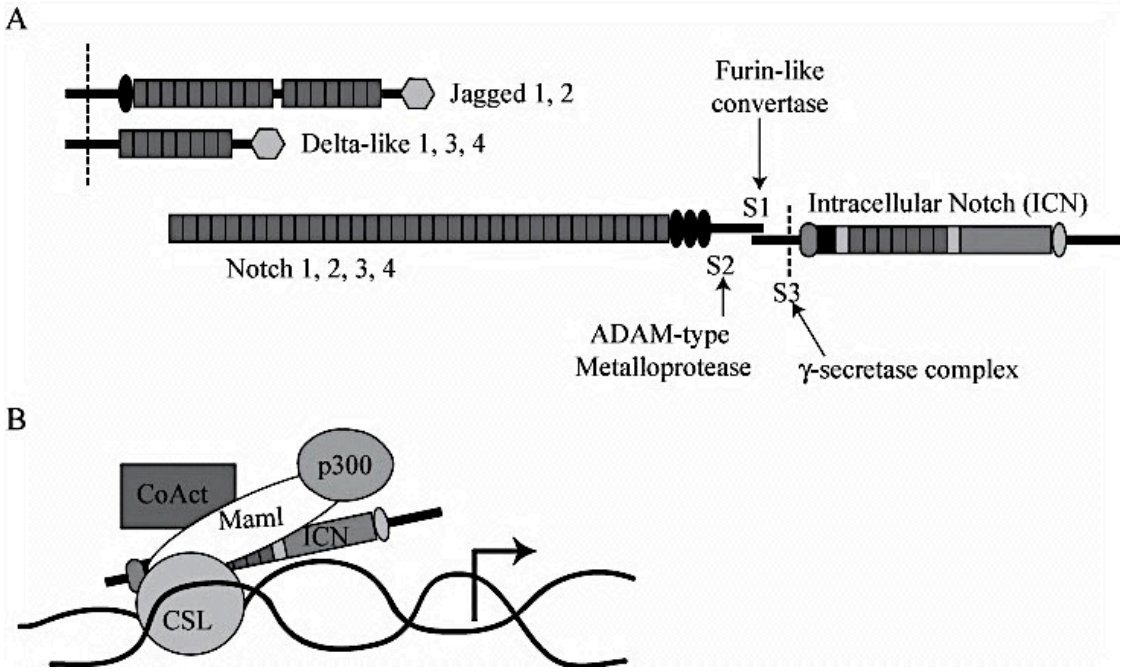

Figure 1.1 Canonical Notch Signaling[1].
(A) Notch signaling occurs upon interaction between one of five ligands from either the Jagged (1, 2) or Delta-like (1, 3, 4) families and one of four Notch receptors (Notch1-4). Notch receptors are cleaved in the Golgi complex by a furin-like convertase (S1), leading to expression of a heterodimeric Notch receptor. Upon ligand binding, the S2 cleavage site is made accessible to an ADAM-type metalloprotease. The last cleavage (S3) is mediated by the γ-secretase complex and releases the Notch intracellular domain (ICN); **(B)** ICN translocates into the nucleus to interact with the DNA-binding protein, CSL/RBP-Jk (encoded by the *Rbpj* gene), a member of the Mastermind-like (Maml) family and other transcriptional coactivators.

The transcriptional activation complex consists of ICN, the DNA-binding factor CSL/RBP-Jκ, a coactivator of the Mastermind-like family (Maml1-3), and other transcriptional activators, like p300 (**Figure 1.1B**).[40-43] Assembly of the core transcriptional activation complex leads to target gene activation. Collectively, this process is referred to as "canonical Notch signaling". Most incidences of Notch signaling in mammals have been attributed to "canonical" Notch signaling, although "non-canonical" Notch signaling has been described in *Drosophila*.[44-48] Others have suggested

[1] Figure taken from:

Sandy AR, Jones M, and Maillard I. 2010. Notch signaling and development of the hematopoietic system. Reichrath J and Reichrath S (Eds.). *Notch signaling in embryology and cancer.* Austin: Landes Bioscience and Springer Science+Business Media.

that "non-canonical" Notch signaling occurs in mammals, but the mechanisms defining this activity of the Notch pathway in mammals are poorly characterized.[49,50]

In the absence of Notch signaling, CSL/RBP-Jκ is part of a repressor complex.[51-54] The functional importance of the repressor complex remains unclear, although some of its components have been elucidated. CSL/RBP-Jκ interacts with SMRT/mSin3A/HDAC-1 (SMRT, Silencing Mediator for Retinoic acid and Thyroid hormone receptor; HDAC-1, histone deacetylase-1), NCor/mSin3A/HDAC-1, CIR/SAP30/HDAC-2, and SHARP/CtBP/CtIP (SHARP, SMRT and HDAC associated repressor protein; CtBP, C-terminal binding protein; CtIP, CtBP Interacting Protein).[51-54]

Given the potent capacity of the Notch pathway to drive transcriptional output and major phenotypic consequences, the generation and half-life of ICN needs to be tightly controlled. The C-terminal PEST domain of ICN is targeted by E3 ubiquitin ligases, resulting in proteasome-mediated ICN degradation. For example, Sel10/Fbw7, Numb, and Itch have been shown to regulate degradation of ICN through ubiquitin modification of the PEST domain.[55-58] The importance of the PEST domain and the E3 ubiquitin ligase, Fbw7, in turning off Notch signaling is exemplified by the recurrent mutations that occur in the PEST domain and Fbw7 in T-ALL.[20,59]

Altogether, Notch signaling is a highly controlled signal transduction pathway with complex regulation at the level of receptor ligand/expression, ligand/receptor affinity, receptor proteolysis, and transcriptional complex formation.

1.3 Notch signaling in the hematopoietic system

In the hematopoietic system, Notch signaling is used recurrently in multiple contexts to drive different biological outcomes.[60,61] For instance, Notch signaling is essential for the emergence of hematopoietic stem cells during definitive but not primitive hematopoiesis.[62-64] At subsequent stages of hematopoiesis, Notch signaling is required to drive development of different lineages of the immune system **(Table 1.1)**. For instance, Notch signaling drives T cell generation at the expense of B cell development from multipotent bone marrow progenitors that migrate into the thymus.[29,65,66] Notch2 and Dll1 are required for the generation of marginal zone B cells in

a dose-dependent manner, as heterozygous loss of *Notch2* or *Dll1* results in a 50% reduction in marginal zone B cells.[28,30] Moreover, Notch signaling is important for the generation of specific dendritic cell subsets. Notch-CSL/RBP-Jκ signaling is required for the development of $CD8^-$ dendritic cells in the spleen, and Notch2 is required for splenic $CD11b^+Esam^{High}$ and intestinal lamina propria $CD11b^+CD103^+$ dendritic cells.[67,68] Recent evidence also indicated that Notch signaling regulates the generation of megakaryocytes.[69] Additionally, Notch was shown to restrict myeloid lineage differentiation, as inactivation of *Nicastrin*, encoding a component of the γ-secretase complex resulted in accumulation of granulocyte/monocyte precursors and a myeloproliferative disorder.[24]

More recently, emerging data suggested that Notch signaling also promotes the differentiation of subsets of innate lymphoid cells (ILCs). The recently described ILC classification mirrors that of T helper subsets.[70-72] For example, ILC1, natural killer cells being the prototypical members, produce abundant IFN-γ. ILC2 express IL-5, IL-13, IL-9, and some IL-4, while ILC3 produce IL-17A and/or IL-22. Similar to T helper subset responses, ILC1 are important for clearance of intracellular pathogens, ILC2 are required for allergic/asthma responses, and ILC3 mediate anti-microbial immunity. All three ILC lineages have been suggested to be Notch-dependent in some capacity **(Table 1.2)**. For instance, transient Notch signaling was suggested to be important for murine and human (umbilical cord blood) natural killer cell/ILC1 development, at least *in vitro*.[73,74] In conflicting reports, Notch signaling was suggested to be dispensable for natural killer cell development using *in vitro* assays.[75,76] Also, Notch signaling was suggested to be important for the generation of $Ror\alpha^+$ ILC2 through *in vitro* gain-of-function assays, and aryl hydrocarbon receptor-dependent $Ror\gamma t^+$ ILC3 through *in vitro* gain-of-function assays as well as *in vivo* loss-of-function studies.[77-79]

Table 1.1 Notch regulation of adaptive immune system components.

Cellular subset	Notch manipulation	Notch effect	Reference
Developing thymocytes	Gain-of-function: OP9-Dll4, ICN overexpression Loss-of-function: *Mx1-cre x Notch1*$^{-/-}$, DNMAML expression, *Lck-cre x DNMAML1*$^{f/f}$, *Lck-cre x Notch1*$^{f/f}$ *Foxn1-cre x Dll4*$^{-/-}$ *Lck-cre x Rbpj*$^{-/-}$	Notch1/Dll4 drive T cell development from ETP-DN3a	19,27,29,33,34,75,80-85
Marginal zone B cells	*Mx1-cre x Notch2*$^{f/f}$ *Cd19-cre x Notch2*$^{f/f}$ *Mx1-cre x Dll1*$^{f/f}$ *Maml1*$^{-/-}$ DNMAML1 expression *Cd19-cre x Rbpj*$^{f/f}$	Notch2/Dll1/ Maml1 drive marginal zone B cell development	28,30,82,86
Dendritic cells	*Mx1-cre x Rbpj*$^{-/-}$ *Cd11c-cre x Rbpj*$^{-/-}$	CSL/RBP-Jk drives development of CD8- splenic DCs	67
	Itgax-cre x DNMAML1$^{f/f}$ *Itgax-cre x Notch2*$^{f/f}$ *Itgax-cre x Rbpj*$^{f/f}$ *Itgax-cre x NICD*	Notch2/Maml1/ RBP-Jk drives $CD11b^{+}Esam^{High}$ and $CD11b^{+}CD103^{+}$ DC development	68
Myeloid cells	*Vav-cre x Nicastrin*$^{f/f}$ *Mx1-cre x Nicastrin*$^{f/f}$ *Mx1-cre x* Notch1IC *Mx1-cre x Notch1*$^{f/f}$*Notch2*$^{f/f}$*Notch3*$^{-/-}$	Notch signaling restricts GMP expansion	24

Table 1.2 Notch regulation of innate lymphoid cell subsets.

ILC subset	Notch manipulation	Notch effect?	References
NK cells/ILC1	OP9-Dll1	*in vitro* NK1.1$^+$ ILC1 generation	74
	ICN overexpression	*in vitro* human CD7$^+$CD56$^+$CD3$^-$ NK cells	73
	human Dll4-Fc, γ-secretase inhibitor, human anti-Notch1 antibody	*in vitro* development of human CD7$^+$CD56$^+$ cytotoxic NK cells	87
	OP9-Jag2, OP9-Dll1, OP9-Dll4	*In vitro* generation of human CD56$^+$CD3$^-$ NK cells	88
	OP9-Dll1, *Presenilin 1/2* inhibitor	Notch inhibited *in vitro* NK cell development from DN1 or DN2 thymocytes	75
	γ-secretase inhibitor, rat recombinant Notch1-Fc	Promotion of rat NK cell development in Notch absence	76
ILC2	OP9-Dll1	*in vitro* nuocyte expansion	79
ILC3	γ-secretase inhibitor	*in vitro* adult Rorγt$^+$ ILC generation	78
	CSL/RBP-Jκ knockout	*in vivo* AHR$^+$NKp46$^+$ ILC accumulation	77
	OP9-Dll1, ICN overexpression	*in vivo* generation of NKp46$^+$ ILC	89

The Notch-dependence of many hematopoietic cell populations highlights the requirement for studying Notch signaling in strict, cell-type specific genetic loss-of-function contexts. The duration and intensity of Notch signaling is tightly regulated. Thus, gain-of-function approaches can result in unintended effects and unphysiological functions of Notch signaling. For example, gain-of-function approaches suggested a role for Notch signaling in adult hematopoietic stem cell (HSC) maintenance, which was correlated with expression of Notch ligands in the bone marrow and assumed to be relevant *in vivo*.[90,91] However, strict loss-of-function approaches revealed that Notch is dispensable for adult HSC maintenance.[92-94] Despite the presence of multiple Notch ligands in the bone marrow, Notch signaling is actively maintained in an off state through

the actions of LRF in repressing *Dll4* expression in erythroblasts.[65,95] Furthermore, the effects of Notch deprivation can be compensated by other signaling pathways *in vivo*. Thus, it is necessary to use *in vivo* loss-of-function approaches to identify the true functions of Notch signaling. The data presented in this thesis will demonstrate a clear role for Notch signaling in mature T cell function by using T cell-specific genetic Notch loss-of-function approaches.

1.4 Regulation of T cell development by Notch signaling

Several genetic loss-of-function approaches were used to determine the effects of Notch signaling during T cell development in the thymus. Notch signaling was first described to regulate thymic αβT cell development. Early studies used complementary gain- and loss-of-function approaches to reveal the role of Notch signaling in developing T cells. Where loss of Notch1 or CSL/RBP-Jκ resulted in significant inhibition of T cell development and ectopic B cell development in the thymus, constitutively active forms of Notch signaling induced extrathymic T cell generation at the expense of B cell development.[29,66,96] Later reports demonstrated a cell-autonomous requirement for Notch1 in developing T cells and Dll4 in thymic stroma for maintenance of T cell development.[27,29] Recent work has started identifying specific targets downstream of Notch signaling, such as *Tcf1* and *Hes1,* that elicit some of the effects of Notch signaling on T cell development.[20,97] However, how Notch signaling interacts or crosstalks with other factors has not been fully elucidated.

Notch signaling is required to progressively restrict thymus-seeding progenitors to the T cell lineage at the expense of other hematopoietic lineages.[29,80,85,98] Notch signaling is highest at the earliest state of T cell development, the early T progenitor (ETP), and is actively maintained through the double negative 2 (DN2) and DN3a stages **(Figure 1.2)**. During the DN3a stage, Notch signaling promotes survival, proliferation, and differentiation of DN3 cells at least in part through the PI3K/Akt pathway.[99] One way Notch signaling is turned off at the DN3a stage is through signaling downstream of the pre-T cell receptor that actively turns off *Notch1* expression through a negative feedback loop involving antagonism of E2A-mediated *Notch1* transcription by Id3.[81,100,101] Failure

of Notch signaling to be turned off during later stages of T cell development can contribute to T-ALL in mouse models and humans.[102] Notch signaling intensity remains very low during the subsequent DN4 and double positive (DP) $CD4^+CD8^+$ stages of T cell development, during which time positive and negative selection occur.[83] Presumably, Notch signaling intensity is kept low at these stages to prevent interference of Notch signaling with positive and negative selection.[83,103]

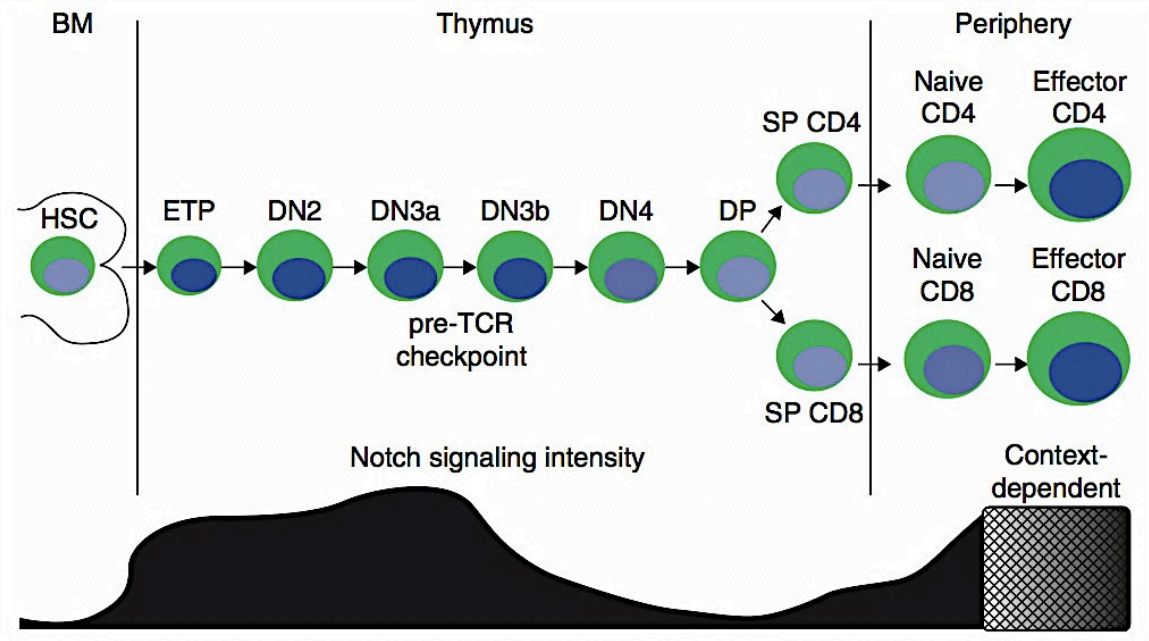

Figure 1.2 Regulation of Notch signaling intensity during T cell development and differentiation[2].
Notch signaling intensity varies throughout T cell development. The Notch target genes *Dtx1* and *Hes1* are expressed at low levels in bone marrow hematopoietic stem cells (HSCs). Upon arrival in the thymic environment, early T progenitors (ETPs) strongly upregulate Notch target gene expression. Expression levels of Notch target genes gradually increase from the ETP stage to the double negative 3a (DN3a) stage. After the β-selection checkpoint, during which Notch signaling is significantly downregulated, Notch signaling intensity steadily decreases from the DN3b stage to the $CD4^+CD8^+$ double positive (DP) stage. Thymic single positive (SP) $CD4^+$ and $CD8^+$ T cells as well as naïve peripheral T cells express low amounts of Notch target genes. Upon T cell activation in the periphery, Notch signaling intensity increases sharply in a context-dependent manner.

[2] Figure taken from:

Sandy AR and Maillard I. 2009. Notch signaling in the hematopoietic system. *Expert Opin Biol Ther*, Nov 9(11): 1383-1389.

Although it has been clearly demonstrated that Notch signaling plays a role in successive stages of T cell development, emerging data suggest that Notch signaling also regulates mature T cell differentiation during antigen-driven immune responses.

1.5 Role of Notch signaling in mature T cell differentiation and function

Notch signaling is emerging as a key player in the differentiation and function of mature $CD4^+$ and $CD8^+$ T cells. Upon encountering activated, mature antigen presenting cells (APCs), $CD4^+$ T cells can differentiate down a number of pathways, including T helper 1 (Th1), Th2, Th17, and T regulatory cells (Tregs).[104] Th1 cells are important for cell-mediated immunity, express the master transcription factor T-bet, and produce IFNγ. In contrast, Th2 cells are required for allergic responses and helminthic immunity. Th2 cells produce IL-4, IL-5, and IL-13 and their differentiation is driven by the transcription factor Gata-3. Anti-microbial immunity and inflammation is promoted by IL-17-producing Th17 cells, driven by the master transcription factor Rorγt. Lastly, Tregs are essential for dampening and controlling immune responses. Mice and humans with mutations in the master transcription factor required for Treg generation, Foxp3, suffer from a multi-organ autoimmune disease, illustrating its importance for Treg suppressive function.[105,106] These $CD4^+$ T cell differentiation pathways are the most clearly defined. However, new T helper subsets are being discovered, and the plasticity of these lineages is only now beginning to be fully appreciated.[104,107]

Upon activation, $CD8^+$ T cells differentiate under the influence of the master transcription factor Eomesodermin, which drives their cytolytic activity towards target cells. Eomesodermin is important for eliciting IFNγ production and cytotoxic molecule expression (Perforin and Granzyme B) in $CD8^+$ T cells.[108,109] Additionally, $CD8^+$ T cells require $CD4^+$ T cell help during the priming phase to respond optimally to pathogens.[110] Despite the significant amount of foundational research on $CD4^+$ and $CD8^+$ effector T cells, pathways that govern their differentiation are still being elucidated.

One such pathway that has the potential to regulate mature T cell differentiation is the Notch pathway. Notch signaling was first suggested to regulate T cell differentiation in a binary fashion, similar to its role in T cell development at the expense of B cells (see

section 1.4).[111] For instance, the Flavell group suggested that Notch regulated a dichotomous switch between Th1 and Th2 differentiation.[111] Using *in vitro* gain- and loss-of-function approaches, it was shown that Dll4 induced Th1 differentiation while Jag1 promoted Th2 differentiation.[111,112] However, it is becoming clear that Notch signaling can regulate mature T cell responses in a context-dependent manner. Through multiple, complementary gain- and loss-of-function approaches *in vivo*, Notch signaling was shown to directly regulate transcription of *Il4* and *Gata3* during Th2 differentiation.[113-116] In contrast, Notch appeared dispensable for clearance of pathogens, like *Leishmania major,* that require Th1 cells for disease control.[115] However, subsequent reports suggested that Notch might also regulate Th1 differentiation. Using γ-secretase inhibitors (GSIs), which target Notch as well as other transmembrane proteins, *Notch1* gain-of-function, and a Notch1 antisense strategy, it was purported that Notch1 directly regulates transcription of *Ifng* and *Tbx21* (encoding T-bet).[117] Others reported that inhibition of Notch signaling using systemic Dll4 blockade decreased or did not change IFNγ production by peripheral $CD4^+$ T cells.[118,119]

Similar studies submitted that Notch signaling may promote Th17 and Treg differentiation.[118,120-123] Using anti-Dll4 blocking antibodies administered systemically, Notch was suggested to either affect or not change IL-17A production.[118,119] Also, *Rorγt*, the master transcription factor for Th17 cells, and the cytokine *Il17a* were proposed to be direct targets of Notch signaling.[121] Using *in vitro* Notch ligand gain-of-function assays and some *in vivo* loss-of-function, Notch was suggested to regulate IL-17 production by direction regulation of IL-9 expression.[124]

Lastly, Treg differentiation and suppressive activity were also suggested to be regulated by Notch signaling.[118,120,122,123] Using *in vitro* and *in vivo* GSI treatment, a *Notch1* antisense strategy, or Notch1 gain-of-function, Notch was suggested to directly regulate Foxp3 expression through cooperation with Smad proteins.[120,123] In contrast, other work using Notch ligand blocking antibodies reported that Notch inhibition promoted Treg development.[118] Overall, Notch signaling seems to regulate mature T cell function based on the immune context, although no unifying mechanism has been found to account for the effects of Notch in $CD4^+$ T cells.

The role of Notch signaling in $CD8^+$ T cells has been less studied in comparison to Notch in $CD4^+$ T cells. Work by Yasutomo's group showed that Notch2 could directly regulates *Eomes* (encoding Eomesodermin), and *Gzmb* (encoding Granzyme B) in cooperation with CREB1 in a $CD8^+$ T cell line.[125] Similarly, it was proposed that Notch1 regulates expression of *Eomes, Gzmb,* and *Prf1* (encoding Perforin).[126] Furthermore, cooperation of T cell receptor and Notch signaling were purported to regulate IFNγ expression in $CD8^+$ T cells through *in vitro* GSI treatment and a *Notch1* antisense strategy.[127]

Collectively, the wide variety of conclusions available in the literature may reflect the different experimental systems used, many of which were gain-of-function strategies or systemic modulation (i.e. GSIs and blocking antibodies) of Notch signaling. As seen in section 1.3, Notch signaling has the ability to regulate a significant number of cellular subsets in the hematopoietic system.[60,61] Thus, approaches that modulate Notch signaling systemically could be altering Notch signaling in multiple cell types, making interpretation of the findings difficult.

In contrast, data generated using strict T cell-specific loss-of-function approaches, such as those described in this dissertation, suggest that Notch regulates T cell responses in a context-dependent manner. For instance, Notch signaling may modulate the threshold of T cell activation resulting in T cell differentiation changes downstream. This concept is reminiscent of T cell differentiation changes as a consequence of costimulation blockade during T cell-mediated immune disorders. Thus, Notch signaling may be modulating T cell responses in a context-dependent manner by acting as a molecule providing costimulation-like signals to T cells. This hypothesis was formulated based on work in this thesis to investigate the role of Notch signaling in two T cell-mediated immune disorders: graft-versus-host disease (GVHD) and experimental autoimmune encephalomyelitis (EAE; discussed in Chapter 5).

1.6 Graft-versus-host disease

1.6.1 GVHD – a historical perspective

In 1944, Medawar performed some of the earliest transplantation experiments using a skin allograft transplantation model.[128] Medawar referred to the rejection of the allogeneic skin tissue as the host-versus-graft (HVG) response.[128] Following Medawar's early experiments, several cellular and genetic contributors to transplantation tolerance and rejection were identified.[129,130] In 1951, injection of bone marrow cells into guinea pigs and mice was shown to rescue them from lethal irradiation injury.[131]

In the late 1940s and 1950s, there was a burst of activity in the field of transplantation. Several groups demonstrated that hematopoietic cells were significant mediators of transplantation tolerance or rejection, depending on the source of hematopoietic cells.[132-138] However, studies in the 1950s and 1960s that sought to translate transplantation into patients were met with significant hurdles. While many patients failed to even engraft the donor cells, a significant number succumbed to severe GVHD, a syndrome initially ascribed to donor immunocompetent cells attacking the host tissues of compromised allogeneic recipients.[139] Despite these failures, there was a renewed sense of hope for transplantation clinical translation with the identification of effective conditioning regimens, in part using dogs as a model transplantation system. Because of their high degree of genetic diversity similar to humans, dogs made an ideal system for transplantation studies.[140] Following results of transplantation in large animals, the late 1960s brought about a flurry of studies translating transplantation into the clinic.

Despite early and more recent efforts, GVHD still remains the most significant complication following allogeneic bone marrow transplantation (allo-BMT).[141,142] In many patients undergoing transplantation for malignant disorders, the goal of transplantation is to achieve efficient anti-tumor activity while minimizing the detrimental GVHD side effects. Patients preparing for transplantation are conditioned with chemotherapeutics and/or irradiation.[143] These conditioning regimens promote the release of pattern associated molecular patterns and chemokines, as well as the release of gut microbiota constituents.[144,145] These "danger signals" activate APCs. GVHD is

initiated when infused donor T cells recognize allo-antigens presented by donor or host hematopoietically or host non-hematopoietically derived APCs.[146-150] These activated T cells produce abundant cytokines, coined the cytokine storm, and elicit GVHD as well as the beneficial graft-versus-tumor (GVT) effects.[151,152] GVHD is divided into two forms: acute and chronic. Acute GVHD is characterized by strong inflammation while chronic GVHD has more autoimmune-like features, as well as chronic tissue inflammation and remodeling involving multiple cell types.[141] For the purpose of this dissertation, I will focus on T cell intrinsic aspects of acute GVHD biology.

1.6.2 Alloreactive T cell differentiation in GVHD

Initially, Th1 cells were thought to be the main aggressor inducing acute GVHD. However, it was later found that all T helper lineages could contribute to GVHD pathogenesis, each with their own tissue tropism and pathogenic effects.[153,154] For instance, IL-17$^{-/-}$ donor T cells had increased differentiation to the Th1 lineage but delayed GVHD onset, although overall GVHD survival was not significantly changed.[154,155] However, deletion of the master transcription factor of Th17 cells, Rorγt, had no measureable effect on survival of allogeneic transplant recipients as compared to WT T cell recipients.[156] In contrast, genetic removal of both T-bet and Rorγt in donor T cells protected from GVHD, partly attributable to increased Treg frequency.[157] These data suggest that both Th1 and Th17 cells differentially contribute to GVHD pathogenesis.

Investigation of Th2 cells in acute GVHD found that knocking out STAT6, an important signal transducer downstream of IL-4 signaling, in T cells resulted in slightly prolonged survival as compared to WT T cell recipients.[153,158] Similarly, IL-4 knockout T cell recipients had improved survival after allo-BMT compared to WT T cell recipients.[159] In contrast to these findings, *in vitro* polarization of T cells to the Th2 lineage before transplantation decreased GVHD severity and serum IFNγ and TNFα levels.[160] Collectively, these data suggest that Th1 cells may dominate the GVHD reaction when Th2 and Th17 responses are intact. Upon manipulation of specific T helper lineages, the balance can tip in favor of the other lineages for inducing GVHD pathology.

Contrasting with the inflammatory environment created by T helper cells during GVHD, Tregs contribute to protection from GVHD. Before the discovery of the master transcription factor of Tregs, Foxp3 (Forkhead box P3), Tregs were identified by high expression of the cell surface marker CD25, the high affinity alpha chain of the IL-2 receptor.[161-166] Despite Foxp3 being a more specific marker for Treg identification, its intracellular location makes it difficult for use in human applications requiring cell separation. Thus, CD25 positivity still remains a reliable marker for Treg-enrichment. Early studies that co-transplanted naïve T cells with $CD25^{+}CD4^{+}$ Tregs demonstrated that Tregs could suppress GVHD at multiple time points post-transplantation.[167-169] Despite the profound suppression of GVHD, co-infusion of Tregs with effector T cells at the time of transplant did not prevent the GVT reaction.[167,169] Altogether, these data highlight the importance of Tregs in suppressing GVHD.

More recent studies have focused on translating Treg-related therapies to the clinic. Correlative studies have shown that there is a direct association between Treg frequency and GVHD severity in patients.[170-172] In fact, clinical trials seeking to therapeutically expand Treg populations in GVHD patients using low-dose IL-2 therapy have shown promise in decreasing GVHD severity.[173-176] In summary, a fine balance of proinflammatory conditions produced by effector T cells and suppressive activities mediated by Tregs regulates GVHD.

1.6.3 T cell costimulatory and coinhibitory pathways in GVHD

Costimulatory and coinhibitory molecules also play a prominent role in GVHD severity and lethality. The immunoglobulin and tumor necrosis factor (TNF) superfamilies are the two main families of cosignaling molecules in T cells. The members of these families can demonstrate a promiscuous interaction pattern whereby one receptor can interact with a wide variety of ligands and one ligand can interact with many receptors. Additionally, interaction between members from different families has been shown, as is the case for the immunoglobulin family member BTLA interacting with the TNF family member HVEM.[177]

A non-exhaustive list of immunoglobulin superfamily members includes CD28, CD80/B7-1, CD86/B7-2, B7-H1/PD-L1, B7-DC/PD-L2, CTLA-4 (Cytotoxic T

lymphocyte antigen-4), PD-1 (Programmed death-1), BTLA (B- and T- lymphocyte attenuator), ICOS (Inducible T cell costimulatory), and ICOS ligand/B7-H2.[178] The TNF superfamily consists of approximately 50 members including HVEM (herpes virus entry mediatory), TNFSF14 (LIGHT), OX40, OX40 ligand, 4-1BB, 4-1BB ligand, CD30, CD30 ligand, CD40, and CD40 ligand.[179]

All the molecules listed above have been studied in the context of GVHD. For instance, inhibiting the costimulatory molecules CD80, CD86, or CD28 with blocking antibodies resulted in reduced T cell expansion, increased survival after transplantation, and largely preserved GVT responses.[180-182] Similar results were shown when T cells were CD28-deficient.[183] Use of a superagonistic anti-CD28 antibody also yielded protection from GVHD and preserved GVT, but the effect was attributed to enhance expansion of Tregs, highlighting the differential sensitivity of effector T cells and Tregs to CD28 signaling.[184,185] Moreover, blocking the costimulatory molecule CD40L or activating the coinhibitory molecule CTLA-4 (CTLA-4 Ig) alone or in combination with anti-CD40L resulted in decreased GVHD, at least partially attributable to tolerance induction in T cells.[181,186-191]

OX40 has also been studied in the context of GVHD elicited by $CD4^+$ and $CD8^+$ T cells. While OX40 deficiency in $CD8^+$ T cells increased GVHD severity, $OX40^{-/-}$ or $OX40L^{-/-}$ $CD4^+$ T cells induced less GVHD.[192] Inhibition of other molecules, like 4-1BB, HVEM, CD30, and ICOS in T cells, has also resulted in reduced GVHD severity after transplantation usually owing to reduced effector T cell function or survival.[193-201] On the other hand, consistent with a role for PD-1 in dampening T cell effector function, inhibition of PD-1 using either anti-PD-1 antibodies or PD-1 knockout T cells evoked increased GVHD severity, at least in part due to heightened IFNγ responses.[202]

These previous approaches seeking to block one or a few effector or costimulatory pathways did not yield a significant survival advantage.[154,155,159,192,195,203-207] Typically, inhibiting one pathway resulted in upregulation of other proinflammatory pathways, which is exemplified in the case of inhibiting certain T cell cytokines.[154] In contrast, work presented in Chapters 2, 3, and 4 will demonstrate that inhibition of Notch signaling in alloreactive T cells significantly inhibits GVHD with broad effects in T cells that are not limited to inhibition of a single T helper lineage. Thus, inhibition of Notch

signaling provides beneficial immunomodulation without global immunosuppression, in contrast to many prior attempts to control GVHD while preserving GVT activity.

Chapter 2

Notch signaling is a critical regulator of allogeneic CD4$^+$ T cell responses mediating graft-versus-host disease[3]

2.1 Abstract

Graft-versus-host disease (GVHD) remains the major barrier to the success of allogeneic hematopoietic stem cell transplantation (HSCT). GVHD is caused by donor T cells that mediate host tissue injury through multiple inflammatory mechanisms. Blockade of individual effector molecules has limited efficacy in controlling GVHD. Here, we report that Notch signaling is a potent regulator of T cell activation, differentiation, and function during acute GVHD. Inhibition of canonical Notch signaling in donor T cells markedly reduced GVHD severity and mortality in mouse models of allogeneic HSCT. Although Notch-deprived T cells proliferated and expanded in response to alloantigens *in vivo*, their ability to produce interleukin-2 and inflammatory cytokines was defective. Notch inhibition decreased the accumulation of alloreactive T cells in the intestine, a key GVHD target organ. However, Notch-deprived alloreactive CD4$^+$ T cells retained significant cytotoxic potential, leading to improved overall survival of the recipients. These results identify Notch as a novel essential regulator of pathogenic CD4$^+$ T cell responses during acute GVHD and suggest that Notch signaling in T cells should be investigated as a therapeutic target after allogeneic HSCT.

2.2 Introduction

Graft-versus-host disease (GVHD) is a life-threatening complication that limits the efficacy of allogeneic hematopoietic stem cell transplantation (HSCT).[141,142,208,209] Despite prophylaxis, GVHD still occurs in many allogeneic HSCT patients. Furthermore, standard immunosuppressive therapy for acute GVHD gives rise to disappointing

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[3] Excerpts taken from:

Zhang Y, **Sandy AR,** Wang J, Radojcic V, Shan GT, Tran IT, Friedman A, Kato K, He S, Cui S, Hexner E, Frank DM, Emerson SG, Pear WS, Maillard I. 2011. Notch signaling is a critical regulator of allogeneic CD4+ T cell responses mediating graft-versus-host disease. *Blood* 117: 299-308.

sustained response rates (< 50%) and impairs graft-versus-tumor (GVT) activity, increasing the risk of tumor relapse.[209,210] GVHD is caused by donor T cells attacking normal host tissues, involving complex interactions of immune cells and inflammatory mechanisms mediating target organ injury.[141,142,208,209] In particular, multiple T cell effector differentiation pathways can induce GVHD.[154,155,159,203-206] Novel strategies that inhibit GVHD while preserving GVT could markedly improve allogeneic HSCT. Whether Notch signaling is critical to allogeneic T cell responses and GVHD remains unknown.

Here, we report that Notch inactivation in donor $CD4^+$ T cells inhibits their capacity to mediate acute GVHD but preserved their cytotoxic potential. Notch-deprived $CD4^+$ T cells expanded in response to alloantigens *in vivo*, but displayed a reduced accumulation in the gut and failed to produce a broad range of effector cytokines. These findings differ from past observations of Notch signaling in mature $CD4^+$ T cells.[60,117,125,126,211-213] Our results indicate that Notch inhibition in alloreactive T cells may be a promising strategy to control GVHD while preserving significant GVT effects after allogeneic HSCT.

2.3 Results

2.3.1 Inactivation of Notch signaling by DNMAML in donor T cells inhibits acute GVHD

To inactivate Notch in mature T cells, we conditionally expressed the pan-Notch inhibitor DNMAML. DNMAML contains the MAML1 Notch-binding domain fused to green fluorescent protein (GFP) and blocks transcriptional activation downstream of all Notch receptors.[82,115] We crossed Cd4-Cre transgenic mice with C57BL/6 (B6) $ROSA26^{DNMAMLf/+}$ mice.[81,115] This resulted in Cre-mediated DNMAML activation in $CD4^+CD8^+$ thymocytes, followed by stable expression in $CD4^+$ and $CD8^+$ lymphocytes. This strategy does not interfere with the requirement for Notch signaling during early T-cell development and produces normal numbers of T cells without baseline abnormalities.[115] All mature $CD4^+$ and $CD8^+$ T cells in these mice cannot respond to Notch signaling, an excellent model to study the role of the Notch pathway during immune responses *in vivo*.

We first tested the impact of Notch deprivation in alloreactive $CD4^+$ T cells using the major histocompatibility complex (MHC)–mismatched B6 anti-BALB/c model **(Fig. 2.1)**. WT or DNMAML $CD4^+$ B6 T cells (CD45.2, H-2^b) were transplanted with T cell-depleted bone marrow (TCD BM) from B6-SJL mice (CD45.1) into lethally irradiated BALB/c mice (H-2^d). Control mice receiving TCD BM remained free of GVHD. All WT $CD4^+$ T cell recipients developed severe acute GVHD with > 50% dying around day 10 and all by day 40 **(Fig. 2.1A)**. In contrast, BALB/c mice receiving DNMAML $CD4^+$ T cells did not develop significant clinical signs of acute GVHD, with 75% surviving > 80 days after transplantation. The survival rate of DNMAML T cell recipients was similar to that of TCD BM recipients. Assessment of the clinical GVHD score indicated markedly decreased acute GVHD severity in DNMAML compared with WT T cell recipients **(Fig. 2.1A)**. This suggested that DNMAML $CD4^+$ T cells induced only mild disease. Histological examination of GVHD target organs showed markedly reduced inflammation in the skin, liver, and intestine of DNMAML compared with WT $CD4^+$ T cell recipients **(Fig. 2.1B)**. To further quantify the protection provided by Notch inhibition, we titrated up the number of donor DNMAML $CD4^+$ T cells and down the number of WT $CD4^+$ T cells **(Fig. 2.1C)**. Mice receiving a very high dose of DNMAML $CD4^+$ T cells (5.0×10^6) remained significantly better protected against GVHD lethality and morbidity than mice receiving only 0.5×10^6 WT $CD4^+$ T cells. Thus, DNMAML expression had more protective effects than a 10-fold reduction in donor $CD4^+$ T cells. DNMAML $CD4^+$ T cells also had significantly preserved anti-leukemic activity *in vivo* (data not shown). Together, these results demonstrate that inactivation of Notch signaling in donor $CD4^+$ T cells prevents acute lethal GVHD in mice.

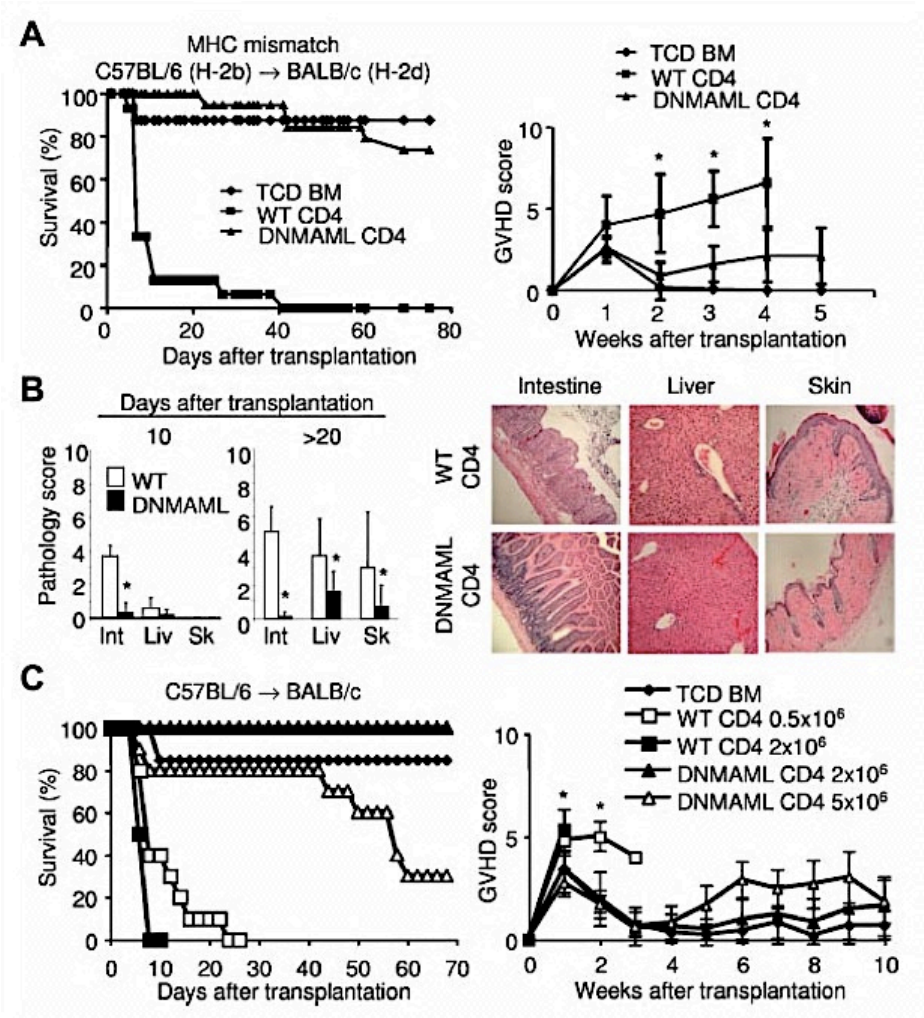

Figure 2.1 Inactivation of Notch signaling in donor CD4$^+$ T cells inhibits acute GVHD in irradiated MHC-mismatched hosts.

(A) Lethally irradiated BALB/c mice (1000 rads) were transplanted with 5x10^6 TCD BM from B6-SJL mice (n=10), with or without 2x10^6 WT B6 CD4$^+$ T cells (WT CD4, n=15) or B6 DNMAML CD4$^+$ T cells (DNMAML CD4, n=19). Survival was assessed over time (P< 0.001, WT versus DNMAML CD4$^+$ T cells). Data shown are pooled from 3 independent experiments (left panel). Right panel, clinical GVHD score (*P< 0.05, WT versus DNMAML CD4$^+$ T cells). Representative data from 1 of 2 independent experiments are presented. **(B)** Histological GVHD score. Tissues were collected at day 10 (n= 4 for each group) or >20 days (range 20-40; WT, n= 8; DNMAML, n= 9) after transplantation (850 rads; left). Int, intestine; Liv, liver; Sk, skin. Right panel, histological analysis of intestine, liver, and skin after transplantation of WT (n= 8) or DNMAML (n= 9) CD4$^+$ B6 T cells (x100), which is representative of 2 independent experiments. **(C)** Dose-response experiment. Lethally irradiated BALB/c mice were transplanted with 5x10^6 TCD BM from B6-SJL mice (n= 13), or TCD BM with 0.5x10^6 or 2x10^6 WT B6 CD4$^+$ T cells (WT CD4, n= 10/group) versus 2x10^6 or 5x10^6 DNMAML CD4$^+$ T cells (DNMAML CD4, n= 10/group). Survival (left panel) and clinical GVHD score (right panel) were assessed over time. Survival (P= 0.012) and GVHD severity (P < 0.01) were worse after administration of 0.5x10^6 WT CD4$^+$ than 5x10^6 DNMAML CD4$^+$ T cells (0.5x10^6 WT versus 5x10^6 DNMAML CD4$^+$ T cells).

2.3.2 Extensive proliferation and expansion of donor DNMAML CD4$^+$ T cells in irradiated MHC-mismatched hosts

To determine the mechanisms by which Notch inhibition in CD4$^+$ T cells prevents GVHD, we first assessed engraftment and expansion of Notch-deprived donor T cells. We tracked DNMAML CD4$^+$ T cells in irradiated BALB/c recipients at day 5 after transplantation **(Fig. 2.2A-E)**. We found modestly but significantly increased donor-derived CD4$^+$ T cell numbers in spleens from BALB/c recipients of DNMAML compared with WT T cells **(Fig. 2.2A, B)**. When CFSE was used to track cell division, both groups had a similar percentage of donor CFSE^{low} T cells (approximately 99%), consistent with extensive proliferation, although the full division history could not be compared due to fluorescence of the DNMAML-GFP fusion protein **(Fig. 2.2C)**. To assess proliferation of DNMAML-expressing CD4$^+$ T cells without GFP interference, we labeled donor T cells with the tracking dye eFluor670 **(Fig. 2.2D)**. This revealed a similar eFluor670 dilution profile in day 5 WT and DNMAML B6 CD4$^+$ T cells. Furthermore, we pulsed BALB/c recipient mice with BrdU and assessed BrdU incorporation by donor-derived CD4$^+$ T cells **(Fig. 2.2E)**. A high percentage (approximately 60%) of both WT B6 and B6 CD4$^+$ DNMAML T cells were found to be BrdU$^+$, consistent with a similar rate of cell cycle entry. To evaluate if Notch inhibition affected the expansion of alloreactive T cells at later stages, we tracked donor-derived CD45.2$^+$ B6 and B6-DNMAML CD4$^+$ T cells in the spleen, BM, liver, and thymus of recipient BALB/c mice at days 14 and 21 **(Fig. 2.2F)**. Higher numbers of donor-derived CD4$^+$ T cells were recovered from these organs in B6-DNMAML compared with WT B6 recipients, most prominently at day 21 after transplantation. Given their similar proliferation rate, this increased expansion could be related to decreased activation-induced cell death of the DNMAML CD4$^+$ T cells.

Because immune-mediated intestinal damage is a prominent cause of mortality in the B6 anti-BALB/c GVHD model, we assessed accumulation of WT or DNMAML donor-derived CD4$^+$ T cells in the small intestine at days 11 and 14 after transplantation **(Fig. 2.2G)**. Few IELs were recovered at these time points in both groups (data not shown). In contrast, a significant number of donor WT CD4$^+$ T cells were recovered from the lamina propria. We observed a significant decrease (4- to 10-fold) in the number of

infiltrating DNMAML CD4$^+$ T cells at both time points **(Fig. 2.2G)**. To assess if this was related to a cell-autonomous effect of Notch inhibition, we coinjected equal numbers of WT and DNMAML CD4$^+$ T cells.

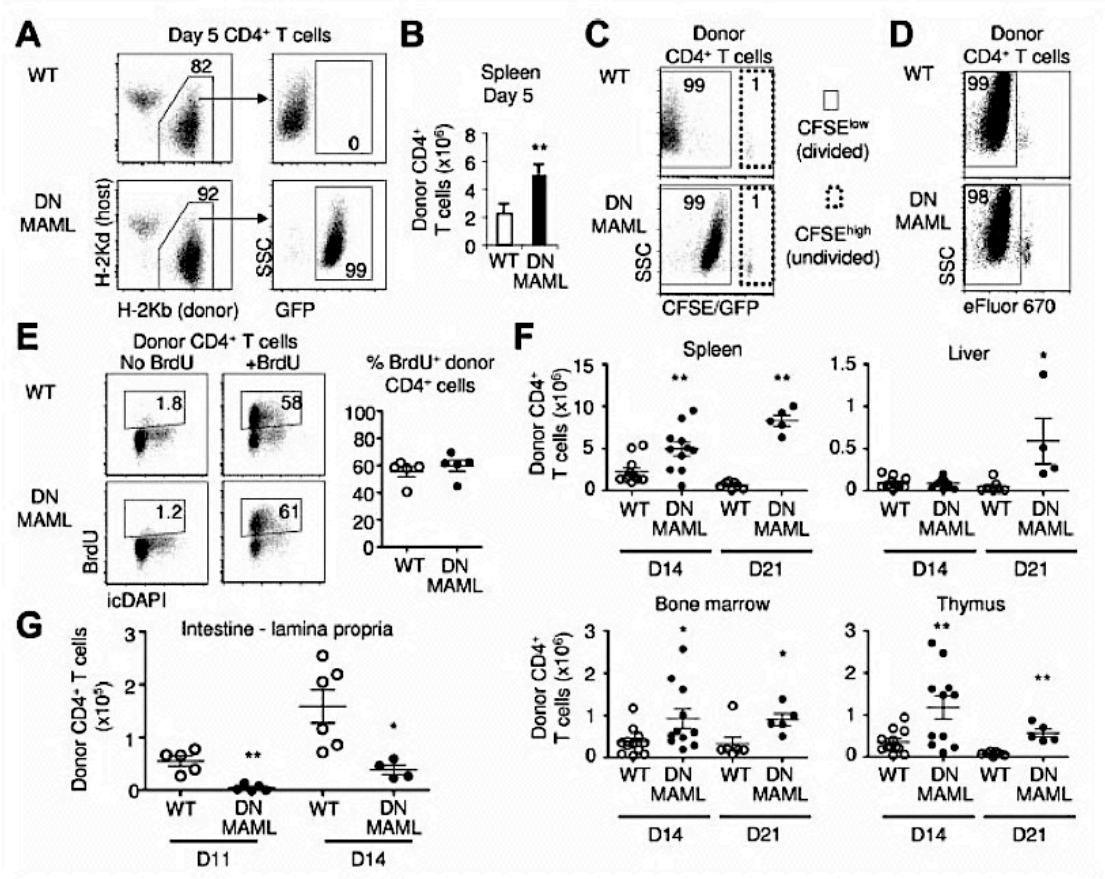

Figure 2.2 Preserved proliferation and *in vivo* expansion of DNMAML alloreactive CD4$^+$ T cells.

(A) Identification of donor-derived CD4$^+$ T cells in the spleen on day 5 after transplantation. DNMAML T cells expressed the DNMAML-GFP fusion protein. Data are representative of 5 experiments. **(B)** Absolute number of donor-derived CD4$^+$ T cells in the spleen at day 5 (mean $\pm$ SD, n= 4). **(C)** Tracking of CFSE-labeled donor CD4$^+$ T cells at day 5. Both WT and DNMAML CD4$^+$ T cells had undergone extensive proliferation, as shown by the high percentage of CFSE^{low} cells. Fluorescence levels appear higher in the DNMAML-expressing cells due to fluorescence of the covalently fused GFP protein. This effect is absent in *Rbpj* knockout mice. **(D)** Tracking of eFluor670-labeled donor WT and DNMAML CD4$^+$ T cells at day 5, showing extensive proliferation in both (% eFluor670^{low} cells). **(E)** BrdU uptake by donor WT and DNMAML CD4$^+$ T cells at day 5. Mice were pulsed with BrdU 6 hours before harvest (n= 5 per group). **(F)** Absolute number of CD45.2$^+$ donor-derived WT and DNMAML CD4$^+$ T cells in spleen, liver, BM, and thymus on day 14 (WT, n= 12; DNMAML, n= 11) and 21 (WT, n= 7; DNMAML, n= 5) post-transplantation (pool of 2 independent experiments). **(G)** Decreased number of donor-derived DNMAML compared with WT CD4$^+$ T cells in the lamina propria of the small intestine on days 11 (WT, n= 5; DNMAML, n= 5) and 14 after transplantation (WT, n= 6; DNMAML, n= 4). *P< 0.05, **P< 0.01 (2-tailed unpaired Student *t* test).

In this context, DNMAML $CD4^+$ T cells were not excluded from the intestine (data not shown), indicating that Notch does not directly control their homing to or retention in the gut. Instead, the decreased accumulation of donor $CD4^+$ T cells in the gut of DNMAML compared with WT recipients may result from a reduced inflammatory response initiated by the alloreactive T cells.

Altogether, Notch-deficient donor $CD4^+$ T cells were able to proliferate and accumulate in lymphoid and hematopoietic tissues of allogeneic HSCT recipients, but showed decreased accumulation in the gut, a key GVHD target organ. This might account at least in part for the decreased GVHD severity and mortality in DNMAML T cell recipients.

2.3.3 Impaired activation and effector functions of alloreactive DNMAML $CD4^+$ T cells

We next assessed the *in vivo* effect of Notch inhibition on the activation and effector functions of alloantigen-stimulated $CD4^+$ T cells. Donor T cells were recovered 5 days after transplantation. CD25 expression was reduced in donor DNMAML $CD4^+$ T cells, suggesting decreased activation **(Fig. 2.3A)**. Intracellular staining showed that production of the inflammatory cytokines IFNγ and TNFα, as well as IL-2, was markedly decreased in DNMAML $CD4^+$ T cells **(Fig. 2.3B)**. Further analysis revealed that DNMAML $CD4^+$ cells displayed impaired production of cytokines associated with Th1 (IFNγ, TNFα), Th2 (IL-4), and Th17 differentiation (IL-17), in addition to IL-2 **(Fig. 2.3C)**. These data suggest that DNMAML-expressing alloreactive $CD4^+$ T cells had a markedly decreased ability to produce a broad range of cytokines. This included cytokines from multiple T helper cell subsets as well as IL-2, an effect of Notch inhibition in mature T cells that has not been previously reported.

Next, we assessed whether DNMAML impaired *in vivo* expansion of Foxp3-expressing $CD4^+$ T cells **(Fig. 2.3D)**. In contrast to decreased cytokine production and CD25 expression, we found a slightly but significantly increased percentage of $Foxp3^+$ cells among day 5 donor-derived B6 DNMAML compared with WT B6 $CD4^+$ T cells. To evaluate if increased T cell–mediated suppression could account for all the effects of DNMAML in preventing acute GVHD, we cotransferred equal numbers of WT and DNMAML B6 $CD4^+$ T cells into irradiated BALB/c mice **(Fig. 2.4A)**. Comparable

expansion of both populations was observed *in vivo* **(Fig. 2.4B)**. Recipients of mixed WT and DNMAML CD4$^+$ T cells still developed severe lethal GVHD, similar to mice receiving only WT cells **(Fig. 2.4C)**. Thus, it is unlikely that an increased suppressive activity explains by itself the inability of DNMAML CD4$^+$ T cells to induce acute GVHD.

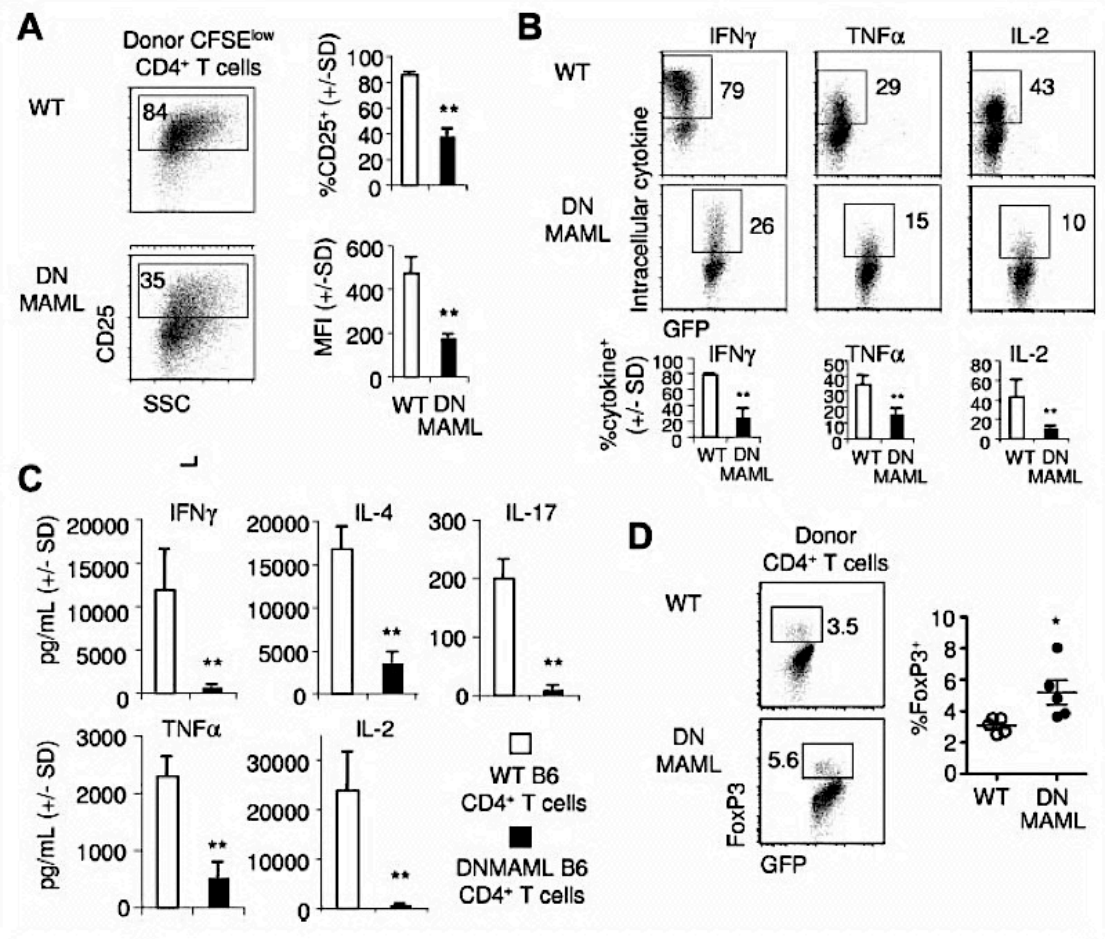

Figure 2.3 Impaired activation and cytokine response in alloreactive DNMAML CD4$^+$ T cells.

WT or DNMAML B6 CD4$^+$ T cells were tracked after transplantation into lethally irradiated MHC-mismatched BALB/c mice (900 rads). **(A)** Decreased expression of CD25 (IL-2Rα) in DNMAML CFSE^{low} donor CD4$^+$ T cells. Bar graphs show the percentage of cells expressing CD25 and the CD25 mean fluorescence intensity (MFI; mean ± SD, n= 3). **(B)** Intracellular staining for IFNγ, TNFα, and IL-2 after *ex vivo* restimulation with plate-bound anti-CD3 and anti-CD28 antibodies. Data are representative of 5 experiments. Bar graphs show mean ± SD (n= 3). **(C)** Cytokine production by 3x10^4 donor-derived CD4$^+$ T cells sort-purified 5 days after transplantation and cultured for 6 hours in the presence of anti-CD3/anti-CD28 antibodies (mean ± SD). **(D)** Intracellular staining for FoxP3 in donor WT and DNMAML CD4$^+$ T cells on day 5. *P< 0.05, **P< 0.01 (2-tailed unpaired Student *t* test).

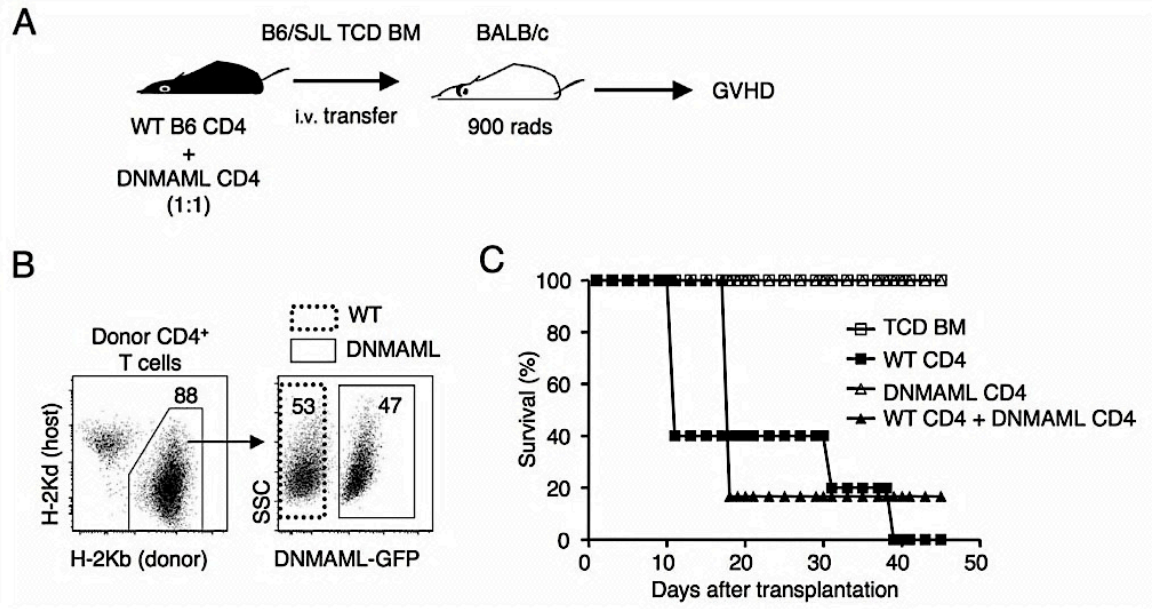

Figure 2.4 Enhanced suppression does not mediate the decreased pathogenicity of DNMAML CD4$^+$ T cells.

(A) Experimental design. T cell depleted bone marrow (TCD BM) was transplanted into lethally irradiated BALB/c recipients, with or without 2x10^6 MACS-purified CD4$^+$ T cells from control wild type B6 mice (WT), DNMAML B6 mice, or a 1:1 mixture of WT and DNMAML CD4$^+$ T cells. **(B)** Tracking of donor-derived T cells at day 5 in the group receiving the 1:1 mixture of WT and DNMAML cells. Close to 50% of donor-derived CD4$^+$ T cells expressed DNMAMLGFP, consistent with similar *in vivo* expansion. **(C)** Overall survival after transplantation. Similarly to recipients of WT CD4$^+$ T cells (n=5), high-grade lethality was observed in the group receiving both WT and DNMAML CD4$^+$ T cells (n=6), indicating that enhanced immune suppression by DNMAML CD4$^+$ T cells did not account by itself for the reduced pathogenicity of these cells.

2.3.4 Loss of CSL/RBP-Jκ impairs the effector functions of alloreactive CD4$^+$ T cells

Notch-mediated transcriptional activation requires CSL/RBP-Jκ, encoded by *Rbpj* .[31,214] To rule out off-target effects of DNMAML, we studied T cells with conditional *Rbpj* inactivation **(Fig. 2.5).**[214] This also allowed tracking of CFSE-labeled T cells without interference from DNMAML-GFP fluorescence. Five days after transfer into BALB/c recipients, *Rbpj$^{f/f}$ Cd4-Cre$^+$* T cells had proliferated extensively, with most cells having divided > 8 times, similar to control cells **(Fig. S2.5A)**. The expansion of CSL/RBP-Jκ-deficient CD4$^+$ T cells was preserved. As with DNMAML, CD25 expression **(Fig. 2.5B)** and production of IFNγ, TNFα, and IL-2 were reduced in CSL/RBP-Jκ–deficient CD4$^+$ T cells **(Fig. 2.5C, D)**. When assessed around the onset of proliferation, CSL/RBP-Jκ-deficient CD4$^+$ T cells displayed normal up-regulation of the

activation markers CD69, CD44, and CD25 **(Fig. 2.5E)**. The equivalent effects of CSL/RBP-Jκ loss and DNMAML expression independently validate the critical role of Notch in alloreactive T cells.

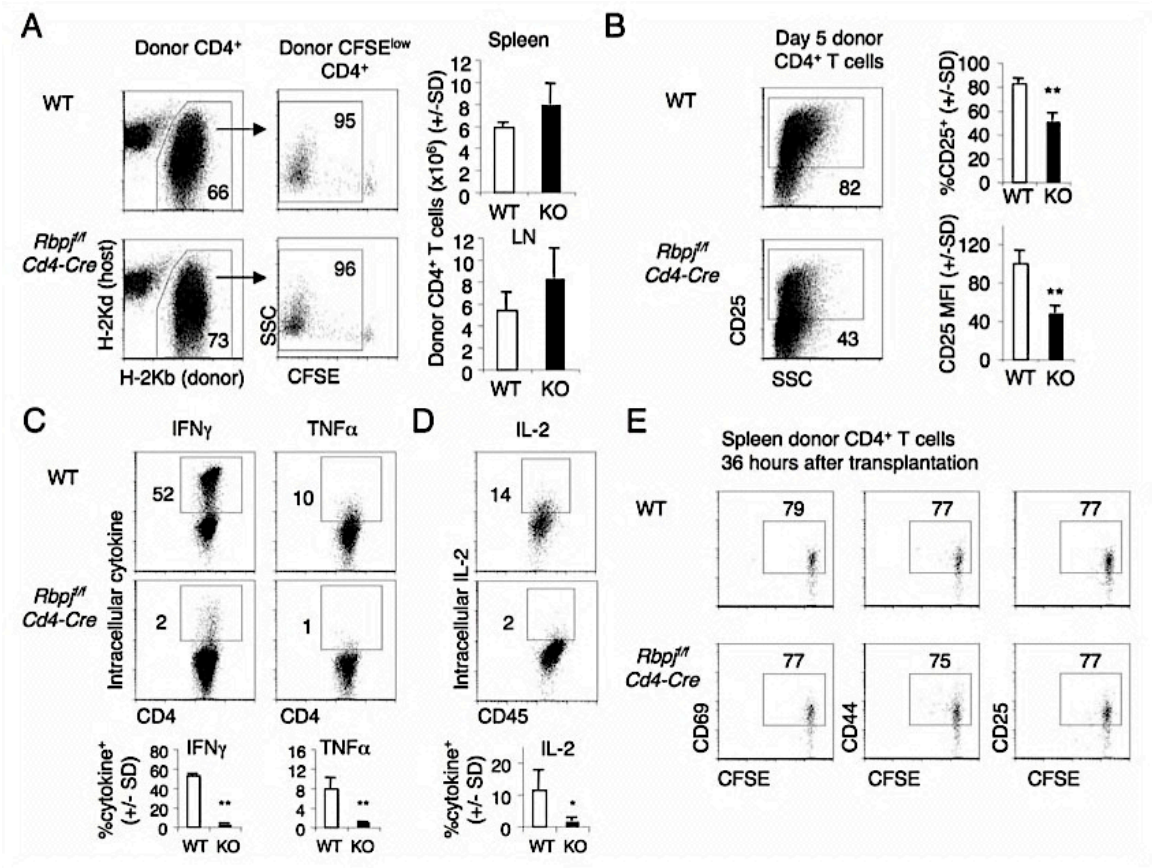

Figure 2.5 Preserved proliferation and expansion but defective function of alloreactive CSL/RBP-Jk-deficient CD4$^+$ T cells.

CFSE-labeled CD4$^+$ T cells were tracked after transplantation of 2x10^6 cells from WT B6 or *Cd4-Cre Rbpj$^{f/f}$* donors (KO, lacking CSL/RBP-Jκ) into lethally irradiated MHC-mismatched BALB/c mice (900 rads). **(A)** Tracking and CFSE dilution profile of donor-derived CD4$^+$ T cells in the spleen at day 5 after transplantation. Absolute numbers of donor-derived CD4$^+$ T cells recovered from the spleen at day 5 (mean ± SD, n=3). **(B)** Decreased expression of CD25 in donor-derived *Cd4-Cre Rbpj$^{f/f}$* T cells at day 5. Bar graphs show the percentage of cells expressing CD25 and the CD25 mean fluorescence intensity (MFI) among CFSE^{low} donor-derived CD4$^+$ T cells (mean ± SD, n=3). **(C)** Intracellular staining for IFNγ and TNFα as well as IL-2 **(D)** after *ex vivo* restimulation with plate-bound anti-CD3 antibody. Data are representative of three experiments. **(E)** Normal early activation of CSL/RBP-Jκ-deficient CD4$^+$ T cells as assessed through upregulation of CD69, CD44 and CD25 36 hours after transplantation. *p<0.05, **p<0.01 (two-tailed unpaired Student t test).

2.3.5 Preserved *in vitro* and *in vivo* cytotoxicity of Notch-deficient alloreactive CD4$^+$ T cells

To assess the global impact of DNMAML expression on CD4$^+$ T cell–mediated cytotoxicity *in vivo*, we quantified the ability of BALB/c recipients of WT B6 or DNMAML CD4$^+$ T cells to eliminate CFSE-labeled allogeneic I-A^{d+} BALB/c targets **(Fig. 2.6A)**. Compared with TCD BM recipients, only a small fraction of infused BALB/c allogeneic targets was recovered from both WT and DNMAML CD4$^+$ T cell recipients, indicating similar and efficient *in vivo* cytotoxic activity. Furthermore, DNMAML alloreactive CD4$^+$ T cells effectively killed A20 leukemic cells in an *in vitro* assay, at least at high effector:target ratios, despite their modestly reduced killing capability compared with WT alloreactive T cells **(Fig. 2.6B)**. Altogether, Notch blockade profoundly impaired the ability of alloreactive T cells to produce effector cytokines, with less drastic effects on their cytotoxic potential.

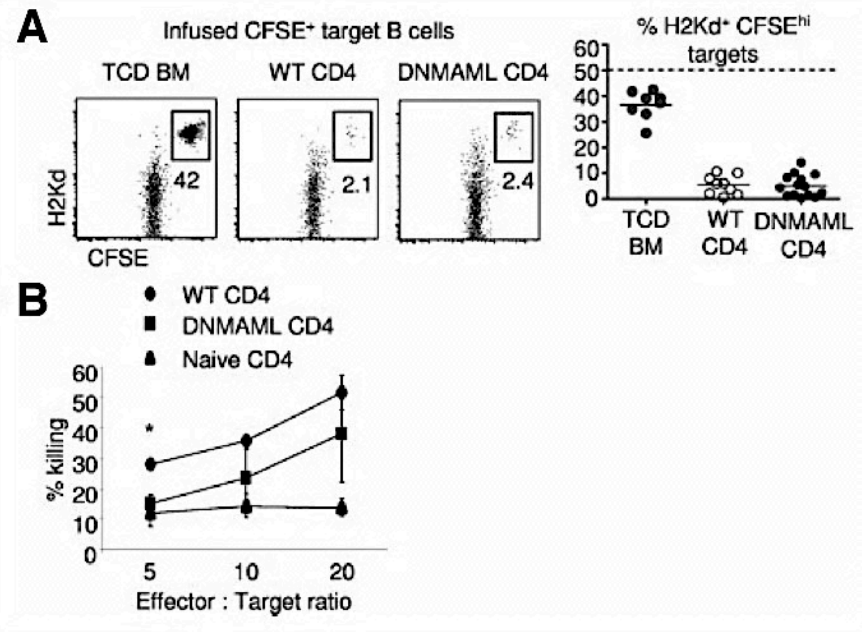

Figure 2.6 Preserved cytotoxic potential of Notch-deprived CD4$^+$ T cells.
A) *In vivo* cytotoxic activity of WT or DNMAML CD4$^+$ T cells against infused CFSE^{high} host-type BALB/c (H-2d) B cell targets, compared with control CFSE^{low} B6-SJL cells (H-2b). Analysis was performed on day 14, 18 hours after infusion of CFSE-labeled targets at a 1:1 ratio (10^7 each). Data are shown as percent residual CFSE^{high}H-2Kd$^+$ cells among all infused CFSE-labeled B cells. The dotted line represents expected recovery in the absence of cytotoxicity. **(B)** *In vitro* cytotoxic activity of WT or DNMAML CD4$^+$ T cells against A20 leukemia cells. *P< 0.05 (2-tailed unpaired Student *t* test).

2.4 Discussion

Identifying the critical signals controlling the complex features of alloimmunity is essential for regulating the balance between harmful and beneficial effects of allogeneic HSCT.[208,210] Our findings demonstrate a critical role for Notch signaling in alloreactive $CD4^+$ T cell activation, differentiation, and function during acute GVHD. Notch inactivation in donor $CD4^+$ T cells preserved their ability to engraft and proliferate, but inhibited their accumulation in the gut and acquisition of selected effector functions during the acute GVH reaction. Notch inhibition decreased the production of multiple cytokines by alloantigen-activated $CD4^+$ T cells, including IL-2, and prevented severe GVHD through mechanisms that differed from past observations of Notch function in mature T cells. However, Notch inhibition preserved significant cytotoxic activity in alloreactive $CD4^+$ T cells.

Multiple studies have shown that inhibition of single effector pathways provides limited therapeutic benefit in GVHD.[154,155,159,203-206] For example, inhibition of single cytokines or inactivation of the master transcription factors controlling individual effector T cell lineages has not translated into a marked survival advantage. In many cases, organ injury mediated by a specific T cell lineage (e.g. Th1 cells) is replaced by another pattern of target organ injury mediated by other lineages (e.g. Th2 or Th17 cells). In contrast, Notch inhibition had functional consequences on multiple $CD4^+$ T helper lineages. As a result, Notch inhibition had a dramatic impact on survival even in otherwise uniformly lethal models of GVHD. However, Notch inhibition did not merely cause immunosuppression, because *in vivo* expansion of alloreactive $CD4^+$ T cells and significant cytotoxic activity were preserved. Thus, we suggest that Notch signaling in $CD4^+$ T cells should be explored as a novel therapeutic target after allo-HSCT.

Past reports using overexpression of Jagged Notch ligands have shown that Jagged can modulate the reactivity of T cells to alloantigens, inducing expansion of Tregs and T cell hyporesponsiveness.[215] This differs from our observations, because we observed a significant trend for enhanced *in vivo* Treg expansion and decreased production of effector $CD4^+$ T cell cytokines. Thus, *in vitro* gain-of-function assays do not predict the *in vivo* function of Notch signaling in alloimmunity. In addition, it is possible that the effects of Jagged ligands in these studies could differ from and even

antagonize those of Delta like ligands, as reported in other experimental systems.[216] In addition, our results also differ from past reports in which GSIs and anti-Notch1 antibodies were shown to inhibit Treg function *in vitro* and in a mouse model of allergic airway inflammation.[122,217] Although the reasons underlying these differences remain to be explored, this also highlights the value of assessing the role of Notch signaling *in vivo* in specific types of immune response using genetic strategies of pan-Notch inhibition.

Although Notch-deficient alloreactive T cells had multiple functional defects, their initial activation, proliferation, and expansion appeared generally normal. Subsequently, significant features of Notch deprivation included decreased expression of CD25 (IL-2Rα). Notch signaling was reported to regulate CD25 at least in developing T cells.[81,218] It is possible that decreased CD25 expression could contribute to impaired activation of Notch-deprived T cells through decreased IL-2 sensitivity. However, we did not observe decreased CD25 expression at all time points and in all models of allogeneic HSCT. For example, decreased CD25 expression was not observed in unirradiated semi-allogeneic recipients, despite multiple changes in DNMAML T cell differentiation (data not shown). Thus, reduced CD25 expression cannot account for all functional defects in Notch-deprived T cells. Furthermore, preserved proliferation despite impaired IL-2 production is consistent with past studies of alloreactive T cells showing that other cytokines such as IL-15, but not IL-2, are most important to sustain their proliferation *in vivo*.[219]

Our work presents the first example of a disease model in which Notch inhibition prevents otherwise uniform lethality. Although systemic Notch inhibition can cause significant side effects, in particular gastrointestinal toxicity due to combined Notch1/Notch2 inhibition, multiple strategies are being developed to overcome this important limitation. This includes targeting of specific Notch receptors and ligands, intermittent administration of Notch inhibitors, or pharmacological strategies that counteract the effects of Notch inhibition in the gut.[61,220-222] In allogeneic HSCT, creative approaches to bypass systemic side effects of Notch inhibition could include manipulation of the cellular product administered to patients so that Notch is inhibited only in infused donor T cells.

In the future, it will be important to determine whether Notch is important only at the onset of allogeneic responses or remains active later. If its main effects are restricted to priming and early differentiation of T cells, it may be desirable to inhibit Notch transiently after allogeneic HSCT, which would avoid consequences of Notch inhibition on extrahematopoietic tissues or de novo T cell generation. Although our observations were made after allogeneic HSCT, they may extend to other types of immune responses with exposure to persistent antigens. For example, Notch may regulate organ rejection (data not shown) and autoimmune diseases (Chapter 5). From a therapeutic perspective, our work points to the Notch pathway as an attractive target to achieve beneficial context-specific immunomodulation in T cell–mediated disorders.

Chapter 3

T cell-specific Notch inhibition blocks graft-versus-host disease by inducing a hyporesponsive program in alloreactive CD4$^+$ and CD8$^+$ T cells [4]

3.1 Abstract

Graft-versus-host disease (GVHD) induced by donor-derived T cells remains the major limitation of allogeneic bone marrow transplantation (allo-BMT). We previously reported that the pan-Notch inhibitor DNMAML markedly decreased the severity and mortality of acute GVHD mediated by CD4$^+$ T cells in mice (Chapter 2). To elucidate the mechanisms of Notch action in GVHD and its role in CD8$^+$ T cells, we studied the effects of Notch inhibition in alloreactive CD4$^+$ and CD8$^+$ T cells using mouse models of allo-BMT. DNMAML blocked GVHD induced by either CD4$^+$ or CD8$^+$ T cells. Both CD4$^+$ and CD8$^+$ Notch-deprived T cells had preserved expansion in lymphoid organs of recipients, but profoundly decreased IFNγ production despite normal T-bet and enhanced Eomesodermin expression. Alloreactive DNMAML T cells exhibited decreased Ras/MAPK and NF-κB activity upon *ex vivo* restimulation through the TCR. In addition, alloreactive T cells primed in the absence of Notch signaling had increased expression of several negative regulators of T cell activation, including *Dgka*, *Cblb* and *Pdcd1*. DNMAML expression had modest effects on *in vivo* proliferation but preserved overall alloreactive T cell expansion while enhancing accumulation of preexisting natural regulatory T cells. Overall, DNMAML T cells acquired a hyporesponsive phenotype that blocked cytokine production but maintained their expansion in irradiated allo-BMT recipients, as well as their *in vivo* and *ex vivo* cytotoxic potential. Our results reveal

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[4] Taken from:

Sandy AR, Chung J, Toubai T, Shan GT, Tran IT, Friedman A, Blackwell TS, Reddy P, King PD, and Maillard I. 2013. T cell-specific Notch inhibition block graft-versus-host disease by inducing a hyporesponsive program in alloreactive CD4$^+$ and CD8$^+$ T cells. *J Immunol*, In press.

parallel roles for Notch signaling in alloreactive $CD4^+$ and $CD8^+$ T cells that differ from past reports of Notch action and highlight the therapeutic potential of Notch inhibition in GVHD.

3.2 Introduction

Using genetic models of Notch inhibition, we discovered an essential role of Notch signaling in $CD4^+$ T cells mediating graft-versus-host disease (GVHD) after allogeneic bone marrow transplantation (allo-BMT), which was the subject of Chapter 2.[223] In mouse allo-BMT models, pan-Notch blockade in donor T cells led to markedly reduced GVHD severity and improved survival.[223] Notch-deprived alloreactive $CD4^+$ T cells had decreased production of inflammatory cytokines, including IFNγ, TNFα, IL-17A, IL-4 and IL-2. Concomitantly, Notch inhibition led to increased accumulation of regulatory T cells (Tregs). However, Notch-deprived $CD4^+$ alloreactive T cells were capable of extensive proliferation, allowing for their enhanced accumulation in lymphoid tissues. Despite reduced cytokine production, Notch-deprived $CD4^+$ T cells retained potent cytotoxic potential *in vitro* and *in vivo*.

However, both $CD4^+$ and $CD8^+$ T cells have pathogenic effects during GVHD. Although we reported an essential function of Notch in $CD4^+$ alloreactive T cells, no information has been available about Notch in $CD8^+$ T cell-driven GVHD. Furthermore, it is unclear if the profound effects of Notch signaling in acute GVHD can be explained by its previously reported effects in T cells, or if new mechanisms are involved. Past work highlighted independent effects of Notch signaling in $CD4^+$ and $CD8^+$ T cells. Notch was shown to regulate *Il4* and *Gata3* expression during Th2 differentiation.[114,115] In Th1 cells, pharmacological inhibitors and a *Notch1* antisense strategy suggested that Notch controlled expression of *Tbx21*, encoding T-bet, a transcription factor regulating *Ifng* transcription.[117] Notch signaling was also shown to influence Th17 and Treg differentiation, as well as $CD4^+$ T cell longevity, at least *in vitro*.[120,121,123,224] In $CD8^+$ T cells, Notch was suggested to act directly at the *Eomes* and *Gzmb* loci, with an impact on differentiation and function.[125,126] However, these findings originate from heterogeneous experimental systems, different immune contexts and variable strategies to manipulate

Notch signaling, including gain-of-function approaches and pharmacological inhibitors. These results can be confounded by off-target effects and may not reflect the physiological functions of Notch in T cells.

Thus, we investigated the cellular and molecular mechanisms underlying the effects of Notch signaling in alloreactive $CD4^+$ and $CD8^+$ T cells during GVHD. Our strategy relied on *in vivo* priming of donor T cells in the presence or absence of all canonical CSL/RBP-Jκ and MAML-dependent Notch signals specifically in T cells, ensuring that T cells were exposed to relevant Notch ligands in the post-transplantation environment. Notch-deprived alloreactive $CD4^+$ and $CD8^+$ T cells shared a profound defect in IFNγ production, suggesting parallel effects of Notch in both T cell subsets. Decreased IFNγ was observed despite preserved or enhanced expression of the transcription factors T-bet and Eomesodermin, consistent with the absence of a classical Th1 or effector $CD8^+$ T cell differentiation defect. Notch-deprived alloreactive $CD4^+$ and $CD8^+$ T cells acquired a hyporesponsive phenotype with decreased Ras/MAPK and NF-κB signaling. Notch inhibition led to increased expression of selected negative regulators of T cell activation. Some of these characteristics have been observed in anergic T cells, suggesting that Notch-inhibited $CD4^+$ and $CD8^+$ T cells acquire an anergy-like phenotype after allo-BMT, resulting in decreased production of inflammatory cytokines. Despite these changes, Notch inhibition preserved alloreactive T cell expansion *in vivo* and only had modest effects on their proliferative potential, while increasing expansion of preexisting natural Tregs and preserving high cytotoxic potential. Altogether, our data demonstrate a novel, shared mechanism of Notch action in alloreactive $CD4^+$ and $CD8^+$ T cells during allo-BMT which differs from all previous reports of Notch activity in T cells. Understanding these effects is essential to harness the therapeutic benefits of Notch blockade to control GVHD after allo-BMT.

3.3 Results

3.3.1 Notch inhibition blocks acute graft-versus-host disease mediated by $CD4^+$ or $CD8^+$ T cells

We previously reported an essential role for Notch in $CD4^+$ T cells during acute GVHD in a major histocompatibility complex-mismatched allo-BMT model (B6 anti-BALB/c).[223] To assess if alloreactive $CD8^+$ T cells were also sensitive to Notch signaling, we used *ROSA26*^{DNMAMLf} *x Cd4-Cre* (DNMAML) mice as source of Notch-deprived $CD4^+$ and $CD8^+$ T cells. These mice express the DNMAML pan-Notch inhibitor in all mature $CD4^+$ and $CD8^+$ T cells. Wild-type (WT) or DNMAML B6 splenocytes were transplanted into irradiated BALB/c mice. Recipients were monitored for survival and GVHD severity. Allo-BMT recipients of B6 T cells died rapidly with severe GVHD **(Fig. 3.1A).** In contrast, recipients of DNMAML $CD4^+$ and $CD8^+$ T cells survived as well as mice infused only with T cell-depleted bone marrow **(Fig. 3.1A)**. We then performed allo-BMT with purified $CD4^+$ or $CD8^+$ T cells. DNMAML expression blocked severe GVHD induced by $CD4^+$ T cells (**Fig. 3.1B**). Purified $CD8^+$ T cells also induced significant lethality post-transplantation with <40% long-term survival **(Fig. 3.1C)**. In contrast, DNMAML $CD8^+$ T cell recipients had >94% survival by day 100, similar to mice receiving no T cells **(Fig. 3.1C)**. These data demonstrate that Notch is an essential regulator of GVHD induced by either or both $CD4^+$ and $CD8^+$ T cells after MHC-mismatched allo-BMT.

To further investigate the role of Notch in $CD4^+$ and $CD8^+$ alloreactive T cells, we used the B6 anti-BALB/c model as well as a minor histocompatibility antigen (miHA)-mismatched model (B6 anti-BALB/b) in which lethal GVHD is mediated by CD4-dependent $CD8^+$ T cells.[225] Together, WT $CD4^+$ and $CD8^+$ T cells induced significant lethality with <20% long-term survival in both models **(Fig. 3.2A, B)**. DNMAML $CD4^+$ and $CD8^+$ T cell recipients achieved ~80% survival **(Fig. 3.2A, B)**. When either $CD4^+$ or $CD8^+$ T cells expressed DNMAML, recipients were protected from severe GVHD with 80-90% of the mice surviving long-term **(Fig. 3.2A, B)**. These data indicate that Notch is

required in both $CD4^+$ and $CD8^+$ T cells to fully induce GVHD in MHC- and miHA-mismatched allo-BMT models.

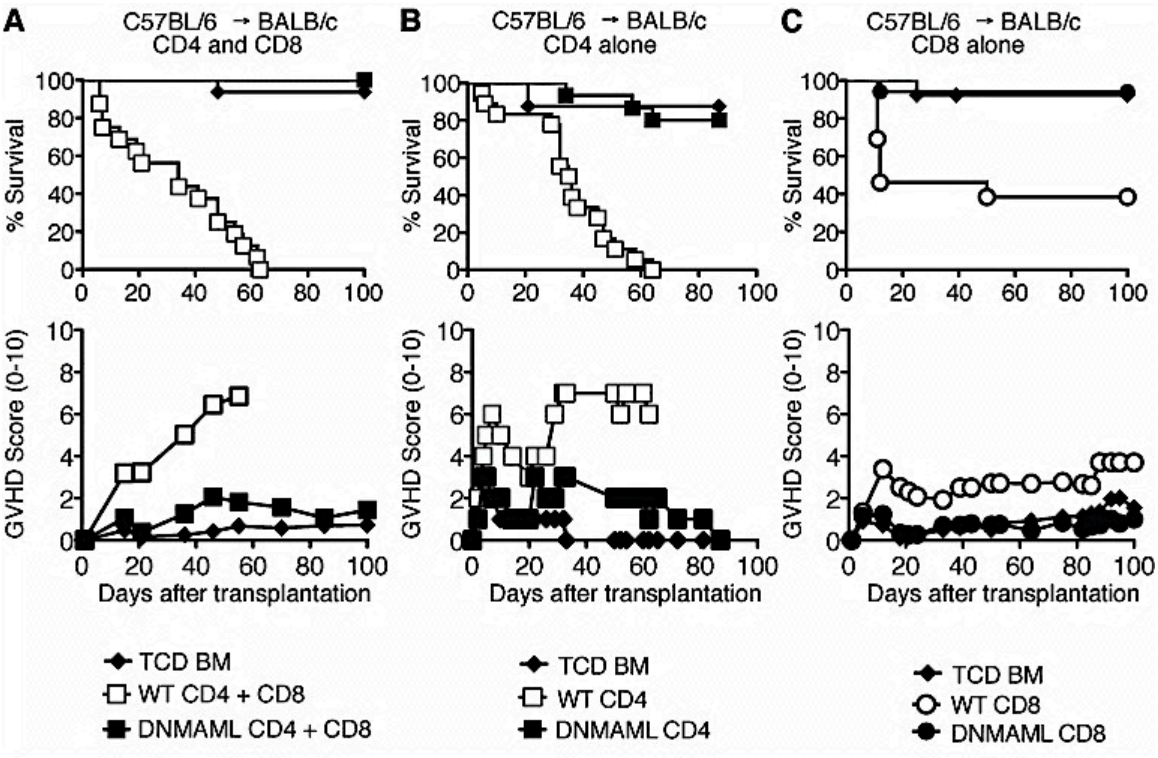

Figure 3.1 DNMAML inhibits graft-versus-host disease mediated by $CD4^+$ and/or $CD8^+$ T cells after MHC-mismatched bone marrow transplantation.
Lethally irradiated BALB/c mice ($H\text{-}2^d$) were transplanted with B6 T cell-depleted bone marrow (TCD BM, $5x10^6$ cells) alone or with **(A)** B6 ($H\text{-}2^b$) splenocytes containing $CD4^+$ and $CD8^+$ T cells from wild-type (WT) or DNMAML mice ($10x10^6$ cells; 16 mice/group) ($p<0.001$ for WT vs. TCD and WT vs. DNMAML survival); **(B)** purified B6 WT or DNMAML $CD4^+$ T cells ($2x10^6$ cells; 8-17 mice/group) ($p<0.001$ for WT vs. TCD and WT vs. DNMAML survival); **(C)** purified B6 WT or DNMAML $CD8^+$ T cells ($5x10^6$ cells; 13-17 mice/group) ($p<0.01$ for WT vs. TCD; $p<0.001$ for WT vs. DNMAML survival). Recipients were monitored over time for survival and GVHD severity after transplantation (clinical GVHD score, 0-10).

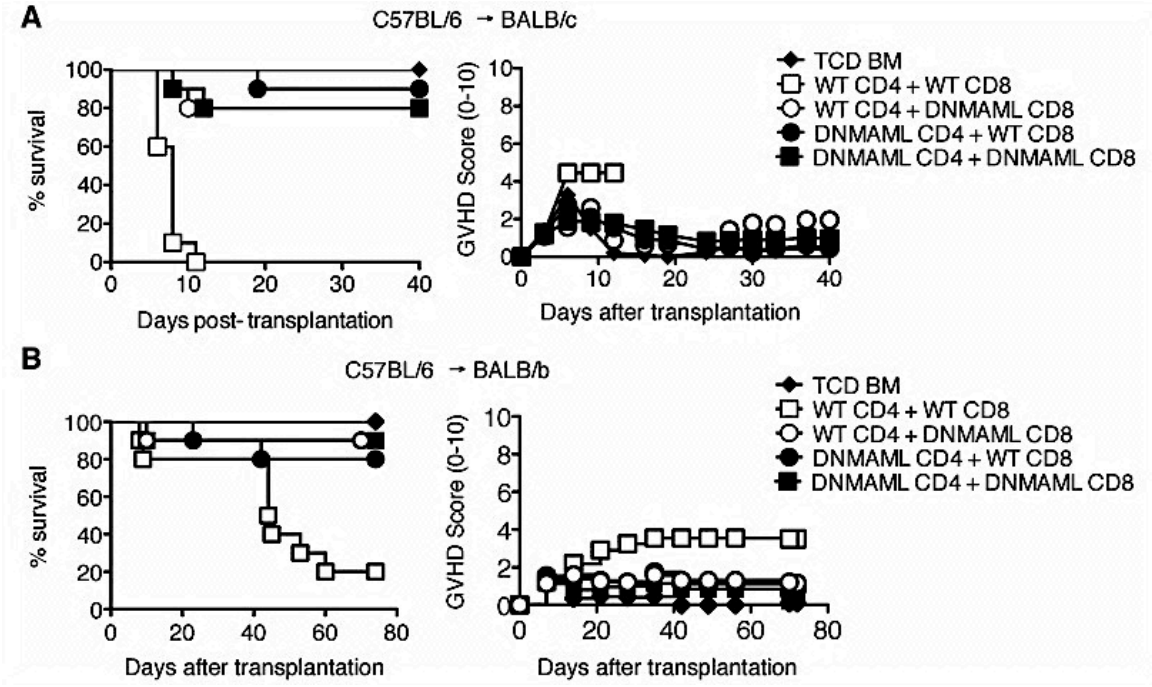

Figure 3.2 DNMAML-mediated Notch inhibition in CD4$^+$ and CD8$^+$ T cells protects against lethal GVHD in major and minor histocompatibility antigen-mismatched allo-BMT models.

(A) Lethally irradiated (900 rads) BALB/c (H-2^d) recipients received B6 T cell-depleted bone marrow (TCD BM, 5x10^6 cells) alone or with 0.4x10^6 CD4$^+$ and 2x10^6 CD8$^+$ T cells ($p<0.001$ for WT CD4$^+$ and CD8$^+$ vs. TCD; $p<0.001$ for WT CD4$^+$ and CD8$^+$ vs. DNMAML CD4$^+$ and CD8$^+$, WT CD4$^+$ and DNMAML CD8$^+$, DNMAML CD4$^+$ and WT CD8$^+$ survival); **(B)** Lethally irradiated BALB/b (H-2^b) recipients were transplanted with B6 TCD BM (5x10^6 cells) alone or with 2x10^6 CD4$^+$ and 2x10^6 CD8$^+$ T cells ($p<0.001$ for WT CD4$^+$/CD8$^+$ vs. TCD; $p<0.01$ for WT CD4$^+$/CD8$^+$ vs. DNMAML CD4$^+$/CD8$^+$ or WT CD4$^+$/DNMAML CD8$^+$; $p<0.05$ for WT CD4$^+$/CD8$^+$ vs. DNMAML CD4$^+$/WT CD8$^+$ survival). Recipients were monitored for survival and clinical GVHD score after transplantation (n=5-10/group, representative of 2 experiments).

3.3.2 Alloreactive DNMAML CD8$^+$ T cells display intrinsic and CD4$^+$ T cell-dependent defects in IFNγ production

Notch inhibition preserves CD4$^+$ T cell expansion after allo-BMT, but profoundly decreases IFNγ production.[223] Since CD8$^+$ T cells are a major source of IFNγ during GVHD, we assessed DNMAML CD8$^+$ T cell expansion and IFNγ production. After transplantation in the B6 anti-BALB/c model (**Fig. 3.3A**), we recovered similar numbers of donor-derived WT and DNMAML CD4$^+$ and CD8$^+$ T cells from lymphoid organs of allo-BMT recipients, indicating that Notch blockade did not prevent *in vivo* expansion of these cells **(Fig. 3.3B-C)**. In contrast, DNMAML expression markedly decreased IFNγ production by both alloreactive CD4$^+$ and CD8$^+$ T cells **(Fig. 3.3D)**, suggesting parallel

effects of Notch in these two subsets. These differences in IFNγ production could not be explained by increased apoptosis of Notch-deprived T cells during the restimulation period (data not shown). To determine if changes in IFNγ production by Notch-deprived alloreactive T cells were biologically relevant, serum IFNγ levels were measured on day 5 after transplantation **(Fig. 3.3E)**. Serum IFNγ levels were markedly reduced in recipients of DNMAML T cells. Since IFNγ production by $CD8^+$ T cells is influenced by cell-intrinsic signals and $CD4^+$ T cell help, we studied DNMAML $CD8^+$ T cells in the presence of WT or DNMAML alloreactive $CD4^+$ T cells (**Fig. 3.3F**). WT $CD4^+$ and $CD8^+$ T cells transplanted together produced abundant IFNγ. In the presence of WT $CD4^+$ T cells, DNMAML expression in $CD8^+$ T cells markedly decreased but did not abolish IFNγ production. When both DNMAML $CD4^+$ and $CD8^+$ T cells were infused, $CD8^+$ T cells had little to no IFNγ production. Thus, Notch promotes maximal IFNγ production by alloreactive $CD8^+$ T cells via cell-intrinsic changes in the $CD8^+$ compartment and effects on $CD4^+$ T cell help.

To verify that Notch signaling was potently inhibited in both alloreactive DNMAML $CD4^+$ and $CD8^+$ T cells, we assessed expression of the Notch target gene *Dtx1* **(Fig. 3.3G)**. *Dtx1* transcripts were significantly reduced in both subsets of alloreactive DNMAML T cells. Collectively, inhibition of Notch signaling in alloreactive T cells preserved their expansion but profoundly reduced T cell-dependent IFNγ production and systemic IFNγ levels.

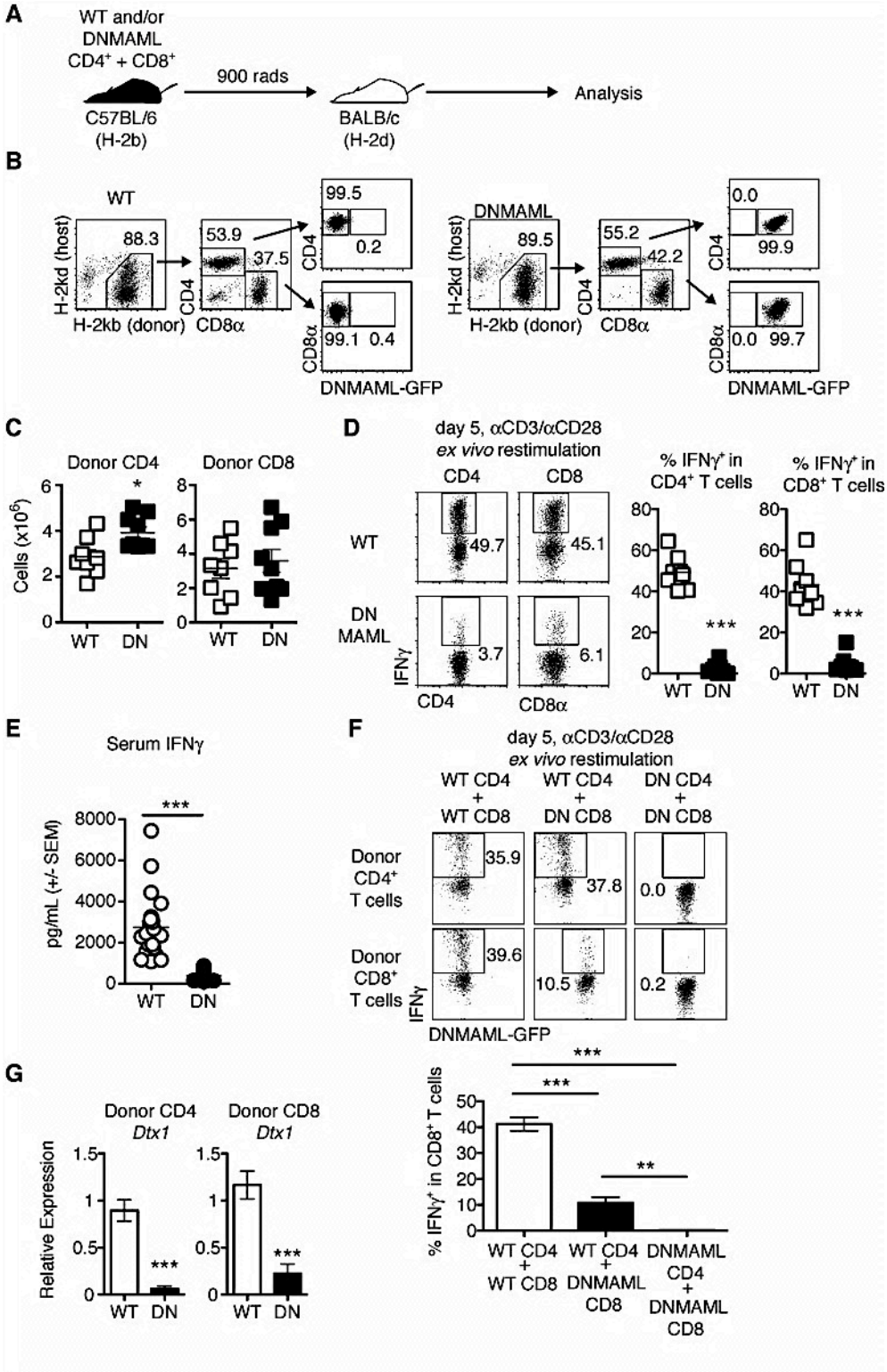

Figure 3.3 Alloreactive DNMAML CD4$^+$ and CD8$^+$ T cells have preserved *in vivo* expansion but markedly decreased IFNγ production.

(A) Experimental design: lethally irradiated BALB/c mice were transplanted with combinations of WT or DNMAML CD4$^+$ and CD8$^+$ B6 T cells; **(B)** Donor-derived T cells were tracked by flow

cytometry based on expression of donor/host MHC class I molecules and DNMAML-GFP; **(C)** Preserved expansion of donor-derived H-2Kb^{+}H-2Kd^{-} DNMAML CD4^{+} and CD8^{+} T cells as compared to WT T cells on day 5 post-transplantation (n=3-5 mice/group, 2 independent experiments); **(D)** Intracellular staining for IFNγ in donor-derived H-2Kb^{+}H-2Kd^{-} CD4^{+} and CD8^{+} spleen T cells after *ex vivo* anti-CD3/CD28 restimulation (n=3-5 mice/group, representative of >3 experiments). DNMAML inhibited IFNγ production by CD4^{+} and CD8^{+} T cells; **(E)** Serum was collected on day 5 after transplantation and IFNγ levels were measured by ELISA (n=5 mice/group, ≥2 independent experiments); **(F)** Mixed populations of WT or DNMAML CD4^{+} and CD8^{+} T cells (2x10^{6} each) were transplanted into lethally irradiated BALB/c recipients. IFNγ production was measured by intracellular flow cytometry on day 5 post-transplantation. DNMAML blocked IFNγ production in CD8^{+} T cells both through cell-intrinsic and CD4-dependent effects (n=3/group, representative of two experiments); **(G)** Abundance of *Dtx1* Notch target gene mRNA in sort-purified alloreactive CD4^{+} and CD8^{+} T cells on day 5 post-transplantation (n=3 mice/group, representative of >3 experiments). **p<0.01; ***p<0.001.

3.3.3 Notch inhibition blocks IFNγ production in alloreactive CD4^{+} and CD8^{+} T cells despite preserved T-bet and Eomesodermin expression.

Notch was suggested to control the expression of *Tbx21* (encoding T-bet) in CD4^{+} T cells and *Eomes* (encoding Eomesodermin) in CD8^{+} T cells.[117,125,126] To assess if these mechanisms accounted for decreased IFNγ production by Notch-deprived CD4^{+} and CD8^{+} T cells after allo-BMT, we measured T-bet and Eomesodermin levels at the peak of the effector response. Alloreactive DNMAML CD4^{+} and CD8^{+} T cells had preserved *Tbx21* and increased *Eomes* transcripts as compared to WT T cells **(Fig. 3.4A**). We used flow cytometry to assess intracellular T-bet and Eomesodermin and established antibody specificity by staining alloreactive T cells from WT mice compared to *Tbx21*$^{-/-}$ and *Eomes*$^{f/f}$ x *Cd4-Cre* mice **(Fig. 3.4B).**[108,226] Consistent with mRNA findings, alloreactive DNMAML CD4^{+} and CD8^{+} T cells had preserved intracellular T-bet and increased Eomesodermin **(Fig. 3.4C)**. As expected, we observed more Eomesodermin protein in CD8^{+} T cells than CD4^{+} T cells, consistent with accurate detection of Eomesodermin.[108] These data indicate that decreased IFNγ production by DNMAML CD4^{+} and CD8^{+} T cells was not caused by decreased Th1 and effector CD8^{+} T cell differentiation resulting from reduced expression of these master transcription factors.

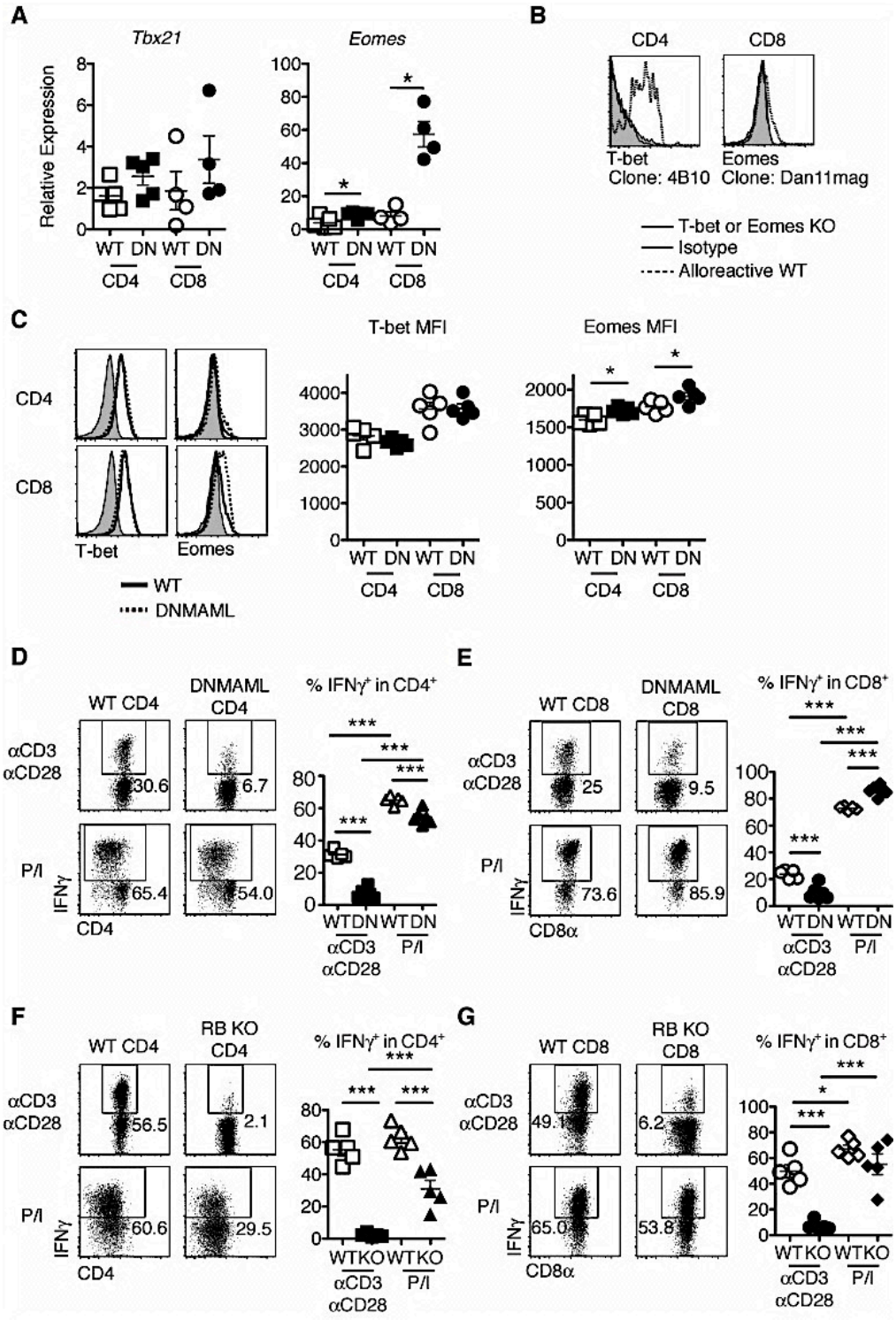

Figure 3.4 Preserved T-bet and enhanced Eomesodermin expression in alloreactive Notch-deprived CD4$^+$ and CD8$^+$ T cells, allowing restoration of IFNγ production after treatment with PMA and ionomycin.

Lethally irradiated BALB/c mice (900 rads) were transplanted with B6-CD45.1 TCD BM ($5x10^6$ cells) and splenocytes ($10x10^6$ cells) from WT or DNMAML B6 mice. **(A)** Preserved *Tbx21* mRNA (encoding T-bet) and enhanced *Eomes* transcripts in DNMAML CD4$^+$ and CD8$^+$ T cells.

Donor-derived H-2Kb^{+}H-2Kd^{-}CD45.2^{+} CD4^{+} and CD8^{+} T cells were sort-purified and subjected to qRT-PCR (day 14, n=4-5 mice, representative of 3 experiments); **(B)** Specific detection of T-bet and Eomesodermin in alloreactive T cells with anti-T-bet (4B10) and anti-Eomesodermin (Dan11mag) antibodies. Splenocytes from WT, B6.129S6-*Tbx21*^{tm1Glm}/J or *Eomes*$^{f/f}$*xCd4-Cre* mice were transplanted into irradiated BALB/c recipients. Histograms show intracellular staining with isotype control or specific antibodies in donor-derived H-2Kb^{+}H-2Kd^{-}CD45.2^{+} CD4^{+} or CD8^{+} T cells (day 5) (n=2); **(C)** Representative intracellular flow cytometry plots and mean fluorescence intensity (MFI) for T-bet and Eomesodermin expression in alloreactive WT and DNMAML CD4^{+} and CD8^{+} T cells (day 14, n=4-5 mice, representative of 4 experiments); **(D-G)** At day 5 after transplantation, spleen and lymph node cells were incubated for 6 hours with either anti-CD3/anti-CD28 (2.5 μg/ml each), or PMA and ionomycin (50 ng/ml and 500 ng/ml, respectively). Percent IFNγ^{+} cells as measured by intracellular flow cytometry in WT and DNMAML donor-derived **(D)** CD4^{+} and **(E)** CD8^{+} T cells, or WT and CSL/RBP-Jk-deficient donor-derived **(F)** CD4^{+} and **(G)** CD8^{+} T cells (n=5 mice/group, representative of $\geq$2 experiments). Representative flow cytometry plots are shown. Numbers indicate the percentage of cells in each quadrant. * $p<0.05$; *** $p<0.001$.

PMA (a diacylglycerol analog) and ionomycin (a calcium ionophore) are often used to elicit cytokine production to study T cell differentiation. This strategy can reveal defective IFNγ production by *Tbx21*$^{-/-}$ T cells.[227] In contrast, restimulation of alloreactive DNMAML T cells with PMA/ionomycin restored IFNγ production by both CD4^{+} **(Fig. 3.4D)** and CD8^{+} T cells **(Fig. 3.4E)** close to levels observed in WT T cells. Partial rescue of IL-2 production was also apparent (data not shown). We confirmed the effects of DNMAML-mediated Notch blockade on IFNγ production using *Rbpj*$^{-/-}$ T cells lacking CSL/RBP-Jκ, a central component of the Notch transcriptional activation complex (**Fig. 3.4F-G**). Altogether, our observations were consistent with the presence of functional T-bet and Eomesodermin capable of activating *Ifng* transcription in DNMAML or CSL/RBP-Jκ-deficient alloreactive T cells, suggesting that PMA/ionomycin restored activation of other pathways regulating cytokine production in these cells.

3.3.4 Notch-deprived alloreactive CD4^{+} and CD8^{+} T cells develop blunted Ras/MAPK and NF-κB activation

One of the major pathways activated by PMA is Ras/MAPK signaling, a key contributor to T cell cytokine gene transcription.[228] To assess if alloreactive DNMAML T cells had decreased Ras/MAPK activation, we measured phosphorylation of Erk1/2 and its upstream kinase Mek1. Notch-deprived CD4^{+} and CD8^{+} T cells were primed *in vivo* before sort-purification and *ex vivo* restimulation through the TCR and CD28 co-receptor.

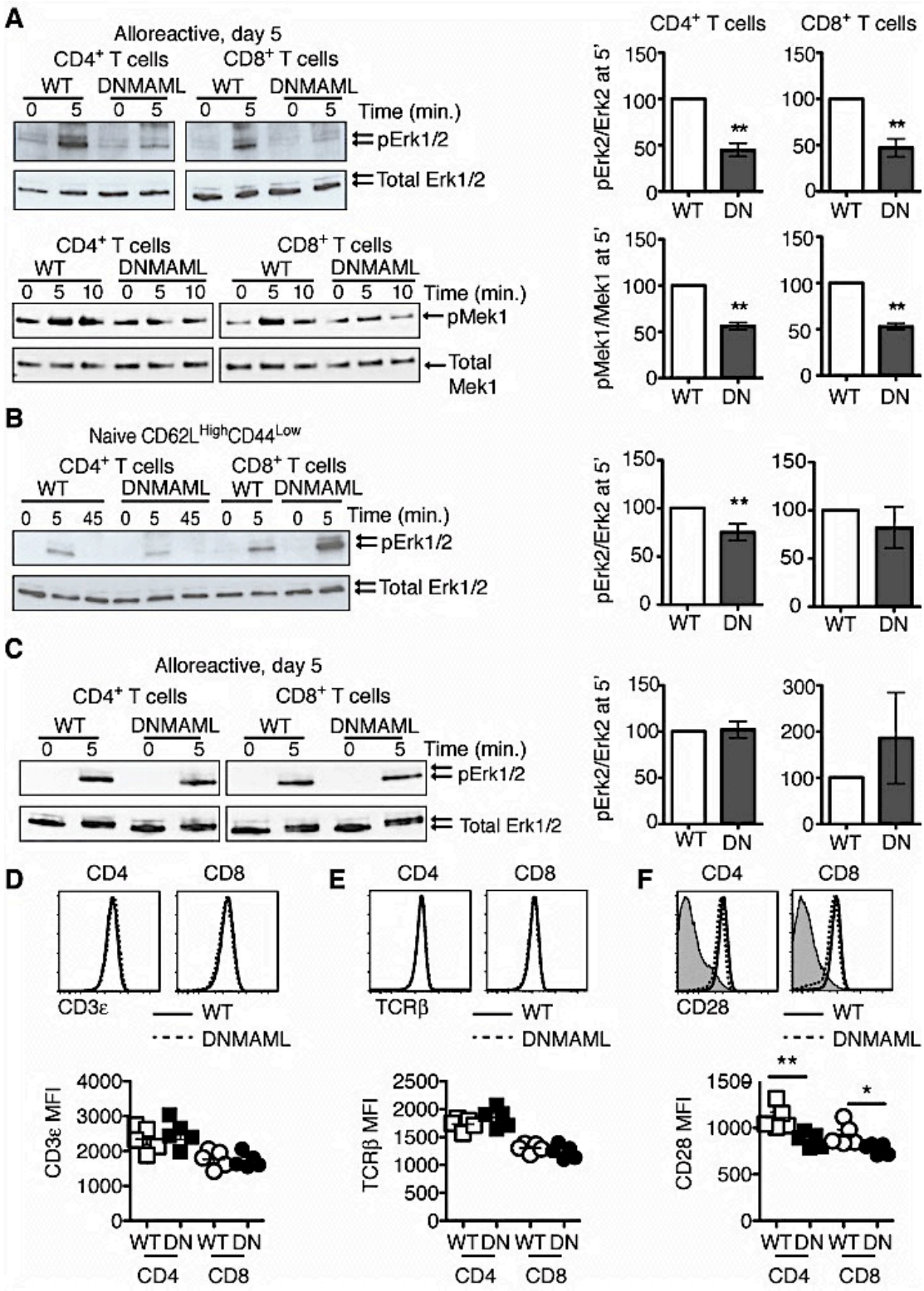

Figure 3.5 Alloreactive Notch-deprived CD4$^+$ and CD8$^+$ T cells have an acquired defect in Ras/MAPK pathway activation that is rescued by PMA.

(A) WT or DNMAML B6 splenocytes were transplanted into lethally irradiated BALB/c recipients. On day 5, H-2Kb$^+$H-2Kd$^-$ alloreactive WT and DNMAML CD4$^+$ and CD8$^+$ T cells

were sort-purified and restimulated *in vitro* for 5-10 minutes at 37°C with anti-CD3/anti-CD28 and IgG crosslinking. Baseline activation was assessed by incubating cells at 37°C with IgG crosslinker alone (0 minute time point). Phosphorylated Erk1/2 and Mek1 were detected by Western blotting as compared to total Erk1/2 and Mek1; **(B)** Naïve $CD62L^{High}CD44^{Low}$ $CD4^+$ and $CD8^+$ T cells were sort-purified from WT and DNMAML mice. Mek1 phosphorylation was assessed after anti-CD3/CD28 restimulation; **(C)** Sort-purified, day 5 alloreactive WT and DNMAML $CD4^+$ or $CD8^+$ T cells were restimulated *ex vivo* with PMA for 5 minutes (or DMSO as negative control). In all experiments, the abundance of phosphorylated proteins was measured by densitometry relative to total protein levels. WT T cells were set to 100% (n=2-4 individual experiments, 6 mice/group in each experiment); **(D)** cell surface CD3ε, **(E)** TCRβ, and **(F)** CD28 levels were assessed in alloreactive WT and DNMAML $CD4^+$ and $CD8^+$ T cells on day 5 post-transplantation. Representative flow cytometry plots and mean fluorescence intensity are shown. **p<0.01.

Both alloreactive DNMAML $CD4^+$ and $CD8^+$ T cells had a significant reduction in Mek1 and Erk1/2 phosphorylation **(Fig. 3.5A)**. Interestingly, naïve $CD62L^{High}CD44^{Low}$ DNMAML $CD4^+$ T cells only had slightly decreased Erk1/2 phosphorylation, while naïve DNMAML $CD8^+$ T cells activated Erk1/2 normally, suggesting that the majority of the Ras/MAPK defect was acquired *in vivo* in the absence of Notch signaling **(Fig. 3.5B)**. Next, we assessed if PMA rescued Ras/MAPK activation in DNMAML alloreactive T cells. PMA induced similar Erk2 phosphorylation in WT and DNMAML T cells, indicating restoration of Ras/MAPK signaling in DNMAML T cells **(Fig. 3.5C)**.

NF-κB is another major pathway activated downstream of diacylglycerol that can regulate *Ifng* transcription in T cells.[229] To capture the overall NF-κB activity in Notch-deprived alloreactive T cells, we bred DNMAML mice to transgenic NF-κB-GFP-Luciferase (NGL) reporter mice and used F1 progeny as donors for allo-BMT **(Fig. 3.6)**.[230] On day 5 post-transplantation, alloreactive NGL and NGL/DNMAML $CD4^+$ and $CD8^+$ T cells were sort-purified and restimulated with anti-CD3/CD28 antibodies. DNMAML $CD4^+$ and $CD8^+$ T cells had significantly reduced NF-κB/luciferase activity. Blunted signal transduction downstream of the TCR occurred in the absence of any changes in TCRβ **(Fig. 3.5D)** or CD3ε **(Fig. 3.5E)** surface expression, although we observed a slight but significant decrease in CD28 expression **(Fig. 3.5F)**. Altogether, both DNMAML $CD4^+$ and $CD8^+$ alloreactive T cells acquired a blunted capacity for Ras/MAPK and NF-κB activation downstream of TCR/CD28 signals after allo-BMT.

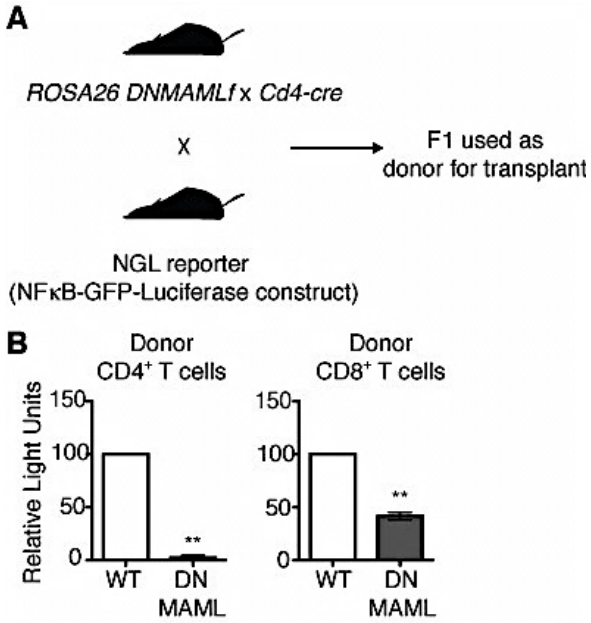

Figure 3.6 Reduced NF-kB activity in Notch-deprived alloreactive CD4$^+$ and CD8$^+$ T cells.

(A) Experimental design. *ROSA26^{DNMAMLf}* x *Cd4-Cre* mice were bred with NF-kB-GFP-Luciferase (NGL) reporter mice. F1 progeny (NGL or NGL/DNMAML) were used as donors for allo-BMT into irradiated BALB/c mice; **(B)** Alloreactive NGL and NGL/DNMAML CD4$^+$ and CD8$^+$ T cells were sort-purified on day 5 after transplantation. Purified CD4$^+$ and CD8$^+$ T cells were restimulated *in vitro* with anti-CD3/anti-CD28 for 16 hours to achieve detectable luciferase activity. NGL/DNMAML CD4$^+$ and CD8$^+$ T cells had significantly reduced luciferase activity (relative light units) as a surrogate for NF-kB activity compared to WT T cells (set to 100%; n=2-3 individual experiments, 6 mice/group in each experiment). **p<0.01.

3.3.5 Alloreactive DNMAML CD4$^+$ and CD8$^+$ T cells have increased expression of multiple negative regulators of T cell activation

Decreased Ras/MAPK signaling leading to reduced cytokine production has been described in certain forms of T cell hyporesponsiveness or anergy.[231,232] Mechanistically, increased expression of diacylglycerol kinases (Dgk) such as Dgkα and Dgkζ was linked to increased degradation of diacylglycerol into phosphatidic acid, resulting in blunted Erk1/Erk2 activation and cytokine production.[233,234] Interestingly, restimulated alloreactive DNMAML CD4$^+$ T cells had elevated levels of *Dgka* and *Dgkz* mRNA, while DNMAML CD8$^+$ T cells had increased *Dgka* and a trend for more *Dgkz* transcripts **(Fig. 3.7A)**. This constellation of effects was reminiscent of T cell anergy. Therefore, we studied a panel of anergy-associated genes, starting with the NFAT-dependent genes, *Egr2/3*.[235,236] *Egr3* but not *Egr2* transcripts were increased in alloreactive DNMAML

$CD4^+$ and $CD8^+$ T cells **(Fig. 3.7B)**. We also observed increased expression of *Rnf128* and *Cblb,* encoding Grail and Cbl-b, two E3 ubiquitin ligases that function as negative regulators of T cell activation.[237,238] In contrast, *Itch* expression was not significantly changed (**Fig. 3.7C**). As co-inhibitory receptors can also regulate alloreactive T cell activation and function, we investigated expression of *Btla*, *Ctla4* and *Pdcd1* (encoding Pd-1) in DNMAML T cells. Although *Ctla4* expression was slightly decreased, alloreactive DNMAML $CD4^+$ T cells had increased *Btla* and DNMAML $CD8^+$ T cells elevated *Pdcd1* mRNA **(Fig. 3.7D)**. These mRNA changes correlated well with protein expression with decreased Ctla-4 and increased Btla and Pd-1 in alloreactive DNMAML $CD4^+$ and $CD8^+$ T cells **(Fig. 3.7E)**. Finally, DNMAML alloreactive T cells had increased intracellular cAMP, a second messenger that provides negative feedback regulation of T cell activation (**Fig. 3.7F**).[239]

Altogether, Notch-deprived alloreactive $CD4^+$ and $CD8^+$ T cells acquired features of hyporesponsive T cells, including increased expression of several negative regulators of T cell activation, some of which are NFAT-dependent. Importantly, naïve DNMAML $CD4^+$ and $CD8^+$ T cells expressed normal levels of these negative regulators (except for *Cblb* and *Itch* which were mildly elevated in naïve DNMAML $CD4^+$ T cells) (**Fig. 3.8**). These findings suggest that Notch deprivation results in changes in naïve T cells that only become apparent after allo-BMT or that Notch inhibition has minimal influence on naïve T cells, but profound effects on T cells *in vivo* during allo-BMT.

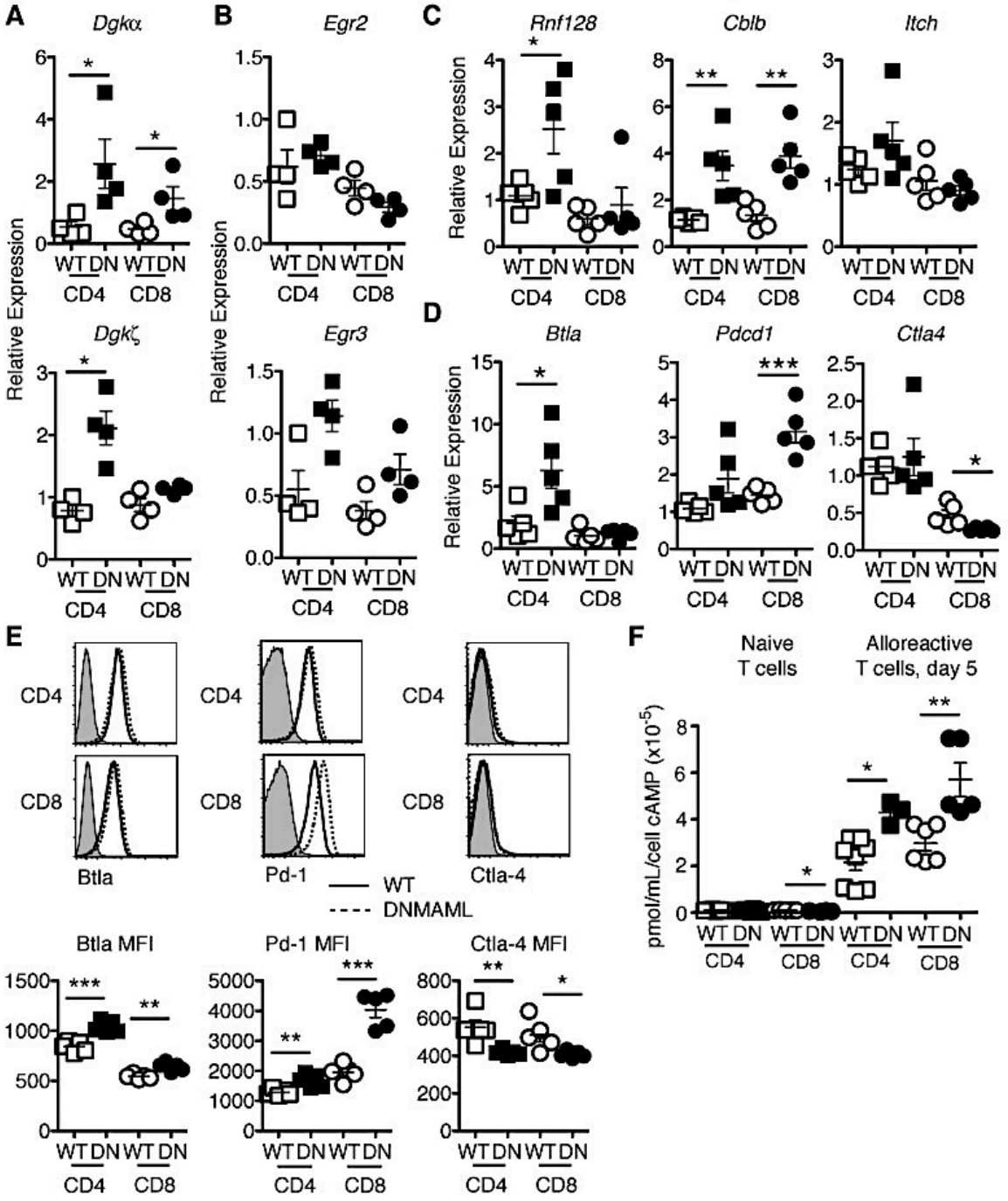

Figure 3.7 Increased levels of *Dgka* mRNA and other negative regulators of T cell activation in alloreactive DNMAML CD4$^+$ and CD8$^+$ T cells.

H-2Kb$^+$H-2Kd$^-$CD45.2$^+$alloreactive WT or DNMAML T cells were sort-purified at day 5 after transplantation. **(A)** Abundance of *Dgka/Dgkz* and **(B)** *Egr2/Egr3* transcripts in anti-CD3/anti-CD28 restimulated alloreactive WT or DNMAML CD4$^+$ and CD8$^+$ T cells (n=4 mice, representative of >2 experiments); **(C)** Expression of anergy-associated E3 ubiquitin ligases *Cblb, Rnf128,* and *Itch* and **(D)** expression of T cell co-inhibitory receptors *Btla, Ctla4, Pd-1* in sort-purified WT or DNMAML CD4$^+$ and CD8$^+$ T cells. Each symbol represents triplicate qRT-PCR data for cells purified from one individual recipient (n=5 mice, representative of 2 experiments);

(E) Cell surface levels of T cell co-inhibitory receptors in WT and DNMAML $CD4^+$ and $CD8^+$ T cells (n=5 mice/group, representative of 2 experiments). Representative flow cytometry plots and mean fluorescence intensity are shown; **(F)** Increased intracellular cAMP in alloreactive DNMAML $CD4^+$ and $CD8^+$ T cells relative to WT T cells on day 5 after transplantation. cAMP levels were very low in naïve cells irrespectively of DNMAML expression (n=3 experiments, 6 mice/group in each experiment). *p<0.05; **p<0.01; ***p<0.001.

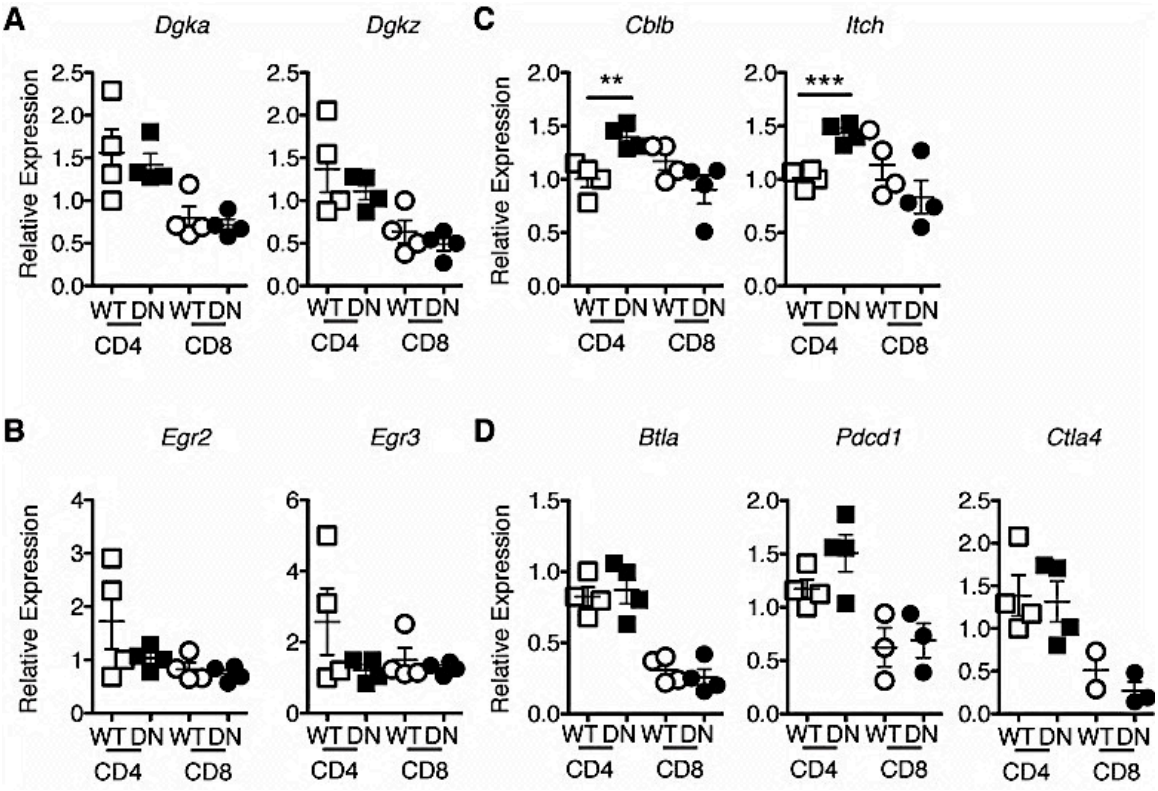

Figure 3.8 Expression of anergy-associated transcripts in naive DNMAML $CD4^+$ and $CD8^+$ T cells.

(A) Abundance of *Dgka*/*Dgkz* and **(B)** *Egr2*/*Egr3* transcripts in sort-purified, anti-CD3/anti-CD28 restimulated naïve ($CD62L^{High}CD44^{Low}$) DNMAML $CD4^+$ and CD8 T cells relative to WT T cells (n=4 mice); **(C)** Expression of E3 ubiquitin ligases associated with T cell anergy, *Cblb* and *Itch*. *Rnf128* was undetected (data not shown); **(D)** expression of the T cell co-inhibitory receptors *Btla, Ctla4* and *Pdcd1* in naïve DNMAML $CD4^+$ and $CD8^+$ T cells compared to WT T cells (n=5 mice, representative of 2 experiments). **p<0.01; ***p<0.001.

3.3.6 Notch inhibition modestly reduces *in vivo* proliferation of alloreactive $CD4^+$ and $CD8^+$ T cells while enhancing expansion of natural Tregs

DNMAML alloreactive T cells acquire increased levels of negative regulators that have previously been associated with decreased proliferation, at least using *in vitro* models of T cell anergy.[240] However, *in vitro* and *in vivo* T cell proliferation are regulated

by different stimuli. To evaluate in detail the impact of Notch blockade on T cell proliferation *in vivo* after allo-BMT, we used *Rbpj*$^{f/f}$ x *Cd4-Cre* mice (**Fig. 3.9**). As DNMAML T cells, CSL/RBP-Jκ-deficient T cells fail to respond to Notch signals, but lack DNMAML-GFP fluorescence, allowing use of CFSE to track proliferation. At day 3 after allo-BMT, Notch-deficient T cells had proliferated slightly less than WT T cells **(Fig. 3.9A)**. However, by day 5, >99% of donor WT and CSL/RBP-Jκ-deficient T cells had proliferated for >6-8 divisions **(Fig. 3.9B)**. Thus, despite modestly reduced initial proliferation in the absence of Notch signaling, Notch-deprived T cells accumulated to levels similar to WT T cells by day 5 after allo-BMT. After this initial burst, *ex vivo* restimulation of Notch-deprived alloreactive T cells revealed only modest cycling defects (**Fig. 3.9C-D**). Similar *in vivo* and *in vitro* results were observed when DNMAML T cells were labeled with eFluor670 (data not shown). To assess ongoing *in vivo* proliferation, we performed a BrdU pulse-chase experiment 5-8 days after allo-BMT (**Fig. 3.9E-G**). CSL/RBP-Jκ-deficient T cells demonstrated slightly reduced initial BrdU incorporation during the pulse phase (**Fig. 3.9F**). During chase, decreased loss of BrdU was apparent in Notch-deprived T cells (**Fig. 3.9G**). Thus, Notch inhibition modestly reduced *in vivo* proliferation of alloreactive $CD4^+$ and $CD8^+$ T cells, while preserving their massive initial expansion.

Traditional immunosuppressants often prevent Treg expansion, which can increase GVHD severity.[241] If inhibition of Notch signaling is to be used to prevent or treat GVHD, we needed to assess the impact of Notch deprivation on Treg expansion. Using *Foxp3-IRES-RFP*$^+$ DNMAML mice, we observed increased accumulation of DNMAML as compared to WT $Foxp3^+$ donor T cells (**Fig. 3.10A**).[242] Furthermore, depletion of Tregs from the donor DNMAML inoculum completely prevented Treg expansion (**Fig. 3.10B-D**). These data indicate that rather than increasing conversion to induced Tregs, Notch blockade enhanced expansion of preexisting natural Tregs after allo-BMT.

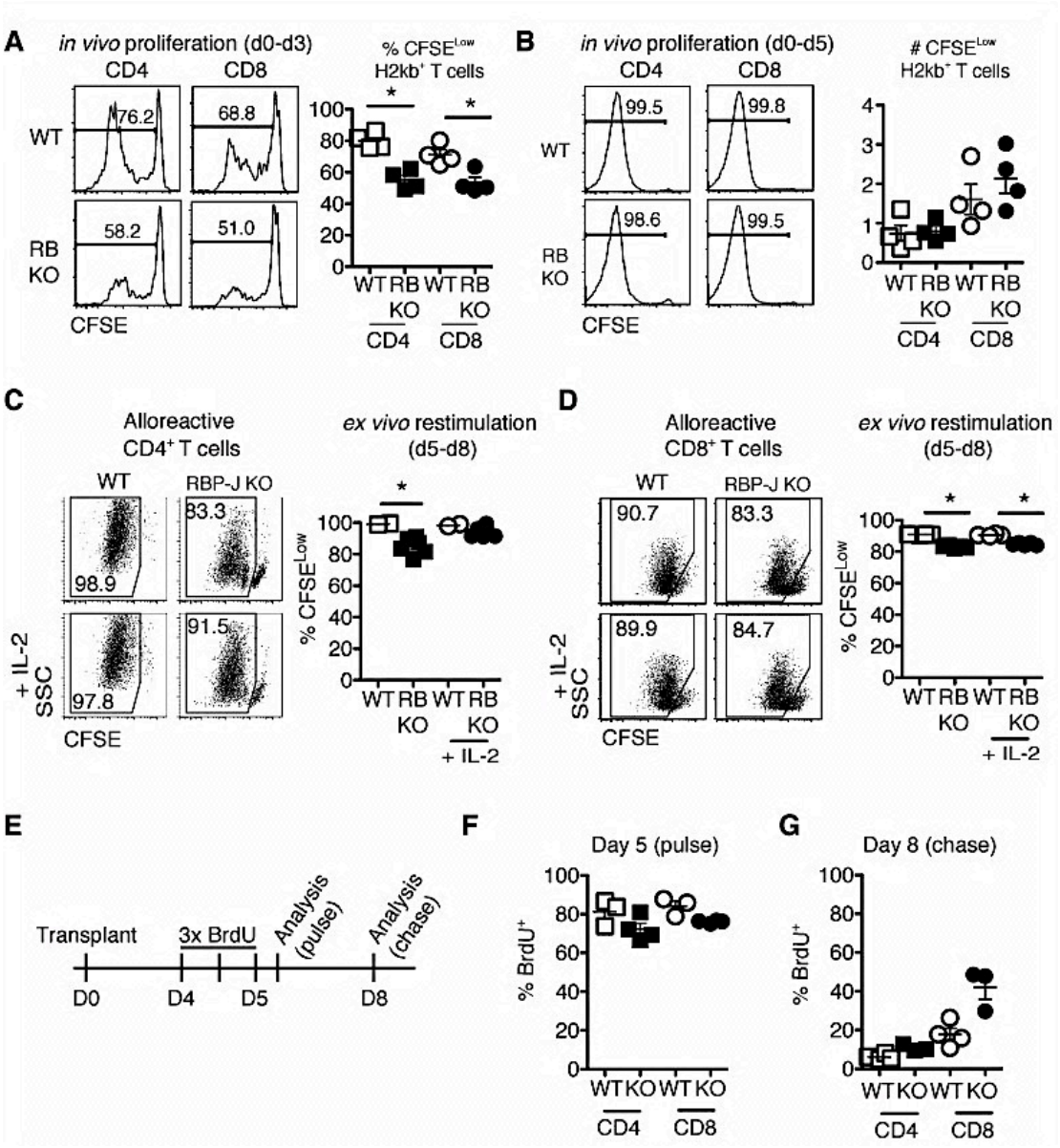

Figure 3.9 Notch-deprived alloreactive CD4$^+$ and CD8$^+$ T cells have a reduced initial proliferative burst and subsequent decreased proliferation *in vitro* and *in vivo*.
CFSE-labeled T cells from WT or *Rbpj$^{f/f}$ x Cd4-Cre* mice (RB KO, lacking all CSL/RBP-Jk-mediated Notch signals) were transplanted into lethally irradiated BALB/c recipients (900 rads). Flow cytometry plots show CFSE dilution at **(A)** day 3 and **(B)** day 5 in donor-derived H-2Kb$^+$H-2Kd$^-$ CD4$^+$ and CD8$^+$ T cells. WT or RBP-Jk KO T cells were transplanted into lethally irradiated BALB/c recipients. On day 5, purified WT and RBP-Jk KO **(C)** CD4$^+$ or **(D)** CD8$^+$ T cells were CFSE-labeled and restimulated *in vitro* for 3 days with anti-CD3/CD28 +/- IL-2. Division history of donor-derived H-2Kb$^+$H-2Kd$^-$ B6 T cells was measured by flow cytometry (n=6-7 mice/group in each experiment, representative of >2 experiments); **(E)** Design of BrdU pulse-chase experiment. Lethally irradiated BALB/c recipients were transplanted with splenocytes from WT or RBP-Jk KO mice. Between days 4 and 5, transplant recipients received 3 doses of BrdU 12 hours apart; **(F)** Four hours after the last BrdU injection, mice were euthanized for day 5 BrdU incorporation analysis (pulse); **(G)** At day 8 after transplantation, residual BrdU

content was assessed (chase). n=5 mice/group in each experiment, representative of 2 experiments. *p<0.05

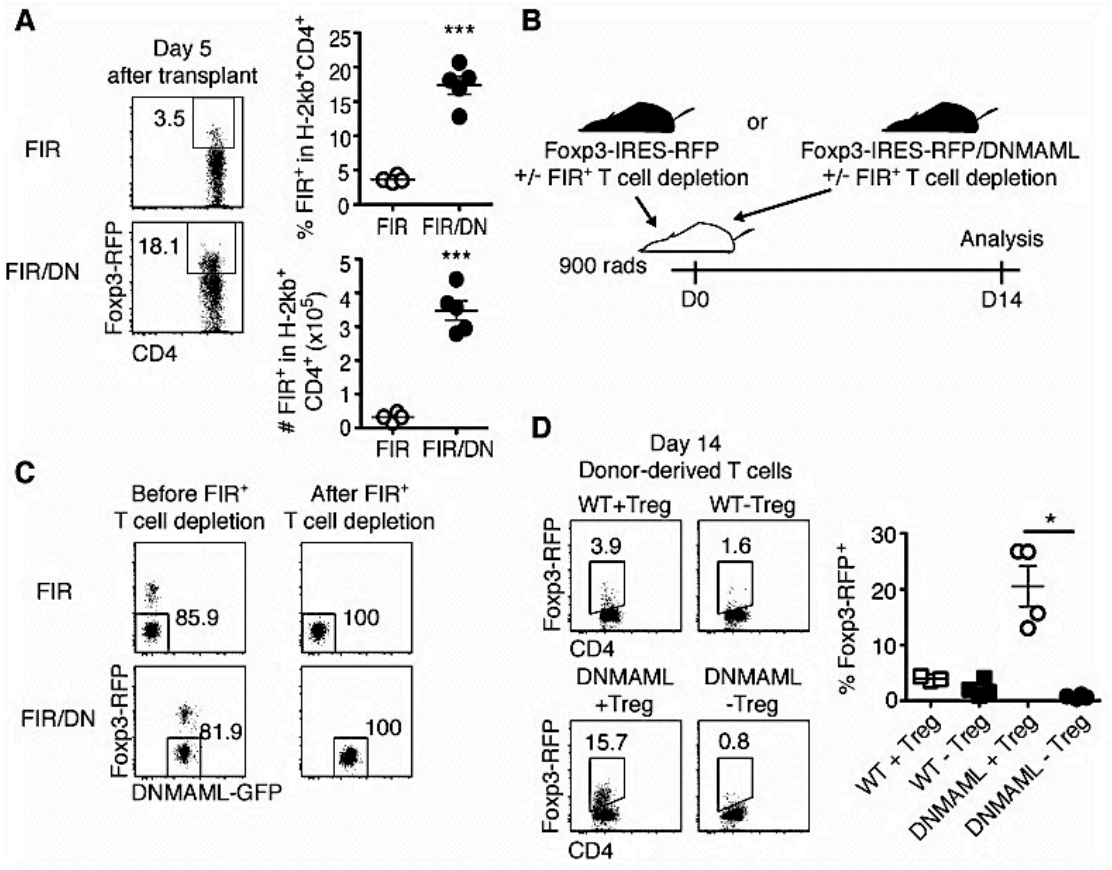

Figure 3.10 Notch inhibition enhances the accumulation of natural Foxp3$^+$ regulatory T cells after allo-BMT.
(A) Detection of live regulatory T cell (Tregs) on day 5 post-transplantation using Foxp3-IRES-RFP (FIR) mice crossed to *ROSA26^{DNMAMLf} x Cd4-Cre* mice, showing expanded Tregs among Notch-deprived alloreactive T cells; **(B)** To determine the origin of the expanded Tregs, lethally irradiated BALB/c recipients were transplanted with WT or DNMAML CD4$^+$ T cells with or without FIR$^+$ Tregs and analyzed on day 14 post-transplantation; **(C)** Post-sort purity of WT and DNMAML CD4$^+$ T cell fractions after depletion of FIR$^+$ cells; **(D)** Frequency of donor-derived FoxP3-IRES-RFP$^+$ T cells as assessed by flow cytometry after transplantation of WT or DNMAML CD4$^+$ T cells, including Tregs (+Treg), or depleted of Tregs (-Treg). Day 14 alloreactive DNMAML Tregs were derived from preexisting Tregs. *p<0.05; *** p<0.001.

3.3.7 Preserved anti-host and anti-tumor cytotoxicity of alloreactive Notch-deprived CD8$^+$ T cells

Our previous work showed that alloreactive Notch-deprived CD4$^+$ T cells had preserved cytotoxicity against allogeneic host and tumor cells after transplantation.[223] To test the overall *in vivo* cytotoxic potential of DNMAML B6 CD4$^+$ and CD8$^+$ T cells,

BALB/c transplant recipients were challenged with a 1:1 mix of $CFSE^{High}$ BALB/c and $CFSE^{Low}$ B6-CD45.1 splenocytes. Cytotoxicity against BALB/c targets was measured by flow cytometry **(Fig. 3.11A).** While recipients receiving TCD BM could not elicit cytotoxicity against the BALB/c targets, mice receiving WT or DNMAML splenocytes showed high and similar cytotoxicity against $CFSE^{High}$ BALB/c targets **(Fig. 3.11A)**.

To determine if alloreactive purified DNMAML $CD8^+$ T cells could lyse allogeneic tumor cells, an *in vitro* cytotoxicity assay was used. T cells were primed *in vivo* in lethally irradiated BALB/c recipients before assessing cytotoxicity *ex vivo*. Incubation of alloreactive WT or DNMAML $CD8^+$ T cells with ^{51}Chromium-labeled allogeneic A20 and P815 ($H2kd^+$) tumor cells showed efficient cytotoxicity, with preserved cytotoxicity against A20 cells and preserved or only slightly reduced cytotoxicity against P815 cells **(Fig. 3.11B).** As expected, syngeneic EL4 ($H2kb^+$) tumor cells were not killed **(Fig. 3.11B)**. Collectively, these data demonstrate that Notch inhibition in alloreactive $CD8^+$ T cells preserved a high degree of cytotoxic potential after transplantation.

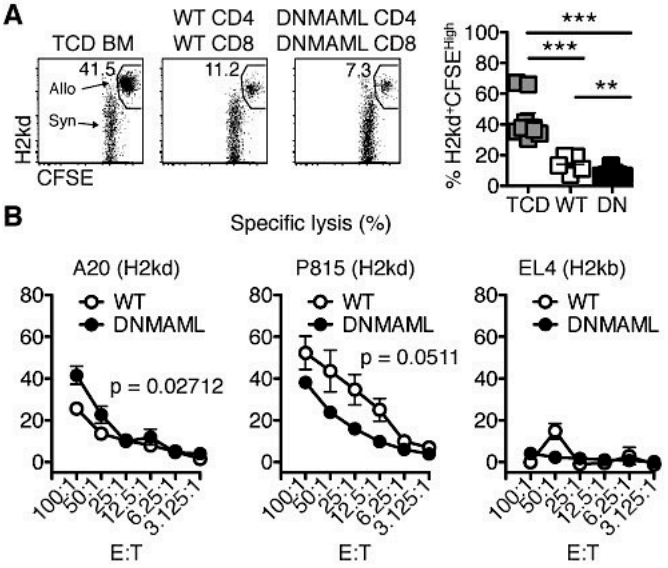

Figure 3.11 Alloreactive Notch-deprived $CD8^+$ T cells maintain high cytotoxic potential.

(A) *In vivo* cytotoxicity assay. Transplanted BALB/c recipient mice were challenged on day 14 with a 1:1 mixture of CFSE-labeled allogeneic targets and control cells ($CFSE^{High}$ H-$2Kd^+$ BALB/c and $CFSE^{low}$ control H-$2Kb^+$ B6-CD45.1 splenocytes, respectively). After 18 hours, elimination of the BALB/c targets was assessed in the spleen by flow cytometry; **(B)** BALB/c recipients were transplanted with TCD BM and WT or DNMAML splenocytes ($5x10^6$ each). On day 8, alloreactive WT and DNMAML and naïve $CD8^+$ T cells were MACS-purified and

incubated with ^{51}Chromium-labeled tumor cells at various E:T ratios for 5 hours (representative of n=3 independent experiments). A20 and P815 cells were allogeneic targets (H2kd). EL4 cells were syngeneic controls (H2kb). **p<0.01; ***p<0.001.

3.4 Discussion

Our findings highlight a new, shared mechanism of Notch action in alloreactive $CD4^+$ and $CD8^+$ T cells that differs from all previously reported Notch functions in T cells **(Table 3.1)**. We used pan-Notch inhibition specifically in T cells to study the effects of Notch signaling in $CD4^+$ and $CD8^+$ T cell differentiation and function in several models of acute GVHD. Notch was absolutely required in both $CD4^+$ and $CD8^+$ T cells to mediate lethal acute GVHD. Notch inhibition preserved *in vivo* T cell expansion, but led to profoundly decreased IFNγ production by both T cell subsets. Decreased IFNγ production was not explained by an overall defect in Th1 $CD4^+$ or effector $CD8^+$ T cell differentiation, as expression of the master transcription factors T-bet and Eomesodermin was preserved or even enhanced in the absence of Notch signaling. In contrast, analysis of signal transduction pathways downstream of the TCR revealed defects in Ras/MAPK and NF-κB activation in Notch-deprived T cells, in addition to increased expression of multiple negative regulators of T cell activation **(Fig. 3.12)**. These features of hyporesponsiveness were observed *in vivo* upon T cell activation without Notch signaling. Importantly, Notch inhibition preserved the overall expansion of alloreactive T cells and efficient cytotoxic potential, while leading to increased accumulation of preexisting natural $Foxp3^+$ Tregs. This constellation of effects led to beneficial immunomodulation, indicating that Notch is an attractive therapeutic target to control GVHD after allo-BMT.

Table 3.1 Summary of biochemical features of alloreactive Notch-deprived T cells.

	Wild-type $CD4^+$ and $CD8^+$ T cells	Notch-deprived $CD4^+$ and $CD8^+$ T cells
IFNγ production	+ + +	+/-
T-bet expression	+ + +	+ + +
Eomesodermin expression	+ + +	+ + + +
Ras/MAPK activity	+ + +	+
NFκB activity	+ + +	+
Negative regulators of T cell activation	+	+ + +
NF-AT target genes	+	+ +

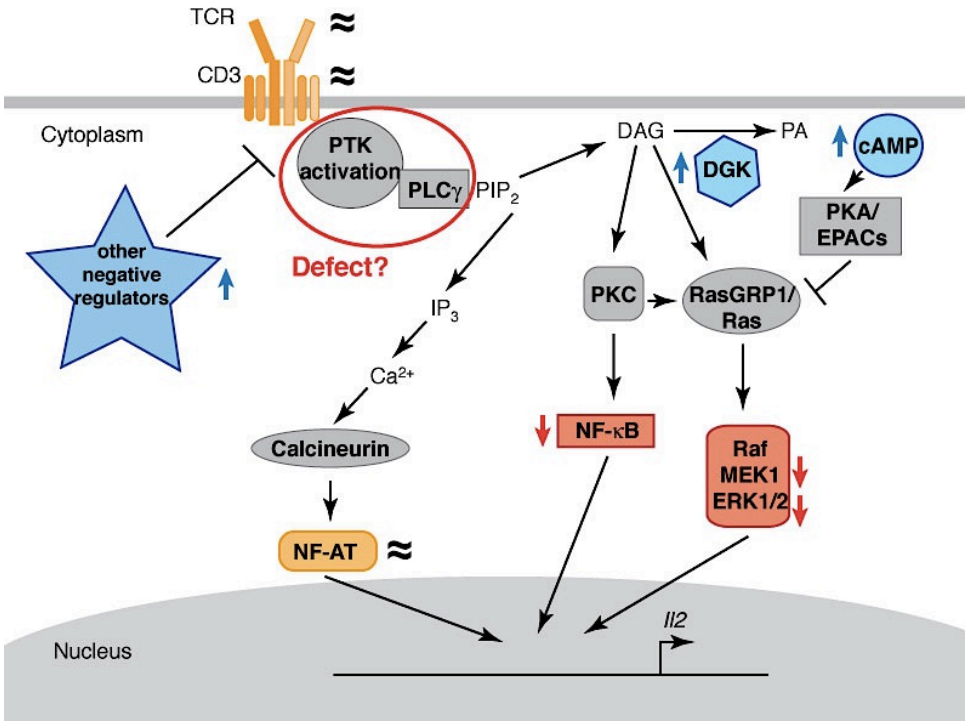

Figure 3.12 Summary of biochemical changes in Notch-deprived alloreactive T cells.

Alloreactive Notch-deprived T cells had decreased Ras/MAPK and NF-κB pathway activity concomitant with increased *Dgka* and *Dgkz* mRNA. Other negative regulators of T cell receptor signaling were also elevated in Notch-deprived T cells, such as cAMP, *Pdcd1* (encoding PD-1), *Cblb,* and *Rnf128* (encoding Grail). Based on expression analysis of NF-AT target genes, NF-AT was seemingly preserved. TCRβ and CD3ε expression levels were also preserved. These data suggest that Notch-deprived alloreactive T cells acquire a predominant defect downstream of the TCR but upstream of DAG. ↓, decreased activity/expression; ↑, increased activity/expression; ≈, preserved activity/expression

Past work in other immune contexts suggested that Notch could directly regulate *Tbx21* transcription in $CD4^+$ T cells and *Eomes* expression in $CD8^+$ T cells.[117,125,126] In contrast, Notch appeared dispensable for transcriptional activation of these genes in alloreactive T cells during GVHD (**Fig. 3.4A-C**).[223] It is possible that use of different experimental systems to manipulate Notch signaling could underlie these discrepant observations. Others reported that Notch receptors could have "non-canonical" effects independent of CSL/RBP-Jκ and MAML, although the nature of these effects is not well defined.[49,50] However, in alloreactive T cells at least, the dominant effects of Notch are mediated by "canonical" signaling and do not involve decreased *Tbx21* and *Eomes* expression. Alternatively, different signaling pathways may be required to activate *Tbx21* and *Eomes* transcription in distinct immune responses. After allo-BMT, T cell exposure to abundant alloantigens in a highly inflammatory environment may bypass the requirement for Notch signaling to activate these genes. Moreover, an interesting feature of Notch-deprived alloreactive T cells was increased *Eomes* expression, a finding that was particularly apparent in $CD8^+$ T cells but also detected in the $CD4^+$ compartment. Although the regulation of *Eomes* expression during T cell differentiation is incompletely understood, decreased IL-12 signaling and increased Foxo1 activity were reported to enhance *Eomes* at the expense of *Tbx21* expression.[243,244] The effects of Notch deprivation on *Eomes* could be mediated via interference with these pathways. Interestingly, increased *Eomes* expression was reported in memory $CD8^+$ T cells that acquire an enhanced ability for long-term persistence, as opposed to terminal differentiation.[245] This mechanism could contribute to the enhanced expansion and survival of Notch-deprived alloreactive T cells after allo-BMT.

In contrast to past findings suggesting that Notch regulates independent aspects of $CD4^+$ and $CD8^+$ T cell biology, our results revealed similar effects of Notch in the $CD4^+$ and $CD8^+$ T cell compartments during GVHD. Notch-deprived $CD4^+$ and $CD8^+$ T cells shared key functional properties and gene expression changes after allo-BMT. During *in vivo* activation, DNMAML alloreactive T cells acquired elevated levels of several negative regulators of T cell activation, including genes whose activation is NFAT-dependent **(Fig. 3.12)**. A prominent feature of Notch-deprived T cells was decreased activation of Ras/MAPK and NF-κB pathways. Ras/MAPK and NF-κB activation are

controlled by diacylglycerol, whose levels are negatively regulated by the lipid kinases Dgkα/ζ. Elevated *Dgka* and *Dgkz* expression in DNMAML T cells is reminiscent of past observations in models of T cell hyporesponsiveness with decreased Ras/MAPK activation.[233,234,240] However, DNMAML T cells also acquired elevated expression of other negative regulators of T cell activation, including E3 ubiquitin ligases and co-inhibitory receptors, in addition to increased intracellular cAMP. Thus, the functional properties of Notch-deprived alloreactive T cells may not be explained entirely by decreased Ras-MAPK and NF-κB activation. Altogether, our data suggest that the predominant defect of Notch-deprived alloreactive T cells lied downstream of the TCR complex but upstream of diacylglycerol **(Fig. 3.12).**

Despite the profound effects of Notch inhibition on cytokine production and a slight reduction in their initial proliferative burst, the overall accumulation of DNMAML T cells was preserved *in vivo*. These findings contrast with many other interventions that decrease expansion of alloreactive T cells *in vivo*. Interestingly, past work suggested that *in vivo* proliferation of alloreactive T cells was independent of IL-2 and may be controlled by IL-15, at least in MHC-mismatched allo-BMT models.[219] Furthermore, classical models of T cell hyporesponsiveness and anergy have often been examined *in vitro*, a situation in which IL-2 production may play a critical role to support proliferation that does not reflect its effects *in vivo*.[240] Other possible mechanisms accounting for the preserved or even enhanced *in vivo* expansion of DNMAML alloreactive T cells include decreased activation-induced cell death, for example as a result of reduced exposure to IFNγ.[246] In parallel to these effects on conventional alloreactive T cells, Notch inhibition markedly enhanced *in vivo* expansion of $Foxp3^+$ Tregs without inhibiting effector T cell expansion, which was explained by increased expansion of preexisting natural Tregs present in the transplant inoculum. These findings may contribute to the protective effects of Notch inhibition in GVHD and contrast with a shortcoming of many immunosuppressive strategies, including calcineurin inhibitors, which limit Treg proliferation.[241]

Alloreactive Notch-deprived $CD8^+$ T cells had largely preserved cytotoxicity after allo-BMT similar to our previously reported effects of Notch deprivation in $CD4^+$ T cells.[223] An interesting feature of alloreactive Notch-deprived T cells is their decreased

cytokine production but preserved cytotoxic potential. Dissociation of cytokine production from cytotoxicity responses could reflect differential sensitivity of these pathways to Notch inhibition. Preserved or even enhanced T-bet and Eomesodermin expression in Notch-deprived T cells could be responsible for maintaining their cytotoxic potential after allo-BMT. Prior work has shown that T-bet and Eomesodermin are important for transcription of cytotoxic molecules.[109] Collectively, Notch inhibition in alloreactive $CD4^+$ and $CD8^+$ T cells preserved efficient cytotoxicity while minimizing GVHD.

Altogether, our findings reveal a broad effect of Notch signaling in $CD4^+$ and $CD8^+$ alloreactive T cells during GVHD. Notch-deprived alloreactive T cells acquired features previously associated with hyporesponsiveness or anergy. However, this had differential effects on T cell effector functions *in vivo* in the post-transplantation environment, with profound inhibition of cytokine production but preserved T cell expansion, cytotoxic potential, and natural Treg accumulation. Notch-deprived T cells maintained potent cytotoxic activity *in vivo*, suggesting a "split anergy" phenotype.[247] These findings are summarized in Table 3.1. Overall, Notch inhibition in T cells induced a unique combination of effects that potently inhibited GVHD, highlighting the promise of this new therapeutic strategy after allo-BMT. Moreover, Notch signaling may play an important role in other T cell-mediated immune disorders with persistent antigen, such as autoimmune diseases like Multiple Sclerosis (Chapter 5).

Chapter 4

Blockade of individual Notch ligands and receptors controls graft-versus-host disease[5]

4.1 Abstract

Graft-versus-host disease (GVHD) is the main complication of allogeneic bone marrow transplantation. Current strategies to control GVHD rely on global immunosuppression. These strategies are incompletely effective and decrease the anti-cancer activity of the allogeneic graft. We previously identified Notch signaling in T cells as a new therapeutic target to prevent GVHD (Chapter 2 and 3). Notch-deprived T cells showed markedly decreased production of inflammatory cytokines, but normal *in vivo* proliferation, increased accumulation of regulatory T cells and preserved anti-cancer effects. Here, we report that GSIs can block all Notch signals in alloreactive T cells, but lead to severe on-target intestinal toxicity. Using newly developed humanized antibodies and conditional genetic models, we demonstrate that Notch1/Notch2 receptors and the Notch ligands Delta-like1/4 mediate all the effects of Notch signaling in T cells during GVHD, with dominant roles for Notch1 and Delta-like4. Delta-like1/4 inhibition blocked GVHD without limiting adverse effects, while preserving substantial anti-cancer activity. Transient blockade in the peri-transplant period was sufficient to provide long-lasting protection. These findings open new perspectives for selective and safe targeting of individual Notch pathway components in GVHD and other T cell-mediated human disorders.

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[5] Excerpts taken from:

Tran IT, **Sandy AR**, Carulli AJ, Ebens C, Chung J, Shan GT, Radojcic V, Friedman A, Gridley T, Shelton A, Reddy P, Samuelson LC, Yan M, Siebel CW and Maillard I. 2013. Blockade of individual Notch ligands and receptors controls graft-versus-host disease. *J Clin Invest* epub ahead of print.

4.2 Introduction

Allogeneic bone marrow or hematopoietic cell transplantation (allo-BMT) is an essential therapeutic modality for patients with hematological malignancies and other blood disorders. Unfortunately, T cells also mediate damage to normal host tissues, leading to graft-versus-host disease (GVHD).[141,208,248] Current strategies to control GVHD involve T cell depletion from the graft or global immunosuppression.[141,249] Despite these interventions, acute and chronic GVHD still arise in many allo-BMT patients.[141,209] Thus, new approaches are needed to prevent GVHD without eliminating GVT activity in allo-BMT recipients.

We have discovered a critical role for Notch signaling in pathogenic host-reactive T cells after allo-BMT.[223,250] To assess the overall effects of Notch signaling in T cells after allo-BMT, we conditionally expressed a dominant negative Mastermind-like (DNMAML) pan-Notch inhibitor in mature $CD4^+$ and $CD8^+$ T cells.[115,223,250] DNMAML is a truncated fragment of the Mastermind-like1 coactivator fused to GFP that blocks transcriptional activation downstream of all Notch receptors.[82,92,115,251] DNMAML expression in donor T cells led to markedly reduced GVHD severity, without causing global immunosuppression.[223,250] DNMAML alloreactive T cells displayed decreased production of multiple inflammatory cytokines and increased expansion of natural regulatory T cells (Treg), leading to reduced target organ damage. However, DNMAML T cells proliferated and expanded *in vivo* as well, or even better, than wild-type alloreactive T cells. Importantly, DNMAML T cells retained potent cytotoxic potential and cytotoxic activity. Our findings identify Notch signaling in donor T cells as an attractive new target to achieve beneficial immunomodulation and inhibit GVHD after allo-BMT.

Although genetic strategies are invaluable to study the role of Notch signaling in disease models, pharmacological interventions are required to harness the therapeutic potential of Notch inhibition.[221] Here, we report that GSIs blocked Notch signaling in alloreactive T cells during GVHD, but led to severe on-target side effects in the intestinal epithelium after allo-BMT. To bypass this limiting toxicity, we targeted individual Notch ligands and receptors in mice using newly developed potent and specific neutralizing

humanized monoclonal antibodies.[221,252] These antibodies block both mouse and human proteins.[221,252] We found that Notch1/2 and Dll1/4 accounted for all the effects of Notch signaling in alloreactive T cells, with dominant effects for Notch1 and Dll4. In particular, combined blockade of Dll1 and Dll4 was safely achieved after allo-BMT, with no evidence of intestinal side effects. Remarkably, transient Dll1 and Dll4 inhibition was sufficient to provide long-lasting protection against GVHD. Protection was associated with persistent Treg expansion. These findings identify novel strategies to safely and efficiently target individual elements of the Notch pathway after allo-BMT, which could lead to new strategies for GVHD control in human patients. Since aberrant Notch signaling has been linked to many human diseases, our work may have broad implications beyond GVHD towards selective therapeutic targeting of individual Notch pathway components.

4.3 Results

4.3.1 Pharmacological pan-Notch inhibitors block Notch signaling in alloreactive T cells but lead to severe gastrointestinal toxicity

The rate-limiting step in proteolytic activation of the Notch receptors can be targeted with GSIs, a class of compounds available for preclinical and early clinical interventions.[253] To assess the potential of pan-Notch inhibition with GSIs to control GVHD, we used the B6 anti-BALB/c MHC-mismatched model of allo-BMT and treated recipient mice with the GSI dibenzazepine (DBZ). DBZ decreased the production of inflammatory cytokines such as IFNγ and IL-2 by alloreactive T cells to a similar extent as genetic blockade of Notch signaling by DNMAML (**Fig. 4.1A-B**).[223] DBZ-treated WT and vehicle-treated DNMAML T cells had similarly decreased expression of *Dtx1*, a direct Notch target gene (**Fig. 4.1B**), indicating efficient inhibition of Notch signaling. To assess if DBZ-mediated pan-Notch inhibition resulted in improved outcome, we monitored survival of allo-BMT recipients (**Fig. 4.1C**). While DNMAML-mediated Notch blockade in alloreactive T cells drastically reduced GVHD severity and increased survival of allo-BMT recipients[223], DBZ-treated mice died within 4-8 days after transplantation with signs of severe diarrhea, even when no T cells were present in the

donor inoculum (**Fig. 4.1C**). This rapid lethality and intestinal toxicity were much more severe than previously reported with GSIs in steady-state conditions, suggesting an essential role for Notch signaling to support recovery of intestinal integrity after total body irradiation. Thus, GSIs can target Notch in alloreactive T cells but lead to profound intestinal adverse effects after allo-BMT.

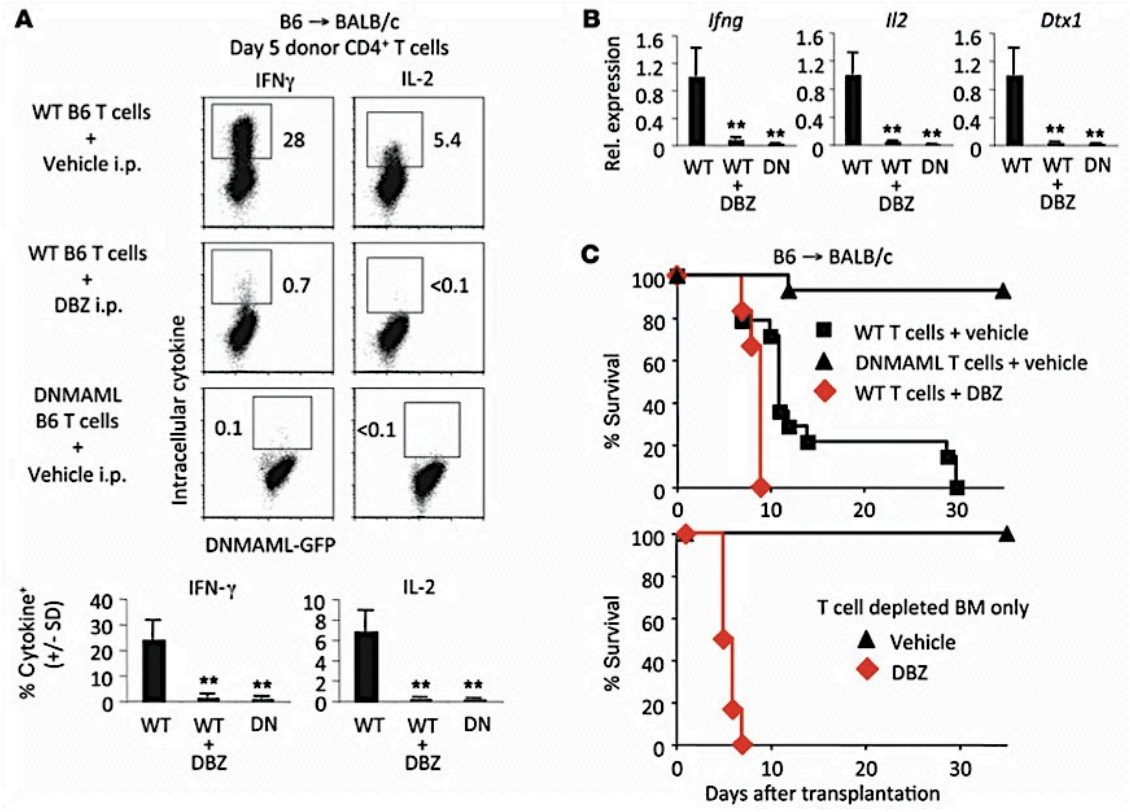

Figure 4.1 Efficient Notch inhibition in alloreactive T cells but severe intestinal toxicity of GSIs after allo-BMT.
Lethally irradiated (900 rads) BALB/c mice were transplanted with B6 TCD BM (5×10^6 cells) with or without WT or DNMAML (DN) B6 T cells (10×10^6 splenocytes). The GSI DBZ was administered daily as compared with vehicle (i.p., 10 μmol/kg). **(A)** Cytokine production by donor-derived $H2Kb^+H2Kd^-$ $CD4^+$ spleen T cells at day 5 after allo-BMT. Representative flow cytometry plots show intracellular IFNγ and IL-2 after anti-CD3/CD28 restimulation. **(B)** Relative abundance of transcripts for *Ifng, Il2,* and the Notch target gene *Dtx1* in day 5 donor-derived $CD4^+$ T cells after anti-CD3/CD28 restimulation. **(C)** Short survival of DBZ-treated mice after allo-BMT, even upon transplantation of TCD BM only ($P < 0.01$, WT vehicle vs. WT DBZ; $P < 0.0001$, TCD vehicle vs. TCD DBZ). In contrast, DNMAML expression in donor T cells led to markedly prolonged survival ($P < 0.0001$, WT vs. DNMAML vehicle) (n = 14 for vehicle-treated, $n = 6$ for DBZ-treated groups).

4.3.2 Notch1 and Notch2 account for all the effects of Notch signaling in alloreactive T cells, with a dominant role for Notch1

In steady-state conditions, *Notch1* and *Notch2* exert largely redundant functions in the gut epithelium.[17,221,254] Thus, targeting only Notch1 or Notch2 could be safer than pan-Notch inhibition after allo-BMT. We studied *Notch1-4* expression in naïve and alloreactive $CD4^+$ and $CD8^+$ T cells (**Fig. 4.2**). Both *Notch1* and *Notch2* transcripts were present (**Fig. 4.2A**). *Notch1* mRNA was more abundant than *Notch2* mRNA. *Notch3* and *Notch4* transcripts were not detectable, even in activated alloreactive T cells (**Fig. 4.2B-C**). To assess the respective role of Notch1 and Notch2 functionally, we used humanized antibodies that target the extracellular negative regulatory region of each receptor to prevent Notch activation.[221] As a control for the quality of these reagents, we found that *in vivo* administration of anti-Notch1 or anti-Notch2 antibodies led to profound depletion of Notch1-dependent thymocytes and Notch2-dependent marginal zone B (MZB) cells, respectively, with no crossreactivity (data not shown).[29,30] These findings indicate high efficacy and specificity.

We tested the impact of Notch1 and/or Notch2 inhibition after allo-BMT, using cytokine production as a surrogate endpoint and DNMAML T cells as positive control for efficient pan-Notch inhibition (**Fig. 4.3**). Combined Notch1 and Notch2 blockade reduced IFNγ (**Fig. 4.3A**) and IL-2 (**Fig. 4.3B**) production by alloreactive T cells to a similar extent as DNMAML expression. Notch1 inhibition alone was sufficient to partially block IFNγ and prevent IL-2 production. Notch2 blockade had minor effects on the mean fluorescence intensity of IFNγ staining and on IL-2 production. This indicated a dominant role for Notch1 with additional contribution from Notch2. To verify that Notch receptors exert cell-autonomous effects in T cells, we studied alloreactive T cells with genetic inactivation of *Notch1*, *Notch2* (**Fig. 4.4**), or both (**Fig. 4.5**). Production of IFNγ and IL-2 in $CD4^+$ T cells and IFNγ in $CD8^+$ T cells was profoundly decreased upon DNMAML expression or combined Notch1/2 inhibition (*Notch1* inactivation with systemic Notch2 blockade, *Notch2* inactivation with systemic Notch1 blockade, or *Notch1/Notch2* inactivation). Thus, cell-autonomous effects of *Notch1* and *Notch2* accounted for all the effects of Notch signaling in alloreactive $CD4^+$ and $CD8^+$ T cells, with a dominant role for *Notch1*.

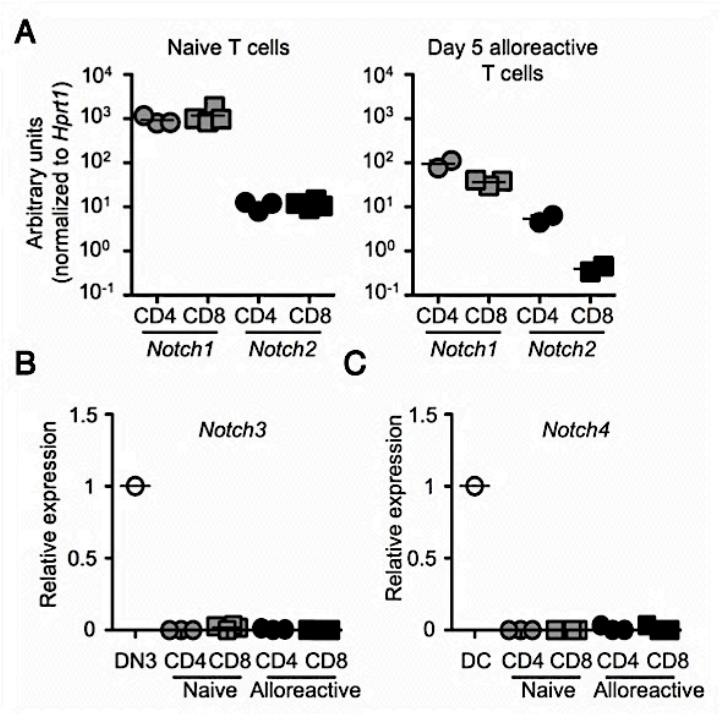

Figure 4.2 Expression of *Notch1* and *Notch2* mRNA and absence of detectable *Notch3/4* transcripts in naïve and alloreactive T cells.

Naïve $CD44^{low}CD62L^{high}$ $CD4^+$ and $CD8^+$ T cells were sort-purified from B6 mice. Alloreactive T cells were purified as donor-derived $H2Kb^+H2Kd^-$ $CD4^+$ and $CD8^+$ T cells from the spleen of BALB/c allo-BMT recipient mice at day 5 after transplantation. Quantitative RT-PCR was performed. **(A)** Absolute quantification of *Notch1* and *Notch2* mRNA using a titration curve generated from known amounts of each PCR product. This allowed for direct comparison of *Notch1* and *Notch2* transcript abundance. Data are expressed as arbitrary units after normalization to *Hprt1* mRNA. **(B)** Relative quantification of *Notch3* transcripts using the ΔΔCt method. $Lineage^-CD44^{low}CD25^{high}$ "double negative 3" (DN3) B6 thymocytes were used as a positive control for *Notch3* expression. **(C)** Relative quantification of *Notch4* transcripts using the ΔΔCt method. $I\text{-}Ab^+CD11c^+$ spleen dendritic cells (DC) were used as a positive control for *Notch4* expression. Each symbol represents data obtained from one individual mouse. Relative rather than absolute quantification was performed in **(B)** and **(C),** as *Notch3* and *Notch4* amplicons were not or only barely detectable in T cells.

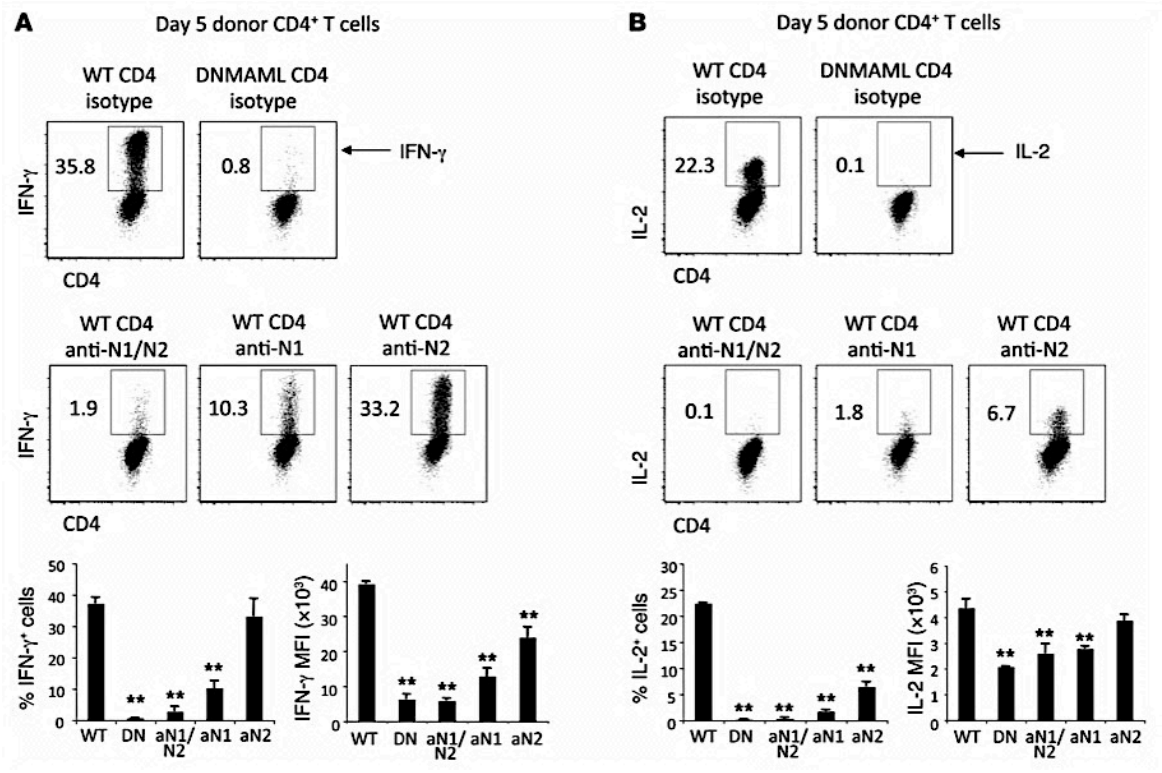

Figure 4.3 Notch1 and Notch2 control the production of IFNγ and IL-2 by alloreactive T cells, with dominant effects of Notch.

WT or DNMAML B6 T cells were transplanted into irradiated BALB/c recipients (900 rads). Isotype control, anti-Notch1 (anti-N1), anti-Notch2 (anti-N2), or both anti-Notch1/Notch2 antibodies were administered at day 0 and day 3. DNMAML T cells exposed to isotype control antibodies were a positive control for pan-Notch inhibition. **(A)** Intracellular staining for IFNγ in donor-derived $H2Kb^{+}H2Kd^{-}$ $CD4^{+}$ spleen T cells after anti-CD3/CD28 restimulation. MFI of the $IFN\gamma^{+}$ cells is shown. **(B)** Intracellular staining for IL-2 under the same conditions. MFI of $IL\text{-}2^{+}$ cells is shown. Representative flow cytometry plots are shown. Numbers indicate the percentage of cells in each quadrant. Bar graphs represent mean ± SD (n = 3) from 1 of 3 representative experiments. $^{**}P < 0.01$.

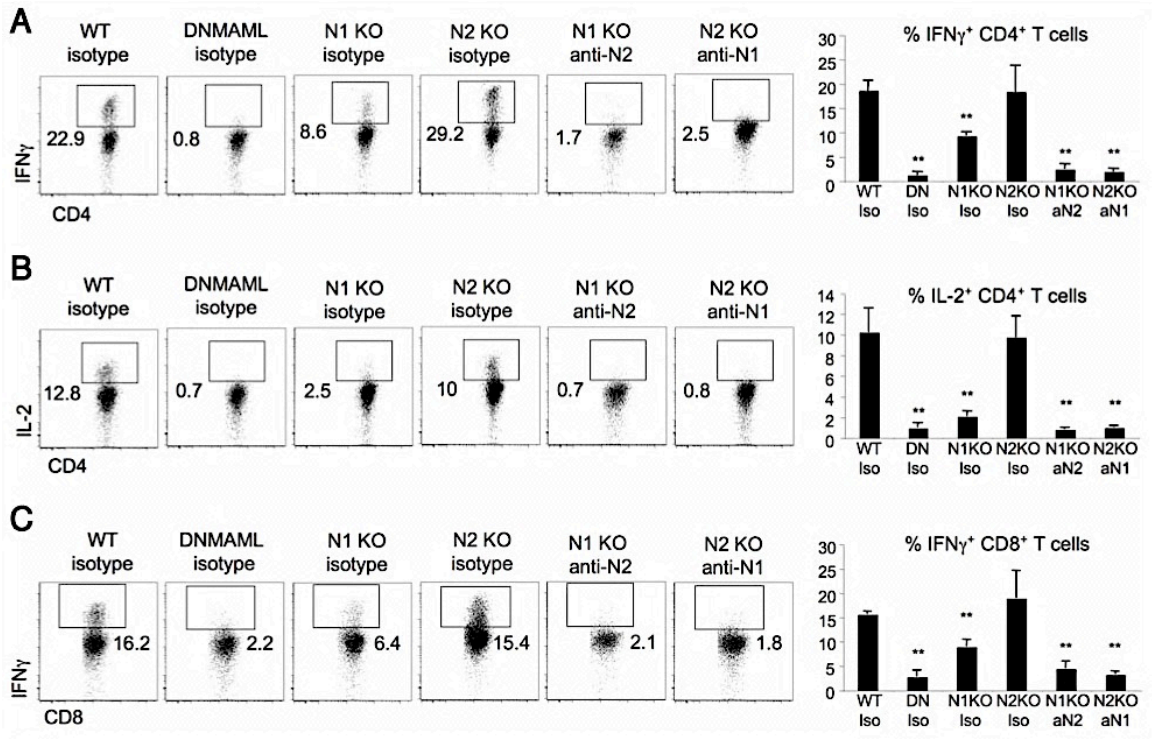

Figure 4.4 Notch1 and Notch2 exert cell-autonomous effects on cytokine production in alloreactive CD4$^+$ and CD8$^+$ T cells.

Lethally irradiated BALB/c mice (900 rads) were infused with TCD BM and WT, DNMAML, *Notch1$^{f/f}$ Cd4-Cre$^+$* (N1 KO) or *Notch2$^{f/f}$ Cd4-Cre$^+$* (N2 KO) spleen T cells. Isotype control, anti-Notch1 or anti-Notch2 monoclonal antibodies were administered i.p. as indicated (5 mg/kg, day 0 and day 3). At day 4.5, splenocytes were restimulated with plate-bound anti-CD3/CD28 antibodies followed by intracellular staining. **(A)** Percentage of IFNγ$^+$ cells among donor-derived H2Kb$^+$H2Kd$^-$ CD4$^+$ T cells; **(B)** Percentage of IL-2$^+$ cells among donor-derived CD4$^+$ T cells; **(C)** Percentage of IFNγ$^+$ cells among donor-derived CD8$^+$ T cells. Representative flow cytometry plots are shown. Numbers indicate the percentage of cells in each quadrant. Bar graphs represent mean +/– SD (n=3-5/group). **p<0.01.

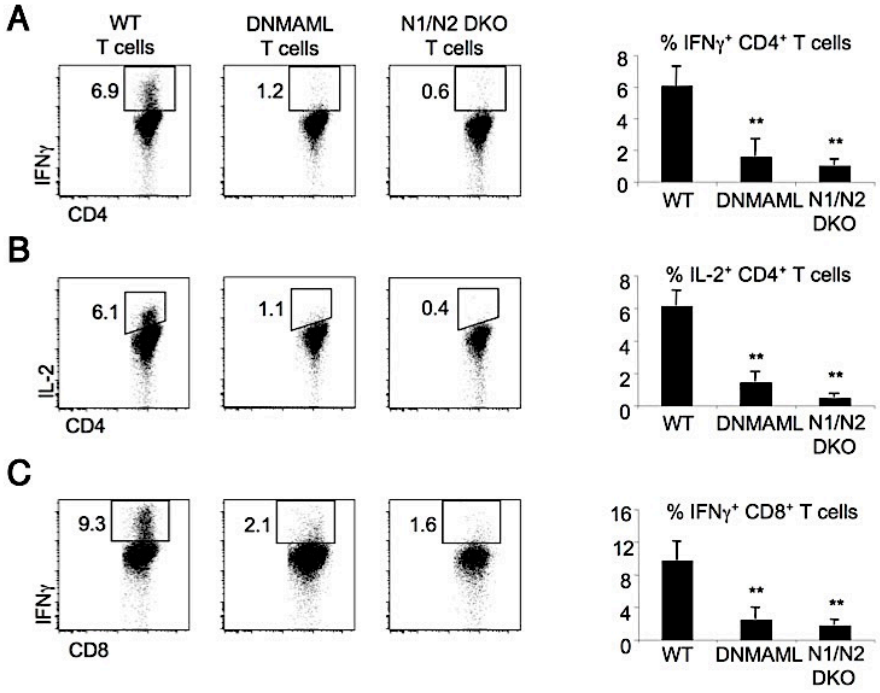

Figure 4.5 Combined *Notch1* and *Notch2* inactivation inhibits cytokine production to a similar extent as DNMAML expression in alloreactive T cells.
Lethally irradiated BALB/c mice (900 rads) were infused with TCD BM and WT, DNMAML or *Notch1*$^{f/f}$*Notch2*$^{f/f}$ *Cd4-Cre*$^{+}$ (N1/N2 DKO) spleen T cells. At day 5, splenocytes were restimulated with plate-bound anti-CD3/CD28 antibodies followed by intracellular staining. **(A)** % IFNγ^{+} cells in donor-derived H2Kb^{+}H2Kd^{-} CD4^{+} T cells; **(B)** % IL-2^{+} cells in donor-derived CD4^{+} T cells; **(C)** % IFNγ^{+} cells in donor-derived CD8^{+} T cells. Representative flow cytometry plots are shown. Numbers indicate the percentage of cells in each quadrant. Bar graphs represent mean +/– SD (n=4/group). **p<0.01.

4.3.3 Dll1 and Dll4 Notch ligands mediate Notch signaling in alloreactive T cells, with a dominant role for Dll4

Past reports suggested a role for Delta-like and not Jagged ligands in promoting Th1-like inflammatory T cell responses, which dominate in GVHD.[111] Thus, we studied the possibility of inhibiting Dll1 and Dll4, the two agonistic Delta-like ligands, using newly developed neutralizing antibodies that target the Dll1 or Dll4 extracellular domain.[31,255] As a control, these antibodies led to profound depletion of Dll1-dependent marginal zone B cells and Dll4-dependent thymocytes, respectively, showing high efficiency and specificity (data not shown).[28,256] After allo-BMT, combined inhibition of Dll1 and Dll4 blocked production of IFNγ and IL-2 to a similar extent as DNMAML expression in T cells (**Fig. 4.6**). Anti-Dll4 antibodies had the most profound effects, while Dll1 blockade only had a minimal impact by itself. Importantly, Dll1/Dll4 blockade

allowed for increased numbers of Tregs to accumulate (**Fig. 4.7**). Thus, transient Dll1/Dll4 inhibition blocked production of inflammatory cytokines (**Fig. 4.6**) and led to increased donor Tregs. Together, compared to the effects of DNMAML-mediated pan-Notch inhibition, Dll1 and Dll4 appeared to account for all the effects of Notch signaling on cytokine secretion by alloreactive T cells, with a dominant role for Dll4.

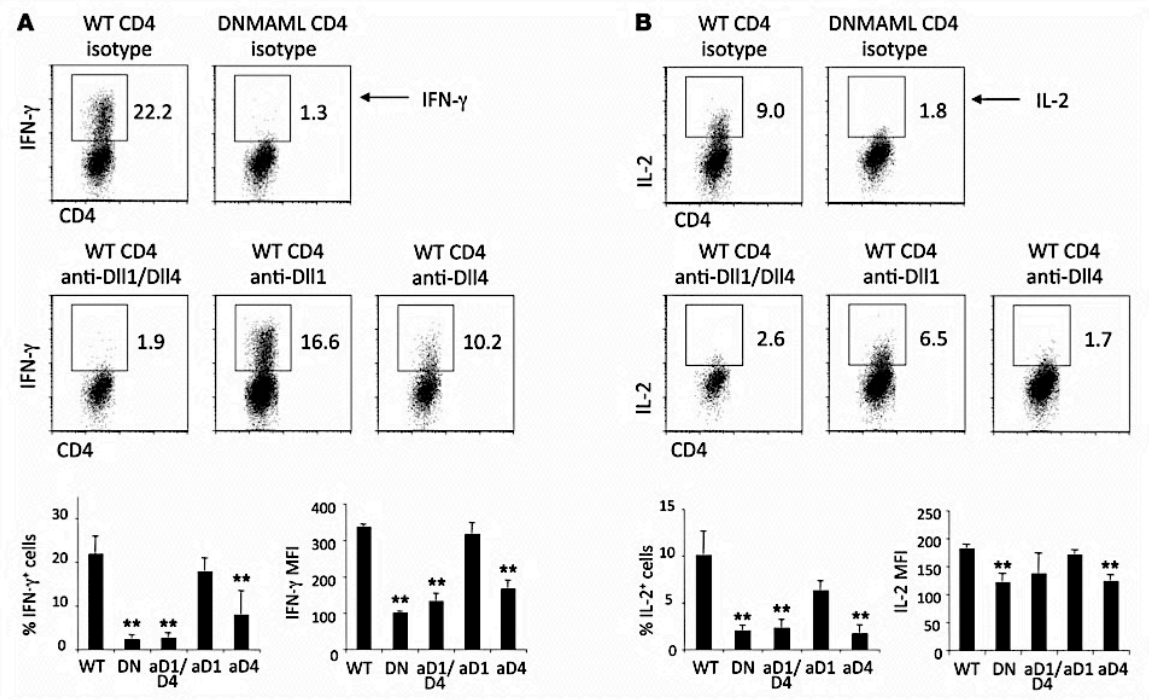

Figure 4.6 Dll1 and Dll4 Notch ligands control the production of IFNγ and IL-2 by alloreactive T cells, with dominant effect of Dll4.
WT or DNMAML B6 T cells were transplanted into irradiated BALB/c recipients (900 rads). Isotype control, anti-Dll1, anti-Dll4, or anti-Dll1/Dll4 antibodies were administered at day 0 and day 3. DNMAML T cells exposed to isotype control antibodies were a positive control for pan-Notch inhibition. **(A)** Intracellular staining for IFNγ in donor-derived $H2Kb^{+}H2Kd^{-}$ $CD4^{+}$ spleen T cells after anti-CD3/CD28 restimulation. MFI of $IFN\gamma^{+}$ cells is shown. **(B)** Intracellular staining for IL-2 under the same conditions. MFI of $IL\text{-}2^{+}$ cells is shown. Representative flow cytometry plots are shown. Numbers indicate the percentage of cells in each quadrant. Bar graphs represent mean ± SD (n = 3) from 1 of 3 representative experiments. **P < 0.01.

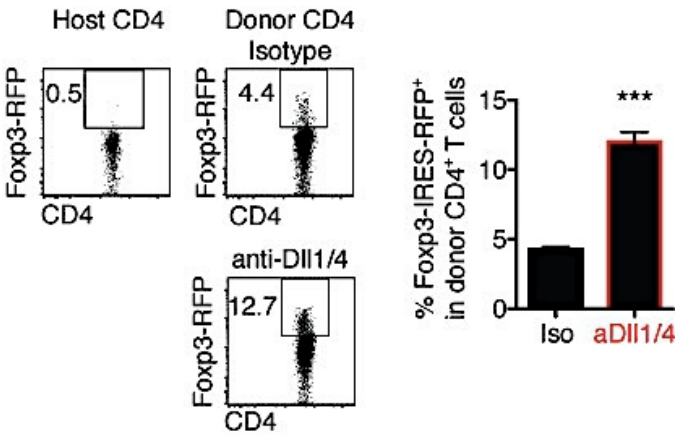

Figure 4.7 Increased accumulation of donor-derived Foxp3$^+$ CD4$^+$ T cells upon Dll1/Dll4 blockade after allo-BMT as revealed using a Foxp3-IRES-mRFP reporter allele.

Lethally irradiated BALB/c recipients (900 rads) were transplanted with TCD BM ($5x10^6$) and $5x10^6$ splenocytes from B6 FoxP3-IRES-mRFP reporter mice (expressing mRFP under control of endogenous *Foxp3* regulatory sequences). Isotype control or anti-Dll1/4 antibodies were administered twice weekly. On day 11, mRFP fluorescence was studied by flow cytometry among H2Kb$^+$ donor-derived CD4$^+$ T cells or in host CD4$^+$ T cells as a negative control.

4.3.4 Dll1 and Dll4 blockade abrogates acute GVHD and preserves cytotoxic potential, even when applied transiently after bone marrow transplantation.

We assessed if Dll1 and/or Dll4 blockade could protect mice from the morbidity and lethality of acute GVHD (**Fig. 4.8**). Individual Dll1 or Dll4 inhibition extended the median survival of allo-BMT recipients by about 25 and 50 days, respectively, although ultimately all animals succumbed to GVHD. However, when both Dll1 and Dll4 were inhibited, we observed increased long-term survival and decreased GVHD severity to an extent similar to that seen with DNMAML-mediated pan-Notch inhibition. Remarkably, short-term Dll1/Dll4 blockade after allo-BMT (day 0-10) conferred as much protection as continuous blockade for 60 days after transplantation. Therapeutic targeting of Notch1 and 2 receptors could not be achieved due to significant toxicity, presumably from on-target gastrointestinal side effects post-irradiation (data not shown). Thus, targeting Delta-like ligands rather than Notch receptors did not induce limiting toxicity and revealed the protective effects of Notch inhibition in GVHD.

DNMAML T cells have preserved cytotoxic activity after allo-BMT.[223,250] Thus, we next assessed the effect of short-term anti-Dll1/Dll4 treatment (**Fig. 4.9**). Overall

cytotoxic potential was captured with an *in vivo* cytotoxicity assay (**Fig. 4.9A-B**). Allo-BMT recipients were challenged on day 14 with CFSE-labeled allogeneic target cells. Efficient elimination of allogeneic targets was observed in recipients of wild-type T cells treated with isotype control or anti-Dll1/Dll4 antibodies. Cytotoxic activity was slightly reduced in anti-Dll1/Dll4-treated recipients. However, it was efficient when compared to recipients of T cell-depleted BM only (**Fig. 10b**). These data demonstrate that Dll1/4 blockade inhibits GVHD while preserving substantial cytotoxic activity.

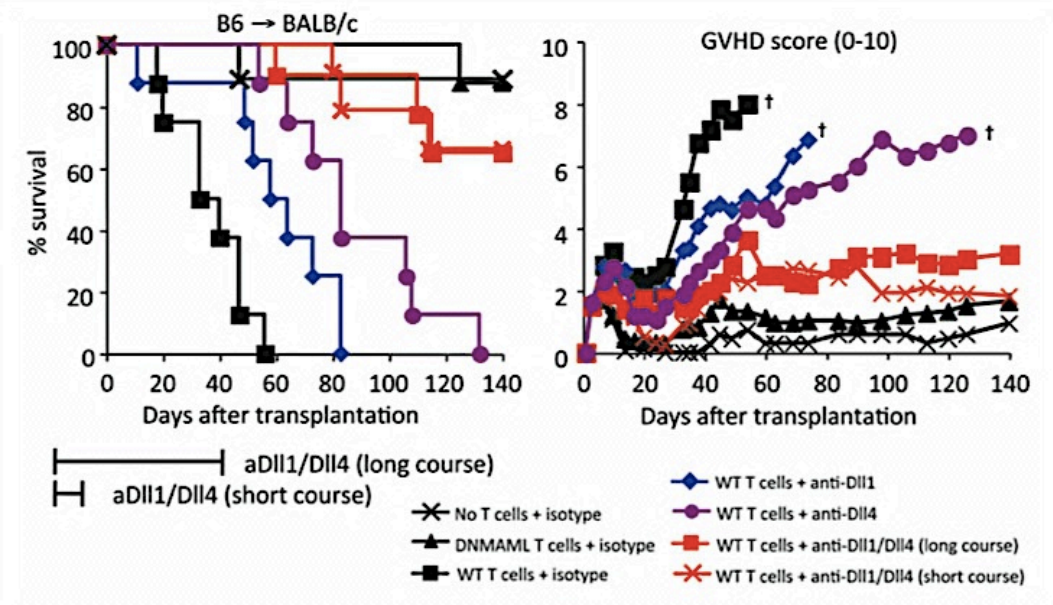

Figure 4.8 Transient blockade of Dll1 and Dll4 protects mice from lethal GVHD.
Survival after transplantation and clinical GVHD score demonstrate increasing protection with Dll1, Dll4, and combined Dll1/Dll4 blockade (P = 0.005, WT vs. anti-Dll1; *P* = 0.0001, WT vs. anti-Dll4; *P* < 0.0001, WT vs. anti-Dll1/Dll4). Anti-Dll1/Dll4 antibodies provided nearly as much protection as DNMAML T cells, even upon short-term administration (P = 0.21, DNMAML vs. anti-Dll1/Dll4 groups) (n = 8/group). Crosses indicate death of all mice by the indicated time point.

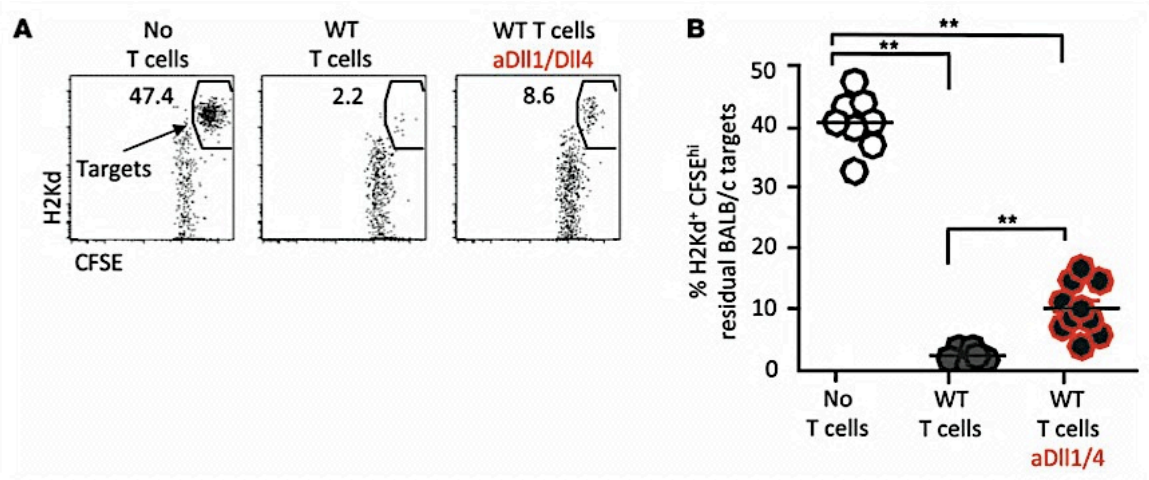

Figure 4.9 Dll1/Dll4 blockade preserved substantial *in vivo* cytotoxicity.
Allo-BMT and transient administration of anti-Dll1/Dll4 or control antibodies (days 0–10) were performed as described in Figure 4.8 legend. **(A)** *In vivo* cytotoxicity assay. Allo-BMT recipient mice were challenged on day 14 with a 1:1 infusion of CFSE-labeled allogeneic targets and control cells ($CFSE^{high}H2Kd^{+}$ BALB/c and $CFSE^{low}$ control $H2Kb^{+}$ B6-CD45.1 splenocytes, respectively). After 18 hours, elimination of the BALB/c targets was assessed in the spleen by flow cytometry. **(B)** Summary of in vivo cytotoxicity data in individual mice (n = 6–10/group). $^{**}P < 0.01$.

4.3.5 Dll1 and Dll4 inhibition does not impair hematopoietic recovery after allo-BMT

To further assess the safety of short-term Dll1/Dll4 blockade, we studied hematopoietic and thymic reconstitution. Blood counts and in particular platelet recovery were not impaired by Dll1/Dll4 blockade (**Fig. 4.10A**). Bone marrow progenitor contents were not affected as compared to mice receiving WT or DNMAML T cells (**Fig. 4.10B**). When CD45.1 was used to track cells derived from the T cell-depleted bone marrow, similar engraftment efficiency was observed (**Fig. 4.10C**). Thus, no major defect in hematopoietic reconstitution was apparent in these conditions.

Thymic output after allo-BMT can be profoundly decreased by GVHD-induced damage to the thymic epithelium (tGVHD).[257,258] However, Dll4 is the physiological ligand driving early T cell development in the thymus.[256] We quantified $CD4^{+}CD8^{+}$ double positive (DP) thymocytes as a measure of thymic function after allo-BMT (**Fig. 4.11**). Recipients of wild-type T cells had markedly reduced DP numbers as a result of severe tGVHD. Infusion of DNMAML T cells preserved thymic cellularity, consistent with markedly decreased tGVHD. Interestingly, Dll1/Dll4 blockade was associated with low DP contents at day 21, consistent with blockade of Dll4-dependent T cell

development. However, after time had elapsed allowing antibodies to be cleared, thymic cellularity increased up to levels seen in DNMAML recipients (day 35). Thus, transient Dll1/Dll4 inhibition protected the thymus from tGVHD, allowing good subsequent immune recovery.

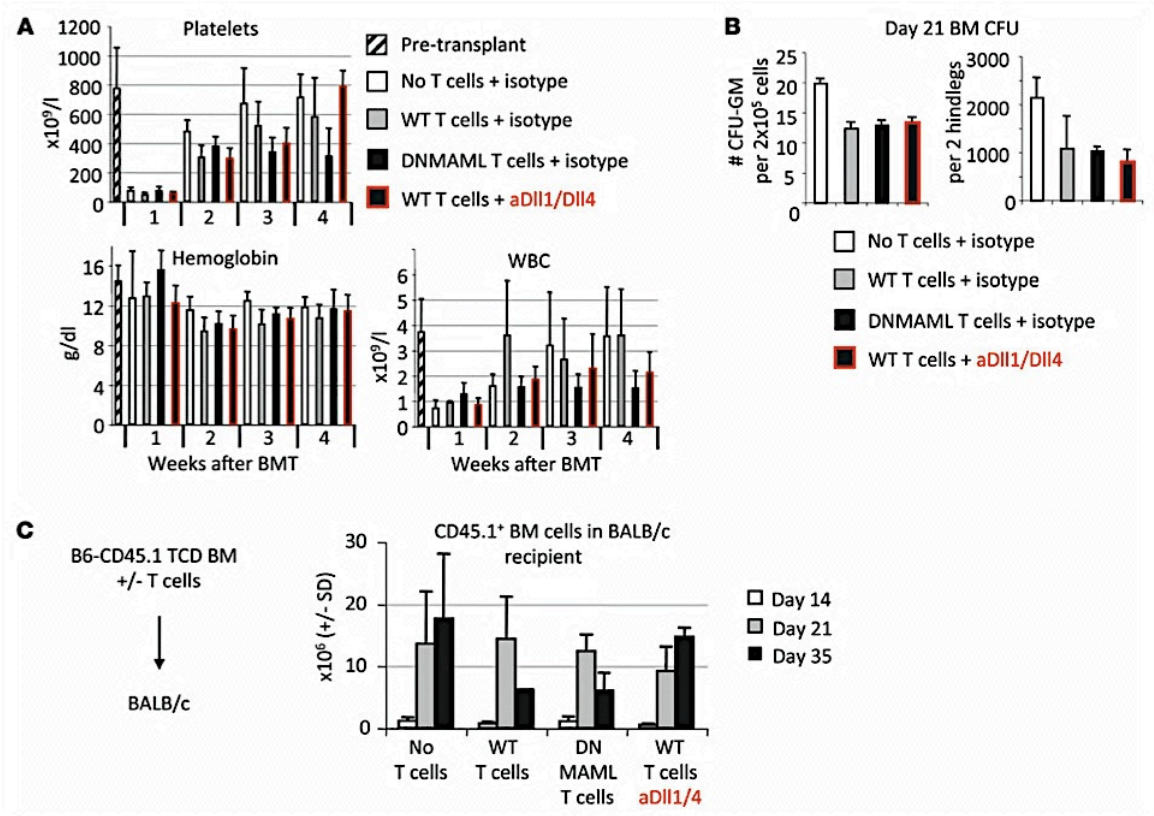

Figure 4.10 Preserved hematopoietic recovery after allogeneic transplantation in mice treated with anti-Dll1/Dll4 antibodies.
Allo-BMT and transient administration of anti-Dll1/Dll4 or control antibodies (days 0–10) were performed as described in Figure 4.8. **(A)** Weekly complete blood counts after allo- BMT showing unimpaired recovery in recipients treated with anti-Dll1/Dll4 antibodies. **(B)** CFU-GM activity in the BM on day 21 after transplantation. **(C)** Absolute numbers of $CD45.1^+$ cells derived from B6-CD45.1 donor TCD BM at days 14, 21, and 35. This showed preserved engraftment and expansion of $CD45.1^+$ donor-derived cells in the BM. Bar graphs represent mean ± SD.

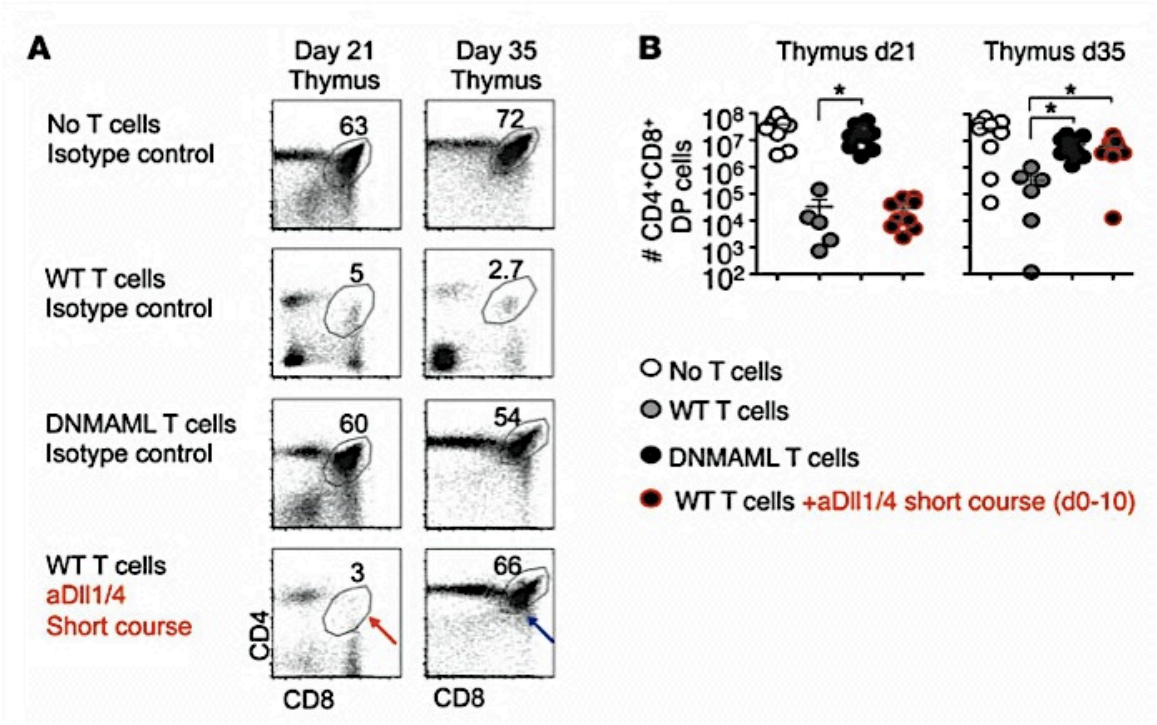

Figure 4.11 Protection from thymic GVHD upon transient systemic Dll1/Dll4 blockade.
Lethally irradiated (850 rads) BALB/c mice were transplanted with TCD BM (5×10^6 cells) with or without WT or DNMAML T cells (10×10^6 splenocytes). Isotype control vs. anti-Dll1/Dll4 antibodies were administered i.p. at days 0, 3, 7, and 10 (short course). **(A)** Thymus was assessed using flow cytometry to identify newly formed $CD4^+CD8^+$ DP thymocytes. At day 21, thymopoiesis was inhibited in the presence of anti-Dll4 antibodies (red arrow). At day 35, after antibody clearance, large numbers of DP thymocytes arose in anti-Dll1/Dll4–treated mice (blue arrow), indicating protection from GVHD-induced thymic damage. **(B)** Absolute number of $CD4^+CD8^+$ DP thymocytes at days 21 and 35 in individual allo-BMT recipients, quantifying preserved thymic recovery at day 35 in anti-Dll1/ Dll4–treated mice. *$P < 0.05$.

4.4 Discussion

Our findings highlight the therapeutic potential of targeting individual Notch receptors or ligands as a new strategy to control GVHD after allo-BMT. We used genetic pan-Notch inhibition as an experimental benchmark to identify non-redundant effects of specific Notch receptors and ligands in alloreactive T cells. Notch1 had dominant effects with a minor contribution from Notch2, while *Notch3-4* transcripts were not detectable in donor T cells. Delta-like ligands appeared to account for all the pathogenic effects of Notch in T cells, with dominant effects of Dll4. In terms of efficacy and safety, short-term combined blockade of Dll1/4 Notch ligands with monoclonal antibodies emerged as

the most promising strategy to harness the beneficial effects of Notch inhibition after allo-BMT in the pre-clinical mouse models that we used.

Past work has described redundant roles for *Notch1* and *Notch2* in T cells in other immune contexts.[113,259,260] Several reasons could explain the more dominant effects of *Notch1* in GVHD. Although both receptors were expressed, *Notch2* was less abundant than *Notch1* mRNA in alloreactive T cells, especially during peak activation. In addition, Notch1 may preferentially interact with the Dll4 Notch ligand during GVHD. This is reminiscent of the specific requirement for Notch1 and Dll4 in early T cell development and neoangiogenesis.[252,256,261] It remains to be determined which cells present Dll1/Dll4 ligands to alloreactive T cells in the post-bone marrow transplant environment. Moreover, Fringe glycosyltransferases could be important by increasing the avidity of Notch receptors for Delta-like as opposed to Jagged ligands.[33,262] This mechanism has not been explored in peripheral T cell immunity, but could account in part for the dominant effects of Delta-like ligands during GVHD.

Early work exploring a role for Notch in alloreactivity relied on Notch ligand overexpression in dendritic cells or in artificial APCs.[215,263,264] This strategy induced hyporesponsiveness of alloreactive T cells, leading the authors to suggest that Notch might be important to induce tolerance after transplantation. In contrast, our work indicates that Notch signaling is required for pathogenic functions of alloreactive T cells *in vivo* after allo-BMT. This is consistent with a recent report describing delayed organ rejection upon Dll1 inhibition in a model of allogeneic heart transplantation, although our observations identified more profound overall effects of the pathway.[265] Our data illustrate the value of studying the effects of Notch signaling *in vivo* in specific immune responses using loss-of-function approaches. *In vitro* studies and gain-of-function approaches may not reliably model the physiological and pathophysiological conditions in which T cells encounter Notch ligands.

Similar to genetic pan-Notch inhibition, Dll1/Dll4 blockade dramatically decreased production of inflammatory cytokines and GVHD without causing global immunosuppression (Chapter 2, 3, and 4 summarized in **Figure 4.12**). Indeed, Notch deprivation preserved *in vivo* proliferation and increased expansion of alloreactive T cells in lymphohematopoietic organs. Because proliferation was not changed, enhanced

accumulation was likely related to decreased activation-induced T cell death upon Notch inhibition. These findings differ from the effects of most interventions used to control GVHD, including calcineurin inhibitors and many methods that decrease proliferation and expansion of alloreactive T cells. We also observed long-lasting expansion of donor-derived Tregs, even when Dll1/Dll4 ligands were blocked transiently after allo-BMT. It is possible that these Tregs helped maintain long-term GVHD control upon Notch inhibition. Mechanistically, our findings indicate that Notch blockade has profound effects on the production of inflammatory cytokines that trigger tissue damage and GVHD. More work will be needed in the future to dissect the differential effects of Notch signaling on distinct effector functions in T cells.

Our findings identify a promising new preclinical strategy of Notch blockade with an improved safety index when compared to systemic pan-Notch inhibition with GSIs. GSIs are currently being explored for their anti-cancer activity in early clinical trials. Gastrointestinal toxicity has emerged as a significant dose-limiting on-target adverse effect in mice and humans.[222,254,266] Concomitant administration of corticosteroids has been reported to decrease gut toxicity in mice.[220] However, corticosteroids induce global immunosuppression, which is an unattractive choice after allo-BMT. Intermittent administration schedules are also being tested to improve the safety of GSIs in cancer patients. However, our observations suggest that minimizing gut toxicity of GSIs may be particularly challenging in allo-BMT patients given the heightened requirement for Notch signaling during intestinal regeneration after irradiation, precisely at the time when Notch exerts its effects on alloreactive T cells. Thus, GSIs are not promising agents for therapeutic intervention after allo-BMT.

The humanized antibodies that we used in this study were designed to block both mouse and human proteins. Thus, our preclinical work could be efficiently translated into new strategies for GVHD control in clinical trials. Although our observations were made after allogeneic HSCT, they may extend to other types of immune responses with exposure to persistent antigens. For example, Notch may regulate organ rejection (unpublished data) and autoimmune diseases (Chapter 5). From a therapeutic perspective, our work points to the Notch pathway as an attractive target to achieve beneficial context-specific immunomodulation in T cell–mediated disorders.

A

	Wild-type $CD4^+$ and $CD8^+$ T cells	Notch-deprived $CD4^+$ and $CD8^+$ T cells
graft-versus host disease	+++	+/-
cytokine production	+++	+/-
in vitro proliferation	+++	+
in vivo proliferation	+++	++
natural Treg expansion	+	+++
in vitro cytotoxicity	+++	++
in vivo cytotoxicity	+++	++

B

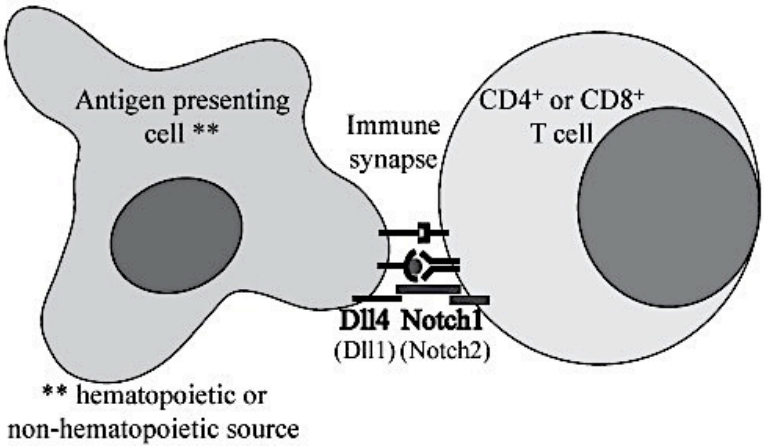

Figure 4.12 Summary of observations after inhibition of Notch signaling in T cells during GVHD.

(A) Notch-inhibited T cells induce significantly less GVHD concomitant with markedly reduce cytokine production and natural Treg expansion. Notch inhibition largely preserved proliferation and cytotoxic potential; **(B)** Infused T cells expressing Notch1 and 2 interact with antigen-presenting cells expressing Dll4 and Dll1. Notch1 and Dll4 play a dominant role in mediating GVHD by T cells.

Chapter 5

Notch regulates T cell accumulation and function in the central nervous system during experimental autoimmune encephalomyelitis[6]

5.1 Abstract

Systemic inhibition of Notch signaling was previously shown to attenuate experimental autoimmune encephalomyelitis (EAE). Different studies attributed these effects to decreased expression of T-bet, enhanced regulatory T cell function, or reduced T cell chemotaxis to the central nervous system (CNS). Interpretation of these heterogeneous findings is difficult, since many cell populations could be affected by systemic Notch blockade. To resolve the role of Notch signaling in T cells during EAE, we used the pan-Notch inhibitor DNMAML and several complementary loss-of-function approaches specifically in myelin-reactive T cells. Notch inhibition in T cells markedly decreased EAE incidence and severity. Notch-deprived myelin-reactive T cells had preserved activation and effector differentiation in secondary lymphoid tissues. However, Notch-deprived T cells failed to accumulate in the CNS post-immunization. Parking wild-type and DNMAML T cells together in bone marrow chimeras increased accumulation of DNMAML T cells in the CNS post-immunization but did not prevent EAE, indicating the absence of dominant suppression by DNMAML T cells. Analysis of CNS-infiltrating Notch-deprived T cells revealed profoundly defective production of IL-17A and IFN-γ, despite preserved expression of T-bet. Thus, Notch controls accumulation and pathogenic functions of $CD4^+$ T cells within their target organ but not in lymphoid tissues during EAE.

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[6] Taken from:

Sandy AR, Stoolman J, Malott K, Pongtornpipat P, Segal B, and Maillard I. Notch regulates T cell accumulation and function in the CNS during experimental autoimmune encephalomyelitis. In preparation.

5.2 Introduction

5.2.1 Etiology and pathogenesis of Multiple Sclerosis

Multiple Sclerosis (MS) is an autoimmune demyelinating syndrome of the central nervous system (CNS). The earliest recorded case of MS was probably described by Auguste d'Este (1794–1848), grandson of George III of England, in his diary.[267] MS may have existed even in the 14th century or before, but the case of August d'Este is the most obvious one based on his detailed account of his own illness.[268] Early microscopic descriptions of MS pathology and plaques were detailed by Rindfleisch and Charcot.[268-270] Rindfleisch noted that

> "nerve fibers primarily lose their myelin and then can be traced a considerable distance into the connective tissue of the lesions as axons, devoid of sheaths."[268]

Today, MS is known as a chronic disease of the brain and spinal cord. The inflammatory plaque in the white matter is the histopathological hallmark of MS.[271] MS is classified as relapsing-remitting, primary progressive, or secondary progressive disease, with the majority of patients experiencing relapsing-remitting MS.[272] The etiology of MS is unclear although there are several hypotheses in the field. Genetics is likely to be a contributing factor to MS. There is a 20-30% and 5% disease correspondence between monozygotic and same-sex dizygotic twins, respectively.[273] The MHC class II gene HLA-DRB1 as well as several non-MHC genes identified through genome-wide association studies seem to confer susceptibility to MS.[274,275] Along with genetics, Vitamin D deficiency, and Epstein-Barr Virus (EBV) infection have also been suggested to be MS risk factors. MS prevalence increases as distance from the equator increases, although decreased exposure to ultraviolet light and vitamin D deficiency effects are difficult to separate.[276] Vitamin D deficiency inversely correlates with relapse rate and disability, and vitamin D administration to mice in a mouse model of MS,

experimental autoimmune encephalomyelitis (EAE), prevents EAE.[277-279] Prior infection with EBV is associated with increased MS prevalence.[280] Moreover, in many cases, EBV seroconversion occurred prior to MS diagnosis.[281] Due to the uncertain etiology of MS, mouse models used to study MS have focused on pathogenic mechanisms rather than etiology.

5.2.2 Th1, Th17, and Tregs in MS and EAE

MS is thought to be primarily mediated by autoreactive $CD4^+$ Th1 and Th17 T cells, with contributions from $CD8^+$ T cells and B cells now becoming apparent. In fact, patients with relapsing-remitting MS seem to have a higher frequency of myelin-reactive $CD8^+$ T cells than chronic progressive patients or healthy controls.[282,283] Studies investigating the role of B cells in MS pathogenesis revealed that B cells found in the cerebrospinal fluid of MS patients have undergone somatic hypermutation to presumably CNS-specific peptides, since these clones are absent from the periphery.[284,285] Indeed treatment of some MS patients with a B cell targeting therapy, Rituximab has shown efficacy.[286,287] Cells of the innate immune system including, dendritic cells, natural killer cells, mast cells, invariant natural killer T cells, and γδ T cells, have also been shown to contribute to MS pathology, at least in animal models.[288] However, for the purpose of this dissertation, I will focus on the contributions of $CD4^+$ T cells to MS pathology.

In EAE, myelin-reactive T cells become activated in the periphery and traffic to the CNS after breakdown of the blood-brain-barrier.[289,290] Upon entry into the CNS, T cells are reactivated by resident and infiltrating activated APCs.[291-293] Cognate interactions between T cells and APCs results in increased inflammation resulting in demyelination and axonal damage and loss.

The contributions of $CD4^+$ Th1 and Th17 cells to EAE are being delineated but some results still do not fit into current paradigms. Before the discovery of Th17 cells in 2006, Th1 cells were thought to be the primary T cell population responsible for promoting EAE. However, this idea was challenged when studies were carried out using a series of knockouts that should render Th1 cells non-functional. For instance, $IFN\gamma^{-/-}$ and $Stat1^{-/-}$ mice were just as susceptible to EAE as WT mice, but $T\text{-}bet^{-/-}$ mice (master transcription factor of Th1 cells) were resistant to EAE.[294,295] These results were

unexpected since patients that received exogenous IFNγ had exacerbated disease.[296] To further investigate the contribution of Th1 cells to EAE, the receptor components of the IL-12 receptor, p40 and p35, were deleted in mice. IL-12 signaling is important for Th1 development.[297,298] Adding to the confusion, while IL-12p40$^{-/-}$ mice were resistant to EAE induction, IL-12p35$^{-/-}$ mice were susceptible, suggesting, at the time, a redundancy in the system for Th1 development.[299] Later it was discovered that another cytokine, IL-23, shared a common receptor component with IL-12, IL-12p40. The IL-23 receptor is composed of the unique p19 subunit and the shared p40 subunit.[300] Remarkably, IL-23Rp19$^{-/-}$ mice (lacking only IL-23) were resistant to EAE induction.[301] These results presented the likelihood that another population of autoreactive pathogenic T cells existed and contributed to EAE. Later, this population, now known as Th17 cells, was characterized as producing IL-17A, IL-17F, IL-6, and TNFα. Adoptive transfer of this population into mice yielded EAE.[302] These data were corroborated by findings that IL-17$^{-/-}$ and Rorγt$^{-/-}$ mice had significantly attenuated EAE.[303,304] These findings did not negate a contribution of Th1 cells to EAE pathogenesis since adoptive transfer studies comparing Th1 and Th17 cells demonstrated that both populations could induce EAE.[305] However, each population induced a unique inflammation pattern in the CNS with a requirement for different subsets of chemokines. Therapeutic targeting of IL-23p19 or IL-12/23p40 has shown efficacy in EAE even during established disease (relapse phase).[306,307] Unfortunately, in this case, animal models did not translate well to treatment of human MS, as clinical trials targeting IL-12/23p40 did not show efficacy.[308] However, these studies do not explain the role of T-bet in encephalitogenic T cell responses during EAE. It has been suggested that T-bet controls the encephalitogenicity of T cell responses during EAE rather than specific cytokine responses or Th1 versus Th17 differentiation.[309] Nevertheless, how T-bet regulates T cell responses during EAE is still unclear and whether its functions translate to MS pathogenesis has yet to be determined.

Tregs have also been shown to play an important role in EAE pathogenesis. For example, myelin basic protein (MBP) TCR transgenic mice crossed to RAG-1$^{-/-}$ mice develop spontaneous EAE due to the absence of natural Tregs.[289] Moreover, adoptive transfer of CD4^{+}CD25^{+} T cells three days before immunization or transferred at the same time as autoreactive T cells significantly reduced EAE severity.[310] However, other work

has shown that even when Tregs accumulate in the CNS, they fail to control EAE, suggesting a requirement for Tregs in dampening the immune response in the periphery before entry into the CNS.[311] Related to these findings in EAE, MS patients have decreased *FOXP3* mRNA and FOXP3 protein in purified $CD4^+CD25^+$ T cells.[312] Moreover, expansion of Tregs has been one proposed mechanism of action of the MS-approved drug, glatiramer acetate.[313]

5.2.3 T cell costimulation in EAE and MS

The role of T cell costimulation in EAE and MS has been another area of active investigation. For instance, polymorphisms of *CTLA4* have been associated with susceptibility to MS.[314] Indeed, blockade of CTLA-4 in EAE results in exacerbation of EAE and lack of clinical remission in a relapsing-remitting model of EAE.[315,316] In contrast, treatment with CTLA-4-Ig reduced EAE severity.[317] However, CD80 and CD86 have been shown to have differential roles in EAE. Treatment with an anti-CD80 F(ab) fragment blocked relapses in a SJL model of EAE.[318] In contrast, treatment with an anti-CD80 antibody resulted in exacerbation of EAE, which conflicts with data showing that a small molecular inhibitor of CD28 decreased disease.[319-321] Anti-CD86 treatment has been shown to exacerbate disease or have no effect on disease outcome.[322,323] Additioanlly, treating with anti-CD86 antibody during the remission phase of a relapsing-remitting proteolipid protein-induced EAE model did not affect disease progression, whereas treatment with anti-CD86 at disease induction exacerbated EAE severity.[318,323]

PDCD1 polymorphisms have also been associated with MS disease progression.[324] Exemplifying this, blockade of PD-1 or PD-L2/B7-DC resulted in exacerbated disease in WT and $CD28^{-/-}$ animals.[325] In contrast, PD-L1/B7-H1 blockade did not alter disease outcome.[325] The presence of PD-L1/B7-H1 and the absence of PD-1 on $CD8^+$ T cells in MS lesions has been suggested to be one pathogenic mechanism in MS.[326,327] Similar to PD-1 blockade, ICOS deficiency exacerbates EAE [328,329].

Very few TNF superfamily molecules have been tested in EAE and whether those observed effects are T cell-intrinsic is unclear. However, those that have been tested have shown a therapeutic benefit. For instance, $OX40^{-/-}$ and $OX40L^{-/-}$ mice are significantly protected from EAE.[330,331] These results are supported by therapeutic use of an OX40-Ig

fusion protein and an anti-OX40L neutralizing antibody, which also protected from EAE.[332,333] Also, an agonist antibody against 4-1BB inhibited EAE.[334] In contrast, immunization of $BTLA^{-/-}$ and $HVEM^{-/-}$ mice exacerbated and prolonged EAE.[335,336] Lastly, MBP TCR transgenic $CD40L^{-/-}$ mice and $CD40^{-/-}$ mice were resistant to EAE compare to MBP TCR transgenic and WT mice, respectively.[337,338] CD40 deficiency was required in both the hematopoietic and non-hematopoietic compartments to achieve full protection from EAE.[338] Similar to $CD40L^{-/-}$ mice, mice treated with an anti-CD40L blocking antibody had significantly reduced EAE even if administration occurred after disease induction.[339] Collectively, these data suggest that costimulatory molecules could be interesting therapeutic targets in EAE and in MS. Data shown in Chapters 2, 3, and 4 suggest that Notch may act as a T cell costimulation receptor during GVHD.[223,250,340] Similarly, data described in this chapter suggest that Notch may behave similarly in EAE and thus may also be an attractive therapeutic target in MS, as suggested in GVHD.

5.2.4 Notch in Experimental Autoimmune Encephalomyelitis

Prior studies showed that systemic Notch blockade can attenuate EAE, but provided conflicting information about the intensity and mechanisms of this effect **(Table 5.1)**. Using GSIs, *Notch1* activation and a *Notch1* antisense strategy, Osborne and collaborators reported that Notch directly regulates expression of *Tbx21* (encoding T-bet) in T cells during EAE.[117] GSIs were also described to enhance remyelination and axonal survival in EAE.[341,342] Another study using GSIs and neutralizing antibodies described Notch3 as a dominant receptor influencing EAE via PKCθ expression in Th1 and Th17 $CD4^+$ T cells.[343] Systemic anti-Dll4 treatment was shown to bolster peripheral Treg function during EAE, while others using a similar approach reported altered T cell differentiation or chemotaxis.[118,119] Another group that used Dll4 blockade reported a mild delay in disease when administered before EAE induction and no protection when administered on day 8 after induction.[344] They attributed their mild protection to reduced cytokine responses in the spleen.[344] Moreover, Jagged1-Fc treatment slightly reduced EAE while Dll1-Fc exacerbated EAE, attributable to altered cytokine responses in secondary lymphoid organs without an effect on Tregs.[345] Jagged2 activation was reported to reduce IL-17A in secondary lymphoid organs and increase Treg responses.[124]

Finally, Notch signaling was recently linked to Th9 differentiation in EAE.[124] At least in part, these discrepant results might reflect the use of heterogeneous experimental systems based on systemic Notch modulation or gain-of-function approaches, which can trigger unintended off- and on-target effects and hinder accurate conclusions about Notch function specifically in T cells. This is particularly important in EAE since Notch affects many immune and non-immune cells that contribute to disease pathogenesis (Chapter 1).[60,346]

Table 5.1 Role of Notch signaling in experimental autoimmune encephalomyelitis.

Notch manipulation	Notch effect(s)	Reference
GSI, Notch1 AS, ICN overexpression	Notch1 directly regulates T-bet expression and decreased IFN-γ	117
Jag1-Fc and Dll1-Fc fusion proteins, Jag1 and Dll1 blocking Abs	Jag1-Fc decreased IFN-γ and EAE, increased IL-10	345
GSI, Notch1 and 3 blocking antibodies	Decreased IFN-γ and PKCθ activation	343
Anti-Dll4 blocking Ab	Decreased IL-17, increased Foxp3/Tregs	118
Anti-Dll4 blocking Ab	Decreased T cell chemotaxis to CNS (Ccr1, 2, 5, 6)	119
Anti-Dll4 blocking Ab	Slightly reduced EAE, reduced IL-17, IL-2, GM-CSF, IL-6	344
Jagged2-Fc, Dll1-Fc, ICN1 overexpression, *Notch1*$^{f/f}$*Notch2*$^{f/f}$ x Cd4-cre	Notch1 directly regulates IL-9 leading to decreased IL-17	124
GSI *in vitro*	Decreased active Notch1 in murine oligodendrocytes, decreased demyelination and increased myelination	341,342

To resolve these conflicting results, we investigated Notch function specifically in mature T cells during EAE using several complementary genetic loss-of-function approaches, including expression of the pan-Notch inhibitor DNMAML and inactivation of Notch receptor genes. T cell-specific Notch inhibition resulted in nearly complete protection from EAE, independent of effects on T cell activation and effector

differentiation in secondary lymphoid organs. Notch-deprived $CD4^+$ T cells failed to accumulate in the CNS post-immunization despite preserved *in vitro* migration. Parking WT and DNMAML $CD4^+$ T cells together in bone marrow chimeras increased accumulation of Notch-deprived $CD4^+$ T cells in the CNS but did not suppress disease. In the CNS, Notch-deprived myelin-reactive $CD4^+$ T cells failed to produce IL-17A and IFN-γ, despite preserved expression of the master transcription factor, T-bet. Our data demonstrate that Notch specifically regulates the secondary response of myelin-reactive $CD4^+$ T cells in the CNS independently of effects on T-bet and Tregs during the primary immune response in lymphoid organs.

5.3 Results

5.3.1 Notch inhibition in myelin-reactive $CD4^+$ T cells prevents EAE.

To overcome limitations of past studies, we used *in vivo* genetic loss-of-function models to investigate Notch signaling specifically in T cells during EAE **(Fig. 5.1A)**. We inhibited the Notch transcriptional activation complex downstream of all Notch receptors in mature T cells by expressing a dominant negative Mastermind-like (DNMAML) mutant or by inactivating *Rbpj*, encoding the DNA-binding factor CSL/RBP-Jκ.[86,115] In selected experiments, DNMAML was introduced into 2D2 transgenic T cells, recognizing MOG_{35-55}.[347] Notch inhibition efficiently prevented EAE, with fewer than 8% of mice developing symptoms upon DNMAML expression or CSL/RBP-Jκ loss in T cells, as compared to ~95% of controls **(Fig. 5.1B, C)**. When DNMAML was expressed in transgenic myelin-reactive 2D2 T cells, disease incidence was also markedly reduced **(Fig. 5.1D, E)**. Decreased EAE correlated with reduced demyelination **(Fig. 5.1F)** and cellular infiltrates **(Fig. 5.1G, H)** in the CNS. Previous approaches yielded significantly less protection, likely because of incomplete Notch blockade or redundancy with other family members when only Notch1, Notch3 or Dll4 was inhibited.[117-119,124,343,344] Thus, Notch inhibition specifically in T cells markedly reduced EAE, even with a high frequency of myelin-reactive T cells in TCR transgenic mice.

5.3.2 Preserved effector differentiation of myelin-reactive Notch-deprived CD4$^+$ T cells in lymphoid tissues.

Previous studies in EAE suggested that Notch modulates effector T cell differentiation in secondary lymphoid tissues.[117,118,124] We assessed T cell responses at peak disease using primarily 2D2 transgenic mice in which myelin-reactive T cells can be identified based on Vα3.2$^+$Vβ11$^+$ TCR expression.[347] DNMAML expression in CD4$^+$ T cells preserved T cell activation in draining lymph nodes (DLN) as measured by increased CD44 expression **(Fig. 5.2A)**. Analysis of DLN IFN-γ and IL-17A-producing cells, two important EAE drivers, revealed no significant difference between WT and Notch-inhibited CD4$^+$ T cells as assessed by ELISpot **(Fig. 5.2B).**[348] By flow cytometry, intracellular IL-17A and IFN-γ expression by activated myelin-reactive 2D2/DNMAML T cells was also largely preserved **(Fig. 5.2C)**. These data suggest that Notch inhibition in peripheral myelin-reactive CD4$^+$ T cells did not significantly impact IFN-γ and IL-17A production. These results are similar to data showing that anti-Dll4 treatment did not alter IFNγ and IL-17A production by proteolipid protein-specific T cells in another EAE model.[119]

Prior studies suggested that Notch1 regulates expression of *Tbx21* (encoding T-bet) in Th1 cells, while Dll4-mediated signaling can increase *Rorc* mRNA (encoding Rorγt) in Th17 cells.[117] However, we found no significant change in *Tbx21* transcripts and a trend for increased *Rorc* expression in activated myelin-reactive 2D2/DNMAML CD4$^+$ T cells post-immunization **(Fig. 5.2D)**. We next studied T-bet expression after verifying antibody specificity in *Tbx21*$^{-/-}$ mice during EAE (**Fig. 5.2E**).[226] We observed a preserved frequency of T-bet$^+$ cells and normal staining intensity among Notch-deprived myelin-reactive 2D2/DNMAML CD4$^+$ T cells, consistent with normal T-bet expression in DLN (**Fig. 5.2F**). Past work suggested that Notch receptors, in particular Notch1, can enhance NF-κB activation in T cells by CSL/RBP-Jκ and MAML-independent pathways.[349] To address this, we studied IFNγ production and T-bet expression in T cells lacking Notch1 or both Notch1/Notch2 during EAE. Disease incidence and severity were drastically reduced in the absence of Notch1 or Notch1/2 (not shown), consistent with the dominance of these receptors in T cells. We observed a normal frequency of IFNγ$^+$ cells (**Fig. 5.2G**) and preserved or even slightly increased T-bet levels (**Fig. 5.2H**) in Notch1-

deficient and Notch1/2-deficient T cells, respectively. Thus, blockade of the Notch transcriptional complex or *Notch1/2* inactivation does not impact T-bet and IFNγ production by DLN T cells during EAE.

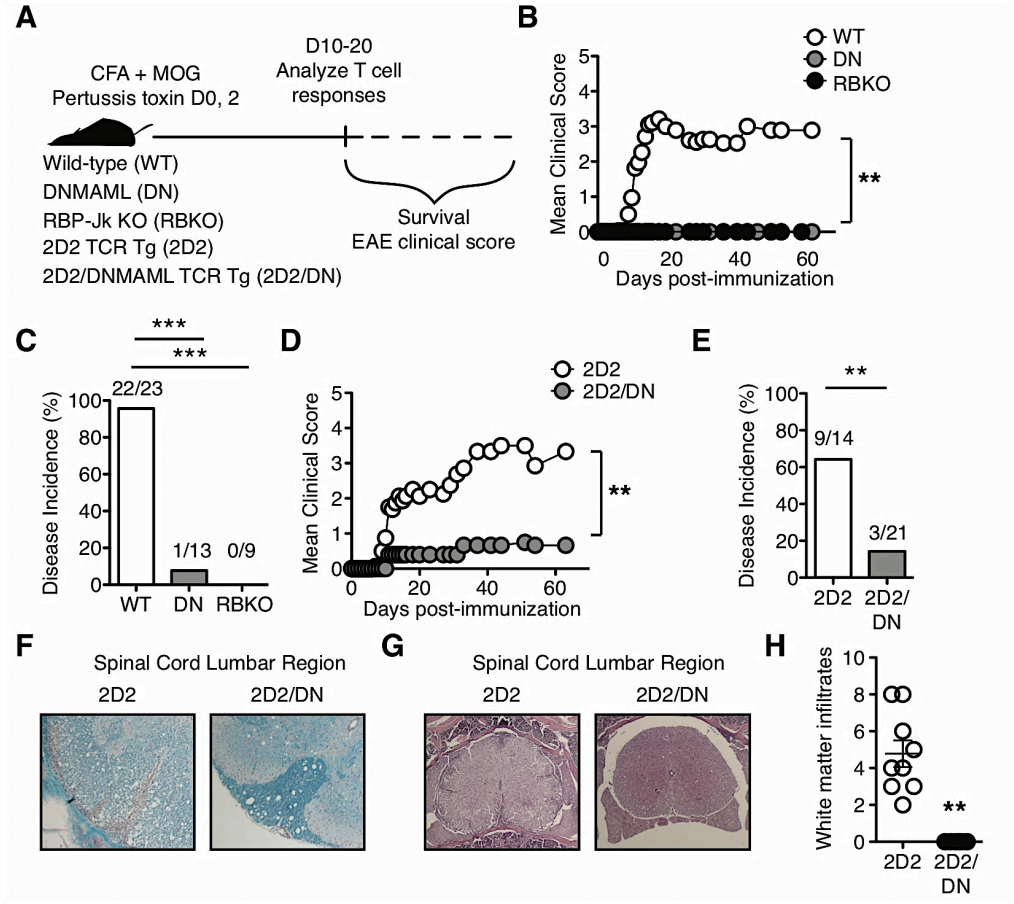

Figure 5.1 Inhibition of Notch signaling in myelin-reactive CD4$^+$ T cells markedly attenuates EAE.
(A) Experimental design; **(B)** Mean clinical EAE score (≥2 experiments); **(C)** Percent disease incidence (score≥2) of immunized WT, DNMAML, or RBKO mice (≥2 experiments); **(D)** Mean clinical score in TCR transgenic 2D2 or 2D2/DNMAML mice (expressing DNMAML in T cells) (≥3 experiments); **(E)** Percent disease incidence (score≥2) of immunized 2D2 and 2D2/DNMAML mice (≥2 experiments); **(F)** Luxol fast blue and **(G)** H&E staining of spinal cord lumbar sections from 2D2 and 2D2/DNMAML mice (representative of n=3 mice/group; 2 experiments); **(H)** Number of white matter infiltrates per H&E section (counted blindly).

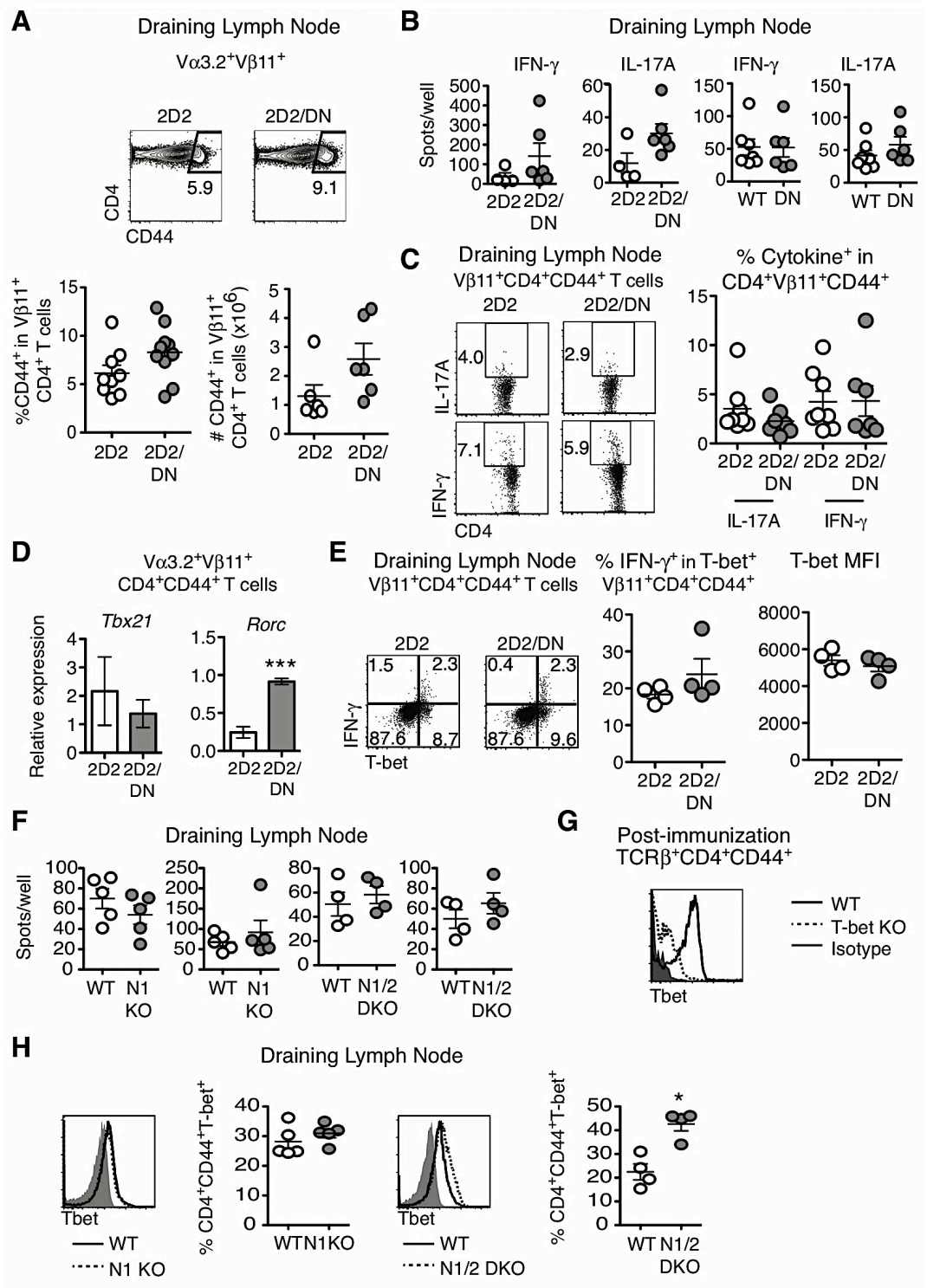

Figure 5.2 Notch inhibition in myelin-reactive CD4$^+$ T cells does not alter initial activation or effector T cell differentiation.

(A) Percent and number of Vβ11$^+$CD4$^+$CD44$^+$ T cells (n=3-4 mice/group; ≥2 experiments). **(B)** Number of IFNγ and IL-17A-secreting cells by ELISpot in DLN from immunized WT, DNMAML, 2D2, and 2D2/DNMAML (n=3-4 mice/group; ≥2 experiments); **(C)** Frequency of IFNγ and IL-17A-producing DLN Vβ11$^+$CD4$^+$CD44$^+$ T cells restimulated with anti-CD3/CD28

and stained for intracellular cytokines (n=3-4 mice/group; 2 experiments); **(D)** *Tbx21* and *Rorc* mRNA in 2D2/DNMAML and 2D2 $CD4^+$ T cells (n=3-4 mice/group; 2 experiments); **(E)** Specificity of T-bet detection. Representative flow cytometry plot; **(F)** Intracellular T-bet in $V\beta11^+CD4^+CD44^+$ T cells by intracellular flow cytometry (2 experiments; n=3-4 mice/group); **(G)** Number of IFNγ and IL-17A-secreting cells by ELISpot in DLN from immunized WT, N1KO and N1/2KO (n=2-3 mice/group; ≥2 experiments); **(H)** Frequency of $T\text{-}bet^+CD44^+CD4^+$ N1KO and N1/2KO T cells measured by intracellular flow cytometry (2 experiments; n=3-4 mice/group).

5.3.3 Notch-inhibited $CD4^+$ T cells fail to accumulate in the CNS despite preserved *in vitro* migration.

Since T cell responses were preserved in lymphoid tissues, we investigated the role of Notch signaling in central nervous system (CNS)-infiltrating $CD4^+$ T cells at peak disease. 2D2/DNMAML $CD4^+$ T cells showed markedly reduced accumulation in the CNS post-immunization **(Fig. 5.3A)**. Similar findings were observed with polyclonal T cells expressing DNMAML or lacking CSL/RBP-Jκ (**Fig. 5.3B**), as well as in the absence of Notch1/2 and to a slightly lesser extent Notch1 (**Fig. 5.3C**). To investigate if Notch-deprived $CD4^+$ T cells failed to traffic to the CNS, α4β1 expression and *in vitro* migration were assessed. The integrin α4β1 drives T cell trafficking into the CNS.[350] We found no difference in α4β1 expression between 2D2 and 2D2/DNMAML $CD4^+$ T cells post-immunization **(Fig. 5.3D)**. Prior work reported that anti-Dll4 inhibits chemotaxis to the CNS due to decreased expression of Ccr2, 5 and 6.[119] In addition, Notch can regulate Ccr7 expression in CNS-infiltrating leukemic T cells.[351] Other chemokine receptors have been linked to $CD4^+$ T cell infiltration into the inflamed CNS, such as Cxcr3.[352] However, we found no significant change in expression of these chemokine receptors by 2D2/DNMAML T cells during EAE (data not shown). Next, we assessed responses of Notch-deprived myelin-reactive $CD4^+$ T cell to all candidate chemokine receptors *in vitro* (**Fig. 5.3E**). Purified, primed 2D2/DNMAML $CD4^+$ T cells migrated at least as well as control 2D2 T cells post-immunization. These data indicate that Notch-deprived myelin-reactive $CD4^+$ T cells can migrate towards chemotactic signals at least *in vitro*, in contrast to results obtained after blocking Dll4 in EAE.[119]

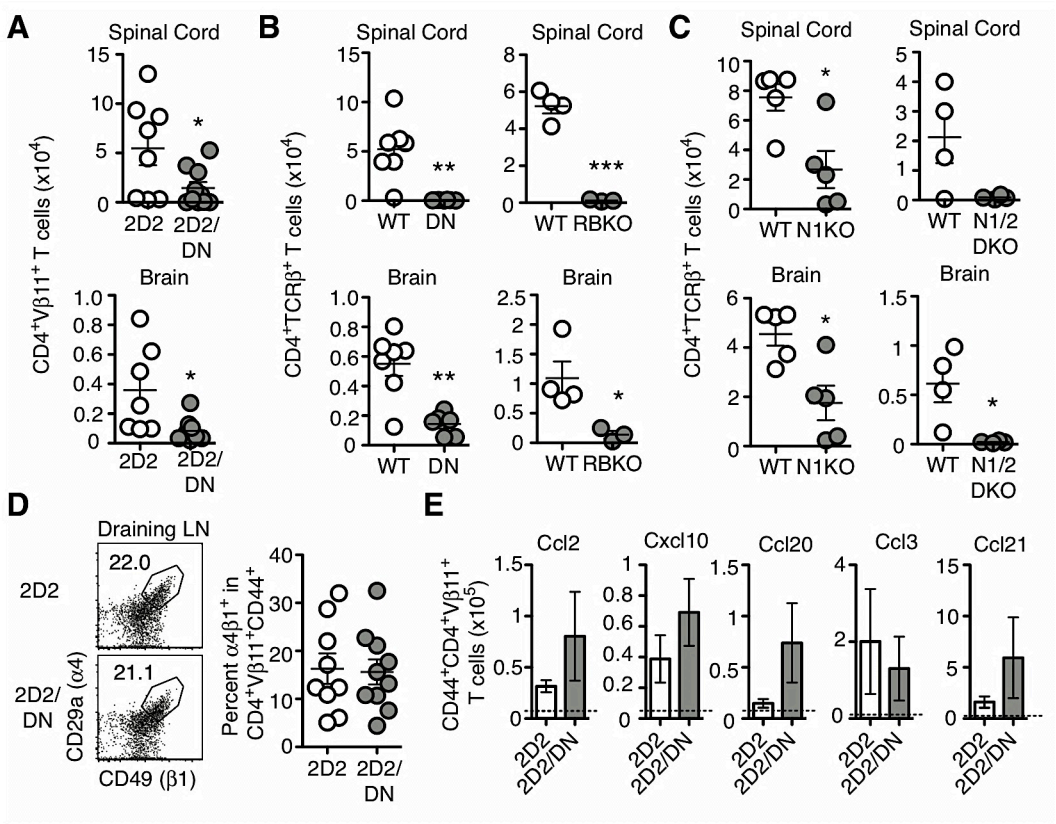

Figure 5.3 Notch-deprived myelin-reactive CD4$^+$ T cells fail to accumulate in the CNS *in vivo* but have preserved trafficking *in vitro*.
Spinal cord and brain infiltrates from immunized **(A)** 2D2, 2D2/DNMAML; **(B)** WT, DNMAML, RBKO; **(C)** WT, N1KO, N1/2KO mice. Numbers of CD4$^+$ T cells were measured by flow cytometry (n=2-4 mice/group; ≥2 experiments); **(D)** Expression of α4β1 in 2D2/DNMAML Vα3.2$^+$Vβ11$^+$CD4$^+$CD44$^+$ T cells measured by flow cytometry (n=3-4 mice/group; 3 experiments); **(E)** Transwell migration of 2D2/DNMAML Vβ11$^+$CD4$^+$CD44$^+$ T cells towards indicated chemokines. Dotted line shows background number of primed 2D2 Vα3.2$^+$Vβ11$^+$CD4$^+$CD44$^+$ T cells migrating without chemokine (n=3-4 mice/group; 3 experiments).

5.3.4 Myelin-reactive Notch-deprived CD4$^+$ T cells do not suppress EAE induced by WT CD4$^+$ T cells.

Past studies using systemic Notch ligand fusion proteins and antibodies suggested that Notch modulation increased Treg frequency, resulting in decreased EAE severity.[118] To assess if increased Treg frequency or function contributed to protection from EAE, we assessed Foxp3 expression, focusing on non-transgenic T cells that have the most Treg activity.[353] No change in Treg frequency was observed in 2D2/DNMAML mice post-immunization **(Fig. 5.4A)**. We observed a slight but significant increase in Treg frequency in DNMAML compared to WT CD4$^+$ T cells post-immunization **(Fig. 5.4B)**.

To assess suppressive capacity of DNMAML T cells post-immunization, we generated mixed bone marrow chimeras to park WT and DNMAML T cells in the same recipients **(Fig. 5.4C)**. WT/DNMAML chimeras succumbed to EAE at the same frequency as mice containing only WT T cells, suggesting the absence of dominant suppressor function in DNMAML T cells **(Fig. 5.4D-E)**.

The very low abundance of DNMAML T cells in the CNS prevented accurate assessment of their effector function. It was previously shown that Ccr6-deficient T cells failed to efficiently traffick to the CNS during EAE.[354] However, bystander WT T cells induced Ccr6-independent effector T cell migration into the CNS. To determine if WT T cells could overcome the inability of Notch-deprived $CD4^+$ T cells to accumulate in the CNS, we measured T cell numbers in the CNS of bone marrow chimeras at peak disease. DNMAML $CD4^+$ T cells partially regained their ability to accumulate in the CNS in the presence of WT $CD4^+$ T cells **(Fig. 5.4F)**. This strategy suggests that bystander WT T cell inflammation can induce Notch-deprived T cells to accumulate in the CNS, allowing us to study their effector function in the CNS.

5.3.5 Inhibition of Notch signaling in myelin-reactive $CD4^+$ T cells in the CNS blocks IL-17A and IFN-γ expression independently of T-bet.

Decreased T cell reactivation in the CNS can result in reduced EAE severity.[292] Since DNMAML $CD4^+$ T cells accumulate in the CNS in the presence of WT T cells **(Fig. 5.4F)**, we studied the impact of Notch inhibition on T cell effector differentiation in the CNS. In immunized mixed bone marrow chimeras, wild-type $CD45.1^+$ competitor T cells were an internal positive control. While DNMAML $CD4^+$ T cells produced IFNγ and IL-17A in DLN **(Fig. 5.2B, C, F, 5.5B)**, they had markedly reduced production of IFNγ or IL-17A in the CNS **(Fig. 5.5A)**. The blunted cytokine response occurred without defect in T-bet expression, as evidenced by the presence of many T-$bet^+IFN\gamma^-$ cells **(Fig. 5.5B)**. Our results suggest that Notch-mediated regulation of IFNγ production is an important feature of its effects in EAE, but through mechanisms that are independent of T-bet and CNS-restricted. Moreover, decreased T cell migration into the CNS could account for some of the effects of Notch inhibition. However, it cannot fully explain

protection from EAE as Notch-inhibited CD4$^+$ T cells that enter the CNS in the presence of bystander wild-type T cells have markedly reduced effector function.

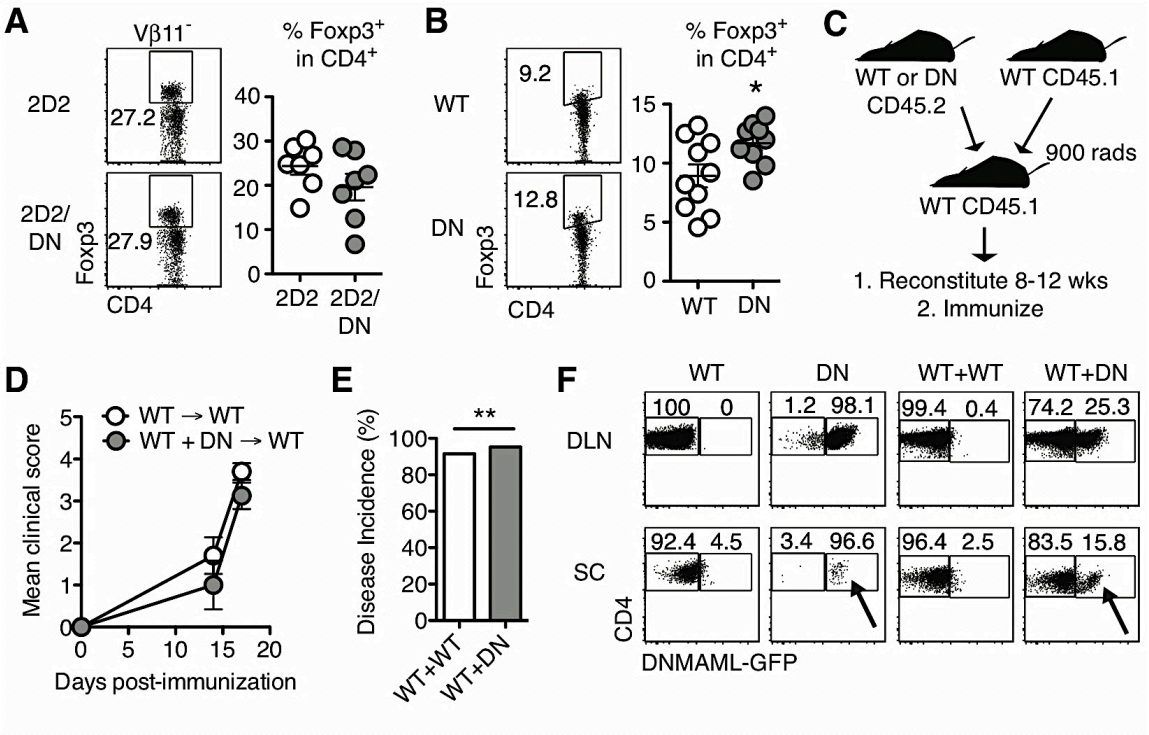

Figure 5.4 CNS accumulation of DNMAML CD4$^+$ T cells is enhanced in the presence of WT CD4$^+$ T cells, but Notch-deprived CD4$^+$ T cells fail to suppress disease.

Foxp3 expression in **(A)** Vβ11$^-$CD4$^+$ 2D2/DNMAML and **(B)** CD4$^+$ DNMAML T cells measured by intracellular flow cytometry (n=3-4 mice/group; ≥2 experiments); **(C)** Experimental design; **(D)** Mean clinical score (representative) (score≥2; 5 experiments); **(E)** Percent disease incidence (score≥2) of immunized BM chimeras (3 experiments); **(F)** Representative flow cytometry plots from BM chimeras. T cells were tracked by CD45.1 and CD45.2 expression at peak disease (n=4-5 mice/group; 3 experiments).

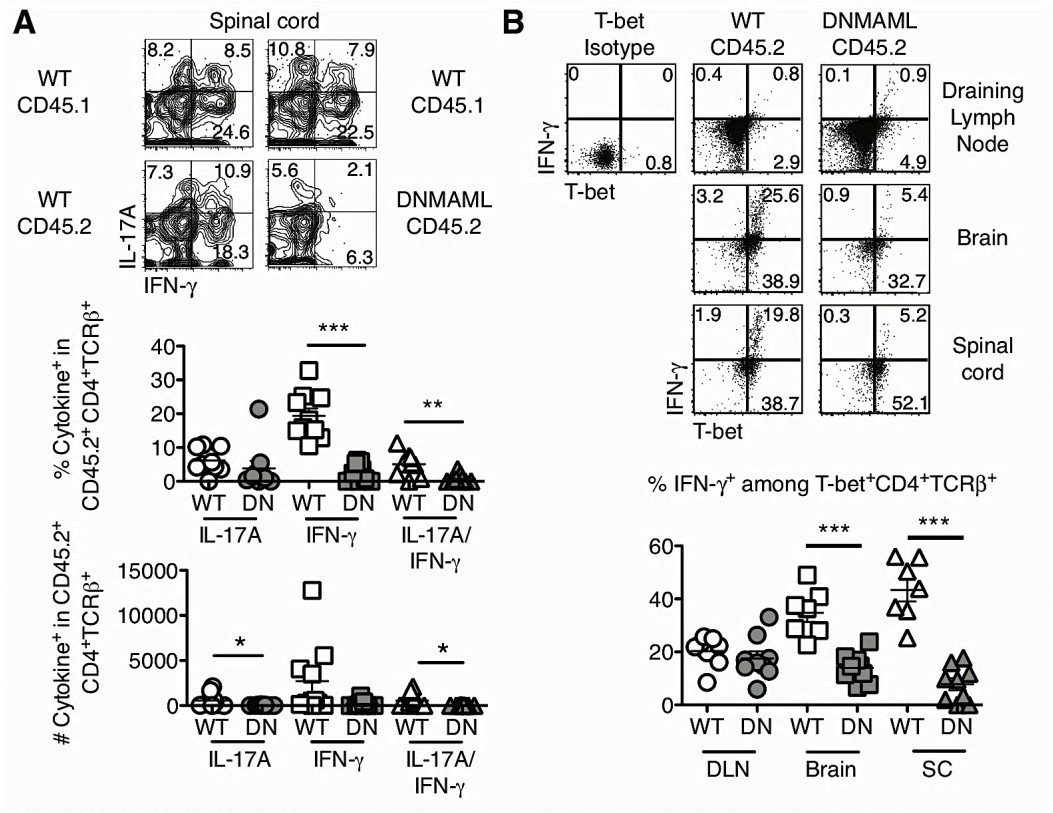

Figure 5.5 Notch-deprived CD4$^+$ T cells have markedly reduced IL-17A and IFNγ production in the CNS despite preserved T-bet expression.
Spinal cord infiltrates from immunized BM chimeras restimulated for 6 hours with anti-CD3/CD28. **(A)** Frequency and number of IFNγ and IL-17A-producing DNMAML CD4$^+$ T cells in the DLN or CNS by intracellular flow cytometry (n=4-5 mice/group; 2 experiments); **(B)** Intracellular T-bet and IFNγ expression by DN CD4$^+$ T cells in the CNS by flow cytometry (n=4-5 mice/group; 2 experiments).

5.4 Discussion

Our findings highlight an essential role for Notch signaling in CD4$^+$ T cells mediating experimental autoimmune encephalomyelitis (EAE). We used pan-Notch genetic inhibition as well as inactivation of individual Notch receptor genes to fully determine the impact of Notch signaling loss-of-function on CD4$^+$ T cells during EAE. We found that pan-Notch inhibition in MOG-transgenic T cells and polyclonal T cells nearly completely protected from EAE. This protection was not due to an effect on myelin-reactive T cell activation or differentiation in secondary lymphoid tissues or a dominant suppressor function of DNMAML T cells. In contrast, Notch-deprived T cells failed to accumulate in the CNS, despite preserved *in vitro* chemotaxis. Parking WT and

DNMAML T cells together in bone marrow chimeras resulted in enhanced accumulation of DNMAML T cells in the CNS. Analysis of accumulated DNMAML T cells in the CNS of bone marrow chimeras revealed a significant defect in IL-17A and IFNγ production, despite preserved T-bet expression. Collectively, Notch signaling regulates myelin-reactive T cells during EAE through mechanisms that have not previously been reported.

Past work suggested that Notch regulates myelin-reactive T cells in secondary lymphoid organs through regulation of T-bet, IFNγ, IL-17A, or Treg function[117,118,124,345] In contrast, our work highlights a function for Notch signaling in myelin-reactive T cells in the CNS and not in secondary lymphoid tissues. Indeed, inhibition of Notch signaling in T cells in secondary lymphoid tissues did not substantially affect T-bet expression, IFNγ and IL-17A production, or Treg function. The reasons for these differences are unclear but may reflect the various methods of Notch signaling modulation used. For instance, many past reports have relied on overexpression of Notch receptors or systemic modulation of Notch signaling with GSIs, blocking antibodies, or agonistic fusion proteins. As discussed in Chapter 1, many cell populations in the immune system require Notch signaling and presence of Notch ligands and receptors does not necessarily lead to active Notch signaling. Thus, using approaches that exceed physiological levels of Notch signaling or systemically modulate Notch signaling may result in bystander effects on other cell types that impact T cells, independently of Notch signaling in T cells. Preserved T cell differentiation in secondary lymphoid tissues was reported with Dll4 blockade during EAE.[119] However, they reported defects in T cell chemotaxis which we did not replicate using genetic loss-of-function approaches in T cells.

Notch signaling has previously been suggested to regulate T-bet expression.[117] Despite drastically decreased IFNγ production in the CNS by Notch-inhibited T cells, myelin-reactive Notch-deprived T cells had preserved expression of T-bet. This is similar to previous work published by our laboratory in which allogeneic Notch-deprived T cells had preserved expression of T-bet but failed to produce IFNγ (Chapter 3).[223,250] Other signaling cascades such as IL-12 and IL-27 have been shown to elicit T-bet expression and could account for preserved T-bet expression in Notch-deprived T cells.[243,355]

Chemotaxis to the CNS is regulated by many redundant signaling cascades.[356] Of these, Notch has been suggested to regulate CCR1, CCR2, CCR5, and CCR6 in EAE, and CCR7 in CNS-homing leukemia cells.[119,351] In contrast, we found that pan-Notch inhibition in myelin-reactive T cells did not affect migration in response to these chemotactic signals, at least *in vitro*. These differences may reflect differences in systemic blockade with Dll4 versus genetic inhibition of Notch in T cells. Inhibition of Dll4 in other populations in secondary lymphoid organs may elicit chemotaxis changes in T cells that are not intrinsic to Notch-inhibited T cells. Additionally, Notch signaling may modulate integrin expression and/or function. For example, the integrin, $\alpha 4\beta 1$, is required for T cell chemotaxis to the CNS during EAE and MS.[350,357] Although Notch-deprived T cells expressed similar levels of surface $\alpha 4\beta 1$, the functionality of this integrin was not determined and could account for their decreased accumulation in the CNS. Prior work has shown that IFNγ induces expression of the $\alpha 4\beta 1$ ligand, VCAM-1, on endothelial cells to facilitate cell adhesion followed by transendothelial migration.[358,359] It is possible that Notch-inhibited T cells acquire a defect in IFNγ production in route to the CNS and fail to trigger VCAM-1 upregulation on endothelial cells. This would be consistent with partial rescue of CNS accumulation in the presence of bystander wild-type T cells. Alternatively, Notch-deprived T cells may have preserved migration to the CNS, which is suggested by our *in vitro* studies, but may fail to survive or proliferate in the CNS. Failure of myelin-reactive T cells to be locally reactivated in the CNS could account for decreased accumulation of Notch-deprived T cells in the CNS.[292,293]

Notch-inhibited myelin-reactive T cells failed to suppress disease induced by WT T cells in mixed bone marrow chimeras. This is in contrast to prior work suggesting that Dll4 blockade expanded Tregs, which resulted in slightly reduced EAE severity.[118] These discrepancies could reflect differences in experimental strategy. Notch-inhibited T cells were mixed with WT T cells in bone marrow chimeras. A mild increase in suppressive capacity intrinsic to Notch-deprived T cells may have been overcome by the large population of WT T cells in the same animal, preventing a complete analysis of Treg-mediated suppression. Also, TCR transgenic mice in which Tregs are present in the non-

transgenic T cell fraction still succumb to EAE, indicating that Tregs may contribute to suppressing disease but are not the only explanation.[353]

Collectively, our results suggest a function for Notch in T cells during EAE that is compartment-specific and CNS-restricted. Inhibition of Notch signaling in mature T cells preserved T cell differentiation in secondary lymphoid organs but not in the CNS. The reason for this difference is unclear but could be explained by local exposure to Notch ligands during T cell restimulation in the CNS. In contrast, Notch may signal in T cells in secondary lymphoid organs or vasculature with the importance of the Notch signals only becoming apparent in the CNS **(Figure 5.6)**. Future experiments will work to localize the source of Notch ligand(s) responsible for T cell function during EAE.

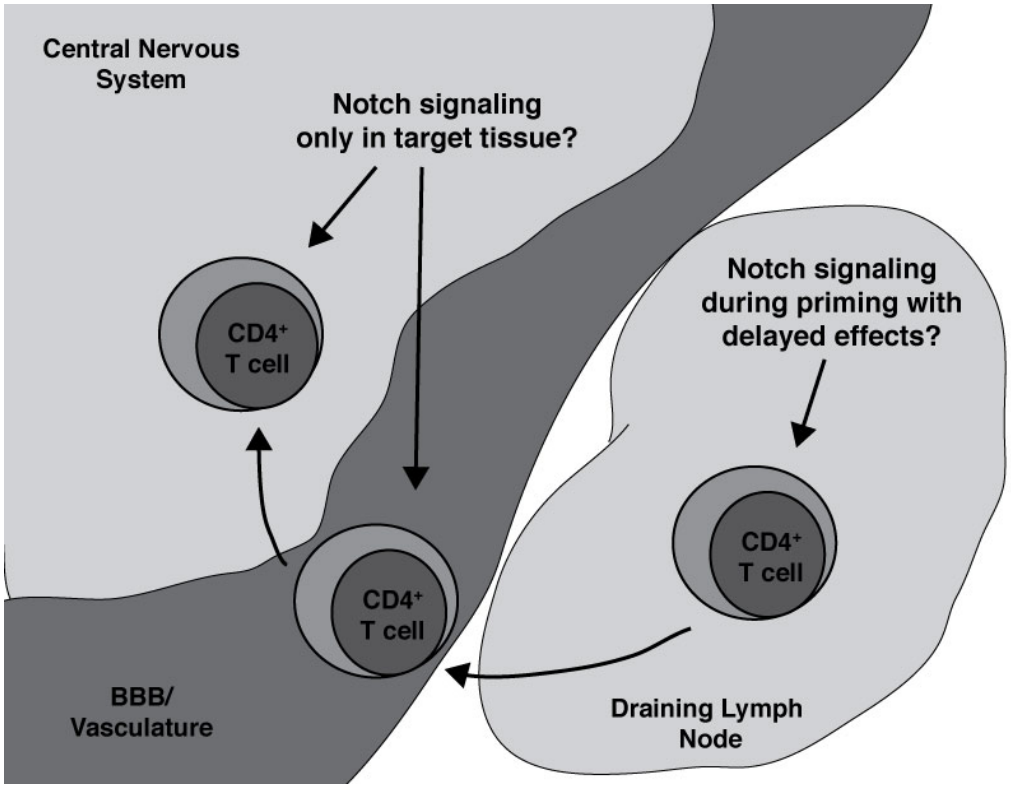

Figure 5.6 Potential sources of Notch signaling during experimental autoimmune encephalomyelitis.
Notch signaling may act on T cells in secondary lymphoid tissues with effects only appearing once they are in the CNS. Alternatively, T cells may receive Notch signals in the blood stream, at the blood-brain-barrier (BBB), or in the CNS that results in potent cytokine production in the central nervous system independently of T-bet.

In summary, we provide clear definitive evidence for a role for Notch signaling in myelin-reactive $CD4^+$ T cells mediating EAE that differs from all prior reports of Notch function in T cells during EAE. Since we inhibited the Notch transcriptional activation complex in T cells, future work will focus on identifying target genes downstream of Notch signaling that elicit these effects. With new humanized Notch ligand and receptor specific antibodies being made recently available, it will be interesting to determine the critical Notch receptors and ligands mediating EAE.[221] This knowledge will contribute towards therapeutic strategies for targeting Notch signaling in MS to decrease MS severity and clinical relapses.

Chapter 6

Conclusions and Perspectives

6.1 Summary

Collectively, the data presented in this thesis demonstrate that Notch signaling modulates T cell responses in two medically relevant T cell-mediated immune disorders: GVHD and EAE. In Chapters 2, 3, and 4 we demonstrated that inhibition of Notch signaling in alloreactive $CD4^+$ and $CD8^+$ T cells significantly reduced GVHD severity and lethality in CD4- and CD8-dependent MHC and miHA models of GVHD (Summarized in **Figure 4.12**).[223,250,340] Notch-deprived T cells had preserved activation but significantly reduced production of several proinflammatory cytokines. Despite blunted IFNγ production by Notch-deprived $CD4^+$ and $CD8^+$ T cells, Notch-deficient T cells had preserved or even enhanced expression of T-bet and Eomesodermin, two drivers of IFNγ production in $CD4^+$ and $CD8^+$ T cells, respectively. These data demonstrate that Notch-deficiency did not result in a classical Th1 or $CD8^+$ T cell effector differentiation defect. However, defective IFNγ production could be partially to fully rescued by bypassing more proximal steps of TCR signaling pharmacologically. Downstream of TCR signaling, we found a significant defect in Ras/MAPK and NF-κB pathway activation in Notch-deprived $CD4^+$ and $CD8^+$ T cells. Additionally, Notch-deprived $CD4^+$ and $CD8^+$ T cells had several features that were reminiscent of anergic T cells **(Fig. 3.12)**.[233,234,240] Concomitant with reduced proinflammatory cytokine secretion and an anergy-like phenotype, Notch-deprived $CD4^+$ T cells contained a higher frequency of natural Tregs after allo-BMT. Lastly, Notch-deprived $CD4^+$ and $CD8^+$ T cells maintained cytotoxic potential in *in vitro* and *in vivo* cytotoxicity assays, suggesting a "split-anergy" phenotype. Together, these data illustrate that inhibition of Notch signaling dissociates cytokine production from cytotoxic function and may be an attractive therapeutic target after allo-BMT.

Analysis of the receptors and ligands mediating Notch effects in T cells revealed a dominant role for Notch1 and Dll4 with minor roles for Notch2 and Dll1 in mediating GVHD.[340] Therapeutic targeting of Dll1 and Dll4 using neutralizing humanized

monoclonal antibodies demonstrated that even a short two-week burst of treatment was able to protect mice long-term from acute, lethal GVHD. Remarkably, treatment with anti-Dll1 and Dll4 did not inhibit hematopoietic recovery post allo-BMT. Moreover, anti-Dll1/Dll4 treatment preserved the cytotoxic potential of the T cells after BMT. Thus, therapeutic inhibition of Notch signaling after allo-BMT achieves beneficial immunomodulation without global immunosuppression.

In Chapter 5, we investigated the role of Notch signaling in EAE. We observed that inhibition of Notch signaling in myelin-reactive T cells significantly protected from EAE, more so than previously reported approaches to inhibit Notch signaling. In contrast to prior results with systemic Notch inhibition or Notch gain-of-function, the protection from EAE was not explained by an effect on T cell activation or differentiation in secondary lymphoid tissues. We observed preserved IL-17A and IFNγ production and expression of T-bet (for Th1 cells) and Rorγt (for Th17 cells) in myelin-reactive $CD4^+$ T cells in secondary lymphoid organs. However, Notch-deprived T cells failed to accumulate in the CNS post-immunization, despite preserved *in vitro* chemotaxis. Parking WT and Notch-deprived T cells together in bone marrow chimeras enhanced the accumulation of Notch-deprived T cells in the CNS. Additionally, Notch-deficient T cells were unable to prevent disease induced by WT T cells, suggesting the absence of a dominant suppressor function of Notch-inhibited T cells. Analysis of Notch-deprived T cells that did accumulate in the CNS of bone marrow chimeras revealed a significant defect in their production of IL-17A and IFNγ, despite preserved expression of T-bet. These data are the first to clearly define a role for Notch signaling in myelin-reactive T cells during EAE and promote Notch as a potential therapeutic target in MS.

Our results in GVHD and EAE differ from all prior reports of Notch function in mature T cells, demonstrating the benefit of using T cell-specific loss-of-function approaches to study Notch signaling in mature T cells. Moreover, our data reveal that Notch signaling regulates T cell responses in a context-dependent manner.

6.2 Mechanistic insights into Notch regulation of T cell function

Prior studies in the hematopoietic system have sought to determine a role for Notch signaling in T cell differentiation and function. For instance, Notch has been suggested to directly regulate a large array of cytokines and effector molecules. These results were achieved through a variety of approaches, many of them being gain-of-function or systemic modulation of Notch signaling. These findings highlight several questions about the mechanism(s) of Notch action in mature T cells. First, does Notch signaling play the same role in T cells regardless of the immune context? Second, do varied results between different experimental systems and immune responses appear to be similar when in fact some effects may have been attributed to the incorrect mechanisms of Notch action? Third, does Notch truly regulate immune responses in a context-dependent manner?

Early work on Notch in mature T cells attempted to frame Notch into its historical context as a lineage determinant. For instance, studies in *Drosophila* demonstrated that Notch was pivotal for specifying one cellular fate at the expense of another. Similarly, investigations of Notch in T cells showed that Notch signaling specified the T cell fate at the expense of B cells. Fitting into this paradigm, initial work on Notch in T cell differentiation suggested that Delta ligands promoted Th1 development while Jagged ligands elicited Th2 development.[111] However, our laboratory's work and data presented in this thesis suggest that Notch does not simply drive lineage decisions based on its regulation of one transcription factor or cytokine over another. In fact, Notch signaling may integrate with larger signaling networks, such as TCR signaling and costimulation, to impact T cell differentiation and function in a context-dependent fashion.

Detecting ICN or CSL/RBP-Jκ bound to transcription factor or cytokine target gene loci has often been sufficient to suggest that Notch signaling regulates these loci. However, there is new insight from Aster and colleagues who performed ChIP-Seq on the genomes of three T-ALL cell lines.[360] They showed that only 36% of CSL/RBP-Jκ binding sites overlapped with Notch1 binding sites. Moreover, 97% of genes with Notch1 binding in the promoter did not respond significantly to Notch signaling. This result is similar to data in yeast showing that only 3% of occupied promoters responded to disruptions of transcription factors.[361] Since these data were performed in T cell lines that

were grown *in vitro,* they may not accurately reflect the function of Notch signaling *in vivo* in mature T cells. However, it does raise the question of whether simply finding Notch or CSL/RBP-Jκ bound to a gene translates to functional and meaningful regulation of that gene. The data from Aster and colleagues would suggest that indeed it does not, although these data do not rule out the possibility that many new targets downstream of Notch signaling may still exist. Despite this possibility, it is unlikely that Notch signaling independently regulates specific T cell differentiation transcription factors and cytokines. Instead, Notch signaling may intercept larger signaling pathways to elicit context-dependent effects. For example, Notch crosstalks with pre-TCR and PI3K/Akt signaling to elicit changes in DN3 thymocytes, perhaps through direct regulation of HES1.[362] In GVHD, Notch signaling context-dependently modulates TCR signals to dampen cytokine responses. In EAE, Notch signaling does not alter T cell function in secondary lymphoid organs, but in the specific context of the CNS, dampens cytokine responses potentially through crosstalk with TCR signaling and costimulatory molecules.

Several possibilities emerge that could explain the ability of Notch signaling to regulate immune responses in a context-dependent manner. First, exposure to specific Notch ligands could differ in various immune responses. For instance, in the highly inflammatory environment of GVHD, Notch ligands could be differentially expressed and regulated in comparison to other immune responses where inflammation is more spatially restricted. Second, Notch signaling could be actively maintained in an off state in certain tissues despite the presence of Notch ligand. For example, in B cell development in the bone marrow, Notch signaling is actively maintained off through the inhibitory actions of LRF on Dll4 expression in erythroblasts.[65] During EAE, Notch signaling could be prevented in T cells in secondary lymphoid organs through an LRF-like mechanism in a T cell-interacting cell but the absence of this factor in CNS-resident cells could promote Notch signaling in T cells in the CNS. Third, expression of specific Notch receptors or post-translational modification of these receptors by fringe glycosyltransferases could modify the response of T cells to Notch ligands. In developing thymocytes, Notch1, 2, and 3 are all expressed, but Notch1 preferentially interacts with Dll4 due to actions of Lunatic Fringe.[34,363] In GVHD, Notch1 and Dll4 are the dominant receptor and ligand, respectively, despite other Notch receptors and ligands being present.

[340] Fringe modification of Notch1 or spatial and temporal availability of Notch ligands could explain the context-dependent regulation of GVHD by Notch signaling. Fourth, certain inflammatory contexts or factors produced during different immune responses could bypass the requirement for Notch signaling. For instance, clearance of *Leishmania major* in mice does not require Notch signaling in T cells.[115]

Collectively, Notch signaling may regulate immune responses in a context-dependent manner. Evidence from studies in different hematopoietic cells suggests that the context-dependence of Notch regulation is determined by availability of Notch ligands, presence of active negative regulators of Notch signaling, post-translational modification of Notch receptors, and the inflammatory context of the immune response.

6.3 Notch signaling as a modulator of costimulation pathways?

Our data revealing a role for Notch in T cells during GVHD and EAE paint Notch signaling as a costimulatory pathway or a modulator of signaling pathways that alters T cell function in a context-dependent fashion. In the absence of Notch signaling, T cell cytokine responses are significantly dampened despite preserved expression of master transcription factors for T cell differentiation. In contrast, presence of Notch signaling results in robust pro-inflammatory T cell responses, eliciting severe, lethal GVHD and EAE. Similar roles for other costimulatory molecules have been observed in GVHD and EAE. For example, mice treated with an agonistic 4-1BB antibody had increased GVHD severity and production of IFNγ and TNFα by thoracic duct lymphocytes.[193] In contrast, deficiency in OX40 during EAE induction resulted in significantly reduced IFNγ, IL-2, and IL-6 production and protection from EAE.[330] Thus, similar to these data, Notch may be functioning as a costimulatory molecule or modulating costimulatory pathways in GVHD and EAE to stimulate context-dependent effects in these T cell-mediated immune disorders. For instance, work presented in Chapter 3 suggest that absence of Notch signaling in T cells during GVHD elicits a hyporesponsive phenotype reminiscent of T cells that failed to receive a costimulation signal. Also, data in Chapter 4 demonstrated that short-term inhibition of Notch signaling during the first two weeks of GVHD

resulted in long-lasting protection, suggesting induction of tolerance similar to failed costimulation.

The question remains open as to how Notch signaling crosstalks with other pathways or functions as a costimulatory molecule in T cell-mediated immune disorders. Several possibilities can be considered. First, Notch signaling could directly regulate costimulatory molecule gene expression, which has been suggested in T-ALL.[364] Second, Notch could directly regulate genes required to amplify TCR or costimulatory signals. Third, Notch could directly regulate Hes or Hey proteins, which could turn off negative regulators of TCR signaling or costimulatory pathways, which has been suggested to occur in T cell development with HES1 regulation of PTEN.[362] The idea of Notch modulating another signaling cascade is not a novel idea. For instance, the data we provided in Chapter 3 demonstrates that Notch signaling positively regulates TCR signaling, and in the absence of Notch signaling, TCR signaling is blunted. Another example of Notch signaling modulating another signaling pathway is in T cell development in the thymus. At the pre-TCR checkpoint at the DN3 stage of T cell development, Notch signaling, pre-TCR signals, and PI3K/Akt signaling synergize to promote cellular metabolism and survival.[98,99]

If Notch influences signaling by costimulatory receptors in T cells mediating GVHD and EAE, then it is essential to understand how the expression of Notch ligands is regulated and if this regulation shares similarities with that of costimulatory ligands. Previous studies have shown that TRIF-dependent and –independent TLR signaling can regulate costimulatory molecule expression.[365] Likewise, TLR signaling was reported to enhance Dll4 and Jagged1 expression in antigen presenting cells.[366,367] Thus, conditioning regimens prior to transplantation may promote cellular damage as well as release of gut constituents, leading to enhanced TLR signaling and increased Notch ligand expression in allo-BMT recipients. However, TLR-independent mechanisms of Notch ligand upregulation have also been reported.[368,369] If Notch cooperates with classically defined costimulation pathways, then it is tempting to speculate that Notch ligands would be expressed by the same cells presenting allo- or auto-antigens. For instance, prior work demonstrated that optimal T cell responses occur when the peptide antigen and costimulation machinery are expressed on the same antigen presenting cell.[370] In GVHD

and EAE, it remains unclear exactly which cell(s) is/are presenting the peptide antigen and the Notch ligand(s) and in which compartment these cells reside. It remains possible that distinct cells can present MHC/antigen complexes and Notch ligands to T cells, either simultaneously or sequentially.

In view of our experimental approach using DNMAML, it is clear that the effects of Notch signaling in T cells during EAE and GVHD involve the canonical Notch transcriptional activation complex. We interfered with the Notch transcriptional activation complex, which indicates that Notch is directly regulating a target gene(s) that is (are) then used to alter T cell differentiation and function, perhaps through intercepting another signaling pathway. The direct targets of Notch signaling in alloreactive and autoreactive T cells that would elicit these effects are unknown but it is a focus of future research. Additionally, interfering with the Notch transcriptional activation complex rules out a non-canonical function of Notch signaling. Non-canonical functions of Notch signaling have been proposed to occur in mammals, but the mechanisms underlying these effects are ill defined.[49,50] Our GVHD and EAE work utilized loss-of-function of Notch receptors and Notch transcriptional complex factors, which resulted in the same phenotype. The functional and phenotypic changes we observed in Notch-deprived T cells were the result of decreased expression of a Notch target gene(s).

Thus, there is precedence for Notch modulating other pathways to alter T cell function, and data provided in this thesis suggest that in mature T cells, Notch may synergize with T cell signaling pathways or act as a costimulatory molecule to support T cell function in its context-dependent regulation of T cell-mediated immune disorders.

6.4 Notch as a therapeutic target in T cell-mediated immune disorders?

Data provided in Chapter 4 suggest that targeting individual Notch receptors and/or ligands may be an effective strategy for treating T cell mediated immune disorders. In Chapter 4, we demonstrated that targeting Dll1/4 in GVHD even during a short course provided long-lasting protection from GVHD, while preserving T cell cytotoxicity and hematopoietic reconstitution after allo-BMT. Targeting individual ligands was key to the therapeutic success of this strategy since pan-Notch inhibition with

GSIs resulted in on-target side effects in the gut, leading to lethality due to a severe lack of gastrointestinal tissue regeneration rather than GVHD. These GSI toxicity results are corroborated by clinical trials with GSIs that reported several side effects, including diarrhea and severe fatigue.[371] Although treatment with GSIs after allo-BMT did not lead to goblet cell hyperplasia, which is the usual on-target side effect of Notch inhibition in the gut.[372] Rather, the GI tract was much more acutely sensitive to inhibition of Notch signaling in the post-irradiation environment, likely due to an effect on the intestinal stem cells rather than on cellular differentiation in the gut.[266,373] Likely, in the case of MS, using GSIs as a therapeutic would result in similar gastrointestinal side effects, like diarrhea, as those observed in clinical trials, since the intestine would not be sensitized by irradiation.[371] Thus, targeting individual Notch receptors and ligands appears to be a more advantageous therapeutic approach.

Targeting the individual Notch ligand Dll4 has been explored in angiogenesis based on data showing that *Dll4* haploinsufficiency leads to angiogenesis defects and embryonic lethality.[374-376] Blocking Dll4 as an anti-angiogenic therapy revealed that anti-Dll4 promoted non-productive vasculogenesis in the tumor, inhibiting tumor growth. Normally, Notch1-Dll4 signaling restricts the number of tip cells and promotes stalk cell development whereas Dll4 inhibition induces tip cells at the expense of stalk cells, leading to nonproductive vasculature.[35] Chronic administration of anti-Dll4 antibodies has been associated with adverse events, including vascular hyperproliferation in the liver and vascular neoplasms.[377,378] Thus, chronic inhibition of Dll4 may not be therapeutically advantageous for patients given these side effects. Fortunately, long-term protection form GVHD is achieved with only a short course of treatment, suggesting that these negative side effects could be avoided. In contrast, if Dll4 turns out to be the dominant ligand mediating Notch effects in EAE, these side effects would be more concerning. For instance, chronic inhibition of Notch signaling may be required for therapeutic use of Notch inhibitors in MS. However, short-term treatment could result in long-lasting effects in T cells negating chronic use of Notch inhibitors in EAE as shown in GVHD.

Blocking other Notch ligands and receptors therapeutically besides Dll4 has not been well studied. The most commonly described role for other Notch receptors and ligands is in organ development rather than tissue homeostasis. For instance, Notch2

function is associated with heart, kidney, and eye development.[379] Jagged1 dysfunction is found in Alagille syndrome, which is characterized by liver, heart, eye, skeletal, craniofacial and kidney abnormalities.[380] Presumably, a developmental role for certain Notch ligands and receptors would negate a role for Notch in tissue homeostasis of these organs. In contrast, Notch1 and Jagged2 are important for terminal differentiation of keratinocytes, whereby loss of Notch signaling promotes tumorigenesis.[15,381] These data indicate that chronic inhibition of Notch1 or Jagged2 may not be therapeutically advantageous. As indicated in Chapter 4, we found that the post-irradiation intestine is acutely sensitive to Notch1/2 blockade, making this therapeutic modality unreasonable for treating GVHD.[340] Chronic blockade of Notch signaling could also have implications for immunity to pathogens because of the role of Notch signaling in the innate and adaptive immune system, although there is a lack of studies in support of this hypothesis.

Altogether, the time is ripe for studying inhibition of Notch signaling in T cell-mediated immune disorders and other diseases characterized by dysregulated Notch signaling. Humanized anti-mouse/human antibodies targeting all Notch receptors and ligands have been developed and provide the tools necessary to inhibit Notch signaling selectively while bypassing side effects associated with global Notch inhibition.[221] The immunoglobulin backbone of these Notch receptor and ligand neutralizing antibodies is IgG1.[221] Despite the fact that IgG1 fixes complement, which could lead to target cell lysis, our results in Chapter 5 demonstrate that blocking Notch1, Notch2, Dll1, or Dll4 with these neutralizing antibodies does not elicit target cell depletion.[340,382] In contrast, we observed preserved or even enhanced T cell expansion.[340] Moreover, identification of Notch target genes in T cells during T cell-mediated immune disorders will also aid in therapeutic targeting of Notch signaling. It is possible that with the identification of genes that mediate the effects of Notch signaling, therapies could be developed that more specifically target the effectors of Notch signaling in a particular context rather than the overall effects of the pathway.

Collectively, this thesis demonstrates the utility of studying Notch signaling using strict genetic loss-of-function approaches and then moving towards therapeutic options supported by fundamental biological questions. Our work reveals positive consequences

of Notch blockade in T cell-mediated immune disorders that could lead to new therapeutic interventions in patients.

Chapter 7

Materials and Methods

7.1 Mice

BALB/c (H-2d) and C57BL/6 (B6, H-2b, CD45.2^{+}) mice were from Harlan (Indianapolis, IN); B6xDBA/2 F1 (BDF1, H-2b/d), BALB/b (H-2b), and Foxp3-IRES-RFP (FIR) from The Jackson Laboratory (Bar Harbor, ME) [242]; C57BL/6.Ptprca (B6-SJL, H-2^{b}, CD45.1^{+}) from National Cancer Institute (Frederick, MD). NF-kB reporter mice (NGL) were described previously [230]. B6.129S6-Tbx21tm1Glm/J mice were provided by Dr. Segal (University of Michigan) [226]; Eomes$^{f/f}$ x Cd4-Cre mice by Dr. Reiner (Columbia University) [383]. C57BL/6-Tg (Tcra2D2, Tcrb2D2)1Kuch/J (2D2) T cell receptor transgenic were provided by Dr. Segal (University of Michigan) [347]. ROSA26$^{DNMAMLf/+}$ mice generated as described were crossed to Cd4-Cre transgenic mice before backcrossing to the B6 background (> 8 generations) [82,115]. In some experiments, *ROSA26*^{DNMAMLf} x *Cd4-cre* mice were crossed to 2D2 mice. *Rbpj*$^{f/f}$ mice were kindly provided by Tasuku Honjo (Kyoto, Japan) [86]. *Notch1*$^{f/f}$ mice by Dr. Kopan (St. Louis, MO) and *Notch2*$^{f/f}$ by Dr. Gridley (Scarborough, ME) [380 384]. Because no effect of Cre expression was observed in alloreactive and myelin-reactive T cells (data not shown), Cd4-Cre^{+} or Cd4-Cre^{-} controls were used. 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.

7.2 Antibodies, flow cytometry, and cell lines

The following antibodies were from BioLegend (San Diego, CA): anti-CD3, CD4, CD8α, CD25, CD69, CD44, CD45.1, CD45.2, CD62L, TCRβ, CD3, H-2Kb, H-2Kd, IFNγ, IL-2, TNFα, Vβ11, Vα3.2, CD28 (37.51), CD152/Ctla-4 (UC10-4B9), and CD279/Pd-1 (RMP1-30). Anti-Foxp3, T-bet (4B10), Eomesodermin (Dan11mag), and CD272/Btla (6F7) antibodies were from eBioscience (San Diego, CA). CD49d (α1) and CD29 (β4) were from BD Biosciences. For restimulation, we used anti-CD3 (145-2C11) and anti-CD28 (37.51; 2.5 μg/mL each; Biolegend) or phorbol myristate acetate (PMA;

50 ng/mL) and ionomycin (50 ng/mL; Sigma-Aldrich). Intracellular flow cytometry was performed per manufacturer's instructions after addition of Monensin or Brefeldin A (4 hours) (BD). Analysis/sorting were on FACSCanto or FACSAria II/III (BD). Dead cells were excluded with DAPI (Sigma-Aldrich, St. Louis, MO). Files were analyzed in FlowJo (Tree Star, San Carlos, CA). A20 (BALB/c, H-2^d) lymphoma/leukemia cells expressing luciferase were kindly provided by Marcel van den Brink (Memorial Sloan Kettering Cancer Center, New York, NY) [385]. A20 lymphoma/leukemia cells not expressing luciferase were from ATCC (Manassas, VA).

7.3 *In vitro* and *in vivo* proliferation assays

Splenocytes or $CD4^+$ and $CD8^+$ T cells were MACS-purified according to manufacturer's instructions (Miltenyi) and labeled with 2.5μM CFSE (Sigma-Aldrich). T cells were incubated for 3 days on anti-CD3/CD28+/-hrIL-2 (Peprotech). Splenocytes were transplanted into irradiated BALB/c mice. For BrdU incorporation, mice were pulsed with 1 mg BrdU intraperitoneally 6 hours before euthanasia. For BrdU pulse-chase, starting at day 4 post-transplantation, mice received 3 doses of BrdU (1 mg) intraperitoneally, 12 hours apart. Four hours (day 5) or 3 days (day 8) after the last BrdU, spleens were harvested for BrdU staining (BD), followed by DNA counterstaining (DAPI [4′,6-diamidino-2-phenylindole]).

7.4 Cell preparations

T cell–depleted bone marrow (TCD BM) was prepared with microbead-conjugated anti-CD4/CD8 antibodies [386]. $CD4^+$ and $CD8^+$ lymphocytes were isolated from spleens and lymph nodes using microbead-conjugated antibodies (Miltenyi Biotech, Auburn, CA). Purity was consistently >92%. In some experiments, T cell-depleted bone marrow (TCD BM) was prepared with anti-Thy1.2 antibodies and complement (Cedarlane Labs, Burlington, NC; >95% depletion).

7.5 Induction of GVHD

For the B6 anti-BALB/c model, we irradiated BALB/c recipients using 900 rads from a ^{137}Cs source, 4 hours apart. We transplanted donor B6 TCD BM (5.0×10^6) alone or with whole splenocytes or purified $CD4^+$ or $CD8^+$ T cells into irradiated BALB/c recipients (4-

10 mice/group per experiment). In experiments studying GVHD target organs, 850 rads were used. The GVHD score was assessed as described [387]. GVHD severity was also assessed by histopathological analysis [388,389]. Images were obtained with an Olympus BX41 microscope (10×/0.3 NA lens, 100× magnification, digital DP70 camera).

7.6 Isolation of intestinal lymphocytes

Intraepithelial and lamina propria lymphocytes (IELs, LPLs) were isolated from small intestines as described, with slight modifications [390]. Briefly, intestines were washed, and Peyer patches removed. Fragments (0.5-1 cm) of intestine were incubated in phosphate-buffered saline with 1mM EDTA and 1mM DTT for 45′ while shaking at 37°C. Supernatant was passed through nylon wool columns and IELs were isolated after centrifugation on a Percoll (Sigma-Aldrich) gradient. For LPL isolation, the remaining tissue was incubated in RPMI/fetal bovine serum 5% with 200 U/mL collagenase (Invitrogen) for 100′ while shaking at 37°C. Supernatant was filtered, and LPLs were isolated after centrifugation on a Percoll gradient.

7.7 Cytokine measurements

Protein levels of cytokines were quantified using a Bio-Plex bead-based (Luminex) cytokine assay purchased from Bio-Rad Laboratories. For serum cytokine analysis, serum was collected on day 5 post-transplantation. Serum IFNγ levels were measured by mouse IFNγ duoset (R&D Systems, Minneapolis, MN) per the manufacturer's instructions (Immunology Core, University of Michigan Cancer Center).

7. 9 Quantitative reverse-transcription PCR

RNA was isolated using the RNEasy Micro kit (QIAGEN) or TRIzol (Invitrogen, Carlsbad, CA). cDNA was prepared with Superscript II (Invitrogen). Real-time polymerase chain reaction (PCR) was performed with TaqMan Master Mix or SybrGreen (Fisher, Rockford, IL) on Mastercycler realplex (Eppendorf, Westbury, NY). Transcript abundance was calculated using the ΔΔCt method (normalization with Hprt1). Primer sequences were from Applied Biosystems (Grand Island, NY) or PrimerBank (http://pga.mgh.harvard.edu.proxy.lib.umich.edu/primerbank): Eomes (5738950a2). Additional primers were: Ifng (5′-GGATGCATTCATGAGTATTGC-3′; 5′-

CCTTTTCCGCTTCCTGAGG-3′), Tbx21 (5′-CAACAACCCCTTTGCCAAAG-3′; 5′-TCCCCCAAGCAGTTGACAGT-3′), Hprt1 (5′-CTCCTCAGACCGCTTTTTGC-3′; 5′-TAACCTGGTTCATCATC-GCTAATC-3′).

7.10 SDS-PAGE and Western blotting

Naïve ($CD62L^{High}CD44^{Low}$) and alloreactive $CD4^+/CD8^+$ T cells were negatively selected using an anti-NK1.1/CD19/Gr-1/CD11b/CD11c cocktail. T cells were incubated on ice with anti-CD3/CD28 antibodies (0.2 μg/10^6 cells) followed by cross-linking with anti-Armenian hamster IgG (0.5 μg/10^6 cells, Jackson ImmunoResearch, West Grove, PA) in RPMI-1640 at 37°C. Alternatively, T cells were activated with PMA (Sigma-Aldrich, 50 ng/ml) at 37°C or DMSO. Cells were lysed in Laemmli buffer and 2-ME. Samples were run on 4-20% MiniTGX gels (Bio-Rad) and transferred to Immobilon-P membranes (GenHunter, Nashville, TN) (semi-dry transfer, Bio-Rad). Membranes were blocked in 10% FBS+TBS-T (25 mM TrisBase-pH8, 125 mM NaCl, 0.05% Tween). Antibodies were from Cell Signaling (Danvers, MA): anti-MEK1/2 rabbit (47E6), p44/42 MAPK-Erk1/2 mouse (3A7), phospho-MEK1/2 (Ser217/221) rabbit (41G9), phospho-p44/42 MAPK-Erk1/2 (Thr202/Tyr204) (E10). Secondary antibodies were peroxidase-conjugated goat anti-rabbit IgG(H+L) or donkey anti-mouse IgG(H+L) (Jackson ImmunoResearch). Blots were developed with ECL Substrate (Thermo) and HyBlotCL (Denville Scientific, South Plainfield, NJ).

7.11 Intracellular cAMP assay

cAMP was measured using ELISA, per manufacturer's instructions (Enzo LifeSciences, Farmingdale, NY).

7.12 Luciferase assay for NFκB activity

Sort-purified alloreactive T cells were restimulated with plate-bound anti-CD3/CD28 (Biolegend, 2.5 μg/mL) for 16 hours. Luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI) and read on PerkinElmer Enspire2300 (Waltham, MA).

7.13 *Ex vivo* cytotoxicity assays

CFSE-based cytotoxicity assays were as described [391]. $CD4^+$ T cells were isolated from WT B6 mice (unstimulated) or recovered from spleens and livers of BALB/c recipients of WT or DNMAML $CD4^+$ T cells 14 days after transplantation. On day 14, alloreactive WT and DNMAML $CD4^+$ T cells were sort-purified and co-incubated with CFSE-labeled BALB/c-derived (H2Kd) A20 tumor cells (ATCC, Manassas, VA) for 8 hours. Naïve $CD4^+$ T cells were sorted in a similar manner and used as a control. Specific lysis was calculated as 100-((unlysed A20/bacgkround A20)*100) and normalized with counting beads (Bangs Laboratories, Inc., IN). For ^{51}chromium release assays, spleen and lymph nodes were harvested on day 8 post-transplantation. WT and DNMAML $CD8^+$ T cells were purified by MACS according to the manufacturer's instructions (Miltenyi). A20 (H-2^d) and P815 (H-2^d) cells were used as allogeneic targets, with EL4 (H-2^b) as syngeneic control targets. Tumor targets were labeled with 2MBq of $Na_2{}^{51}CrO_4$ (PerkinElmer Life, Boston, MA, USA) for 2 hours. After washing 3 times, labeled targets were plated at 5×10^3 cells per well in U-bottom 96-well plates (Corning-Costar Corp., Cambridge, MA). Splenocytes were added in triplicate wells at varying E:T ratios, and incubated for 5 hours. ^{51}Cr activity in supernatants was read in a LumaPlate (PerkinElmer, Waltham, MA) in an auto-gamma counter (Packard Instrument Company, Meriden, CT). Maximal and background release were determined by the addition of 2% Triton X-100 (Sigma) or media alone to targets, respectively. The percentage of specific lysis was calculated as 100 × (sample count – background count)/(maximal count – background count).

7.14 *In vivo* cytotoxicity assay

BALB/c allogeneic target splenocytes were labeled with 2.5μM CFSE. Control targets were B6/SJL splenocytes labeled at a low CFSE concentration (0.25μM). On day 13, we infused a 1:1 mixture of CFSE-labeled BALB/c and B6/SJL splenocytes (10^7) into BALB/c recipients. Spleens were harvested 18 hours later to assess killing of BALB/c-derived $CFSE^{High}$ target B cells. In some experiments, elimination of $CFSE^{High}$ BALB/c allogeneic targets was measured by flow cytometry in the spleen.

7.15 EAE induction

On day 0, age-matched (6-14 weeks) and sex-matched mice were immunized with Complete Freund's Adjuvant containing heat-killed *Mycobacterium tuberculosis* (Fisher, Pittsburg, PA) and myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) peptide (MEVGWYRSPFSRVVHLYRNGK; 0.25 μg/site; Biosynthesis, Lewisville, TN). On day 0 and 2, mice received pertussis toxin (Fisher) (300 ng i.p.). Mice were scored for disease severity according to the following scale: 1= limp tail; 2=inability to right oneself; 3=hind limb weakness; 4=hind limb paralysis; 5=moribund. All analyses of T cell function were done at peak disease (score 3-5).

7.16 Isolation of CNS-infiltrating cells.

Mice were perfused with PBS. Brains and spinal cords were digested with Collagenase (2.125 mg/mL; Invitrogen, Grand Island , NY) and DNase I (1mg/mL; Roche. Indianapolis, IN) followed by purification on a 30/70% Percoll (Sigma, St. Louis, MO) gradient.

7.17 ELISpot

Draining lymph node cells (axial, brachial, inguinal) from immunized mice at peak disease were restimulated in MultiScreen HTS Filter plates (Millipore, Billerica, MA) +/- 50 mg/mL MOG_{35-55} for 18 hours. Antibodies used for cytokine detection were from eBioscience (anti-IFN-γ and IL-17A). Streptavidin-Horseradish peroxidase (HRP) was from Southern Biotech (Birmingham, AL). HRP substrate was from Vector Laboratories (Burlingame, CA).

7.18 Transwell migration assays

The following chemokine ligands were from R&D Systems (Minneapolis, MN): Ccl20/Mip-3a, Ccl2/Mcp-1, Cxcl10/Crg-2, Ccl3/Mip-1a, and Ccl21/6Ckine. $CD4^+$ T cells from 2D2 and 2D2/DN immunized mice at peak disease were purified by Miltenyi Magnetic Bead technology (Auburn, CA). Purified $CD4^+$ T cells were resensitized at 37^oC, plated in a NeuroProbe ChemoTx System (Gaithersburg, MD) and allowed to migrate for 4 hours before analysis of migrated $CD4^+V\alpha3.2^+V\beta11^+CD44^+$ T cells in bottom wells by flow cytometry. Number of cells migrated was normalized using a

standard curve of known numbers of T cells and a fixed number of counting beads (Bangs Laboratories, Fishers, IN) by flow cytometry. Specificity of migration was determined by enumerating the number of activated 2D2 $CD4^+V\alpha3.2^+V\beta11^+CD44^+$ T cells that migrated to the bottom well in the absence of chemokines.

7.19 Generation of mixed bone marrow chimeras for EAE immunization

Lethally irradiated (900 rads) B6-CD45.1 mice received CD45.2-WT B6 or CD45.2-DN and CD45.1-B6 bone marrow (mixed at 1:1 or 7:3 ratio). Mice were allowed to reconstitute for 8-12 weeks before EAE induction.

7.20 Statistical analysis

All statistical analyses were performed using GraphPad-Prism (La Jolla, CA). Survival in different groups was compared using the log-rank test. Comparison of 2 means was analyzed using the 2-tailed unpaired Student t test. When less than five data points were available per group, we used the unpaired Mann-Whitney U test. EAE disease incidence significance was determined by Chi-square and Fisher's exact test.

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