Analysis of a transcriptional network involving PU.1, Notch, and Gata3 in the lymphomyeloid lineage decision during early T-cell development

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ABSTRACT

Hematopoiesis is a well-established system used to study developmental choices amongst cells with multiple lineage potentials, as well as the transcription factor network interactions that drive these developmental paths. Multipotent progenitors travel from the bone marrow to the thymus where T-cell development is initiated and these early T-cell precursors retain lineage plasticity even after initiating a T-cell program. The development of these early cells is driven by Notch signaling and the combinatorial expression of many transcription factors, several of which are also involved in the development of other cell lineages. The ETS family transcription factor PU.1 is involved in the development of progenitor, myeloid, and lymphoid cells, and can divert progenitor T-cells from the T-lineage to a myeloid lineage. This diversion of early T-cells by PU.1 can be blocked by Notch signaling. The PU.1 and Notch interaction creates a switch wherein PU.1 in the presence of Notch promotes T-cell identity and PU.1 in the absence of Notch signaling promotes a myeloid identity. Here we characterized an early T-cell cell line, Scid.adh.2c2, as a good model system for studying the myeloid vs. lymphoid developmental choice dependent on PU.1 and Notch signaling. We then used the Scid.adh.2c2 system to identify mechanisms mediating PU.1 and Notch signaling interactions during early T-cell development. We show that the mechanism by which Notch signaling is protecting pro-T cells is neither degradation nor modification of the PU.1 protein. Instead we give evidence that Notch signaling is blocking the PU.1-driven inhibition of a key set of T-regulatory genes including *Myb*, *Tcf7*, and *Gata3*. We show that the protection of *Gata3* from PU.1-mediated inhibition, by Notch signaling and Myb, is important for retaining a T-lineage identity. We also discuss a PU.1-driven mechanism involving E-protein inhibition that leads to the inhibition of Notch target genes. This is mechanism may be used as a lockdown mechanism in pro-T-cells that have made the decision to divert to the myeloid pathway.

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Chapter 1: Introduction

Using hematopoietic cells as a model for answering developmental questions

Blood-cell development is a widely used system to study developmental questions. Refined cell culture techniques used to culture hematopoietic stem and progenitor cells from adult or fetal tissues allow researchers to amass enough material for intricate experiments. The ability to distinguish different populations of blood cells using cell-surface markers allows for the isolation of discrete developmental populations that can be mechanically sorted and used for downstream applications. Studies using hematopoietic cells have enormous clinical potential and can also answer fundamental questions about biology. Here we will give a brief description of blood-cell development up to the migration of T-cell progenitor cells to the thymus where they will initiate early T-cell development.

Blood cells are classified into three major lineages: the lymphoid lineage, the myeloid lineage including macrophages and dendritic cells, and the erythrocyte/megakaryocyte lineage that includes red blood cells (Table 1). The lymphoid lineage includes B-cells and T-cells, which are part of the adaptive immune system, while the myeloid lineage only includes innate immune cells. Hematopoiesis is driven by the stepwise acquisition of cell-specific traits by progenitor cells along with the repression of alternative cell identities. There are two prevailing models describing the loss of cell potentials from the pluripotent hematopoietic stem cell (HSC), to multipotent progenitors (MPP), and finally to committed cells. The classic model of blood-cell development argues that HSC give rise to multipotent progenitors whose progeny are lymphoid or myeloid

restricted (Lai and Kondo, 2008)(Figure 1A). These progeny are termed CLPs (common lymphoid progenitor) or CMPs (common myeloid progenitors) and mark the early partial divergence of the cells from the adaptive (e.g., B-cells and T-cells) and innate (e.g., macrophages and dendritic cells) immune systems. The more recent 'revised' lineage tree depicts the MPP as giving rise to a CMP and a LMPP (lymphoid-primed multipotent progenitor) that has the potential to generate innate (T-cells) and adaptive (macrophages) immune cells, but not cells of the erythroid or megakaryocyte lineage (Adolfsson et al., 2005; Lai and Kondo, 2006) (Figure 1B). The disagreements about a progenitor with lymphomyeloid potential may arise from the differing experimental methods used to test the potential of the cells (Schlenner and Rodewald, 2010; Rothenberg, 2011). The classical model has been upheld in studies where sorted CMP or CLP cells are re-introduced into animals, in cell free culture assays, and in fate-mapping experiments in mice. Fate-mapping experiments consist of using a fluorescent marker that is turned on when a selected gene is expressed (e.g. the cytokine receptor IL7) and these cells are then 'tagged' and their final developmental fates characterized at a later time point. The fate mapping experiment with IL7-GFP showed that IL7+ (GFP+) progenitors in-vivo gave rise to mostly lymphoid cells while IL7- (GFP-) progenitors gave rise to mostly myeloid cells (Schlenner et al., 2010). Alternatively, the revised model has been proven in in-vitro-based assays where the progenitor cells can be exposed to a variety of cytokines and cells signals they may need for their development and survival (Franco et al., 2006; Bell and Bhandoola, 2008; Wada et al., 2008; Chi et al., 2011). The argument that the true potential of a cell cannot be evaluated if it is not given the proper environmental signals needed to adopt a particular cell identity is often used in support of using in vitro systems. Just because a

supposed myeloid restricted progenitor cell that is injected intravenously does not have the proper signaling to home to the thymus does not mean it could not develop into a T-cell if exposed to Notch signals. New insights in to the multi-potentially of progenitors are made often (Yang et al., 2011) and will no doubt continue to be explored as new methods of progenitor cell classification/isolation are developed.

T-cells develop in the thymus, but the identity of the progenitor or progenitors that populate the thymus remains a point of contention among blood cell developmental biologists (Bhandoola et al., 2007; Zlotoff and Bhandoola, 2011) (Figure 1C). Several considerations are discussed when attempting to identify thymus seeding precursors including their expression of molecules, like CCR9 (Uehara et al., 2002), that drive migration to the thymus and their expression of Notch1. Figure 1C shows several of the proposed progenitors that could replenish the thymus. Regardless of which of these multi-, bi-, or –uni -potent progenitors seed the thymus the majority of the cells generated in the thymus are T-cells. Here we will discuss data showing that this is due in part to the restriction of alternative fates by Notch Signaling. This thesis will focus on describing the regulatory gene network of pro-T cells in conditions permissive for T-linage development and identifying changes in the network when the cells are under conditions permissive for diversion to the myeloid lineage. To follow is a brief summary of early T-cell development and a discussion of the retention of alternative lineage potentials during their earliest stages.

Early T-cells and their developmental potentials

Once T-cell progenitors enter the thymus they are classified as double negative (DN) thymocytes since they are devoid of the mature T-cell markers CD4 and CD8. The

majority of the DN thymocytes generate αβ T-cells (have αβ chains in their T-cell receptors) instead of γδ T-cells (have γδ chains in their T-cell receptors). We will focus on αβ T-cell development and lineage potentials. For a review on γδ T-cells see Kreslavsky et al. and Pang et al. (Kreslavsky et al., 2010; Pang et al., 2012). The DN (DN1–DN4) stages are were originally classified according to their cell-surface expression of CD25 and CD44 (Godfrey et al., 1993)(Figure 2). Since then additional markers such as CD27 and cKit are used to better define DN populations in the thymus into DN1 (or ETP), DN2a, DN2b, DN3a, DN3b, and DN4 populations (Taghon T, 2006; Yui et al., 2010) (Figure 2). The difference between the DN2a and DN2b cell populations is that the DN2b cells are committed to the T-cell lineage and have lost the ability to naturally give rise to NK cells, myeloid cells, and mast cells (unless their transcription factor levels are experimentally perturbed). Therefore, commitment to the T-cell lineage is marked by entrance into the DN2b stage (Yui et al., 2010). The DN3a and DN3b populations are distinct in that the DN3b cells have passed an important checkpoint in T-cell development, β-selection. DN3b cells give rise to DN4 cells which can develop into double positive cells and eventually CD4 or CD8 single positive cells (Germain, 2002). Global analysis of gene expression changes between DN1, DN2a, DN2b and DN3 staged cells revealed that major transcriptional changes leading to the acquisition of a T-cell identity occur between the transition from DN2a to DN2b as well as later at the β-selection checkpoint (Zhang et al., 2012). For the work presented in this thesis we will focus on the DN1–DN3 stages of T-cell development.

T-cell progenitors enter the thymus with the ability to differentiate into several blood cell

types when placed under permissive environments. It is thought that the earliest alternative lineage potential lost to pro-T cells in the thymus is the potential to generate B-cells (Heinzel et al., 2007) which occurs sometime in the DN1 stage. This potential is inhibited by the presence of Notch ligand in the thymic microenvironment (Wilson et al., 2001) as well as the epigenetic repression of key B-cell regulators such as Pax5 and EBF1 (Zhang et al., 2012). Thymic DN1 and DN2a cells are able to generate myeloid cells (Bell and Bhandoola, 2008; Wada et al., 2008), Natural Killer cells (NK) (Yui et al., 2010), dendritic cells (DC) (Shen et al., 2003; Yui et al., 2010), and, rarely, mast cells (Taghon et al., 2007). The generation of myeloid cells, mast cells, and NK cells is at least partially blocked by Notch signaling (Franco et al., 2006; Taghon et al., 2007; Yui et al., 2010). The natural ability of pro-T cells to generate other cell types can be understood in terms of the transcription factors expressed at that stage since many of them are also involved in the development of these other lineages. In fact, a pro-T cells multi-potential ability could be enhanced by the over-expression of these transcription factors. For example, the bzip transcription factor C/EBPa is heavily involved in myeloid cell development (Hohaus et al., 1995; Cai et al., 2008) and is expressed at low levels in DN1 cells (Laiosa et al., 2006b; Rothenberg et al., 2008). Over-expression of C/EBPa can up-regulate the myeloid marker, Mac1, in cells expressing the T-cell maker Thy1. However, C/EBPa-driven Mac1 up-regulation was partially blocked by Notch signaling (Laiosa et al., 2006b). Similarly, the transcription factor PU.1, which is expressed in prethymic cells up to the DN2 stage, can also divert cells to DC and myeloid lineages under certain conditions (Anderson et al., 2002b; Laiosa et al., 2006b). PU.1 in the presence of Notch signaling allows T-cell development, but in the absence of Notch signaling diverts pro-T cells to a myeloid fate

(Franco et al., 2006). Transcription factor levels are also important for maintaining T-lineage fidelity. For example, although *Gata3* is important for T-cell development, high levels of *Gata3* can enhance the generation of mast cells from DN1 and DN2 thymocytes (Taghon et al., 2007).

The studies described herein use pro-T cells to examine lineage decisions between the lymphoid and myeloid programs. Although the decision as to which program to adopt is multi-faceted (Laiosa et al., 2006a), we hope to gain insight regarding the mechanisms used by lineage determining factors to modify the activity of multi-lineage factors so that they support a precise cell fate. Specifically, how the activity of the multi-lineage factor PU.1 can be channeled by Notch signaling to support T-cell development in cells that retain the potential for adopting a myeloid identity.

Transcriptional Regulators Driving T-cell Development

The generation of T-cells from progenitor cells depends on the precise temporal expression of several transcription factors in combination with environmental signals (Rothenberg et al., 2008; Rothenberg, 2012). These factors must also be expressed at T-cell permissive levels since most of them are also involved in the development of alternative cell lineages. To follow is a brief summary of some of the important drivers of early T development (also see Fig. 2).

Runx1 is expressed at high levels during the DN stages of development with the highest expression at DN3, but must be moderately down regulated for the proper development of DP (double positive) cells and continues to be important during later stages of T-cell development (Komine et al., 2003; Wong et al., 2010; Lazarevic et al., 2011). Cells deficient in Runx1 were found to have in block in the DN2 to DN3 transition

(Growney et al., 2005). Ikaros is a zinc finger transcription factor expressed at constant levels during early T-cell development and is also important for the development of blood stem cells and myeloid cells (Yoshida et al., 2006; Francis et al., 2011). Gfi1 is involved in the development of hematopoietic stem cells and NK cells (Hock and Orkin, 2006). *Gfi1* is expressed throughout the early DN stages and *Gfi1* deficiency resulted in a block during the DN1 to DN2 transition (Yucel et al., 2003). *Gfi1* was also found to be important later in T-cell development for CD4 T cell maturation (Pargmann et al., 2007). The E-proteins E2A, HEBcan and HEBalt are known to initiate transcription as homo or heterodimers, have been shown to cooperate with Notch signaling in the regulation of genes and have been implicated in the maintenance of T-lineage fidelity (Murre, 2005; Ikawa et al., 2006; Braunstein and Anderson, 2011). *Tcf7* codes for the Tcf1 transcription factor (can also be called Tcf7) and is one of the first T-cell regulatory genes to be expressed in pro-T cells (Hattori et al., 1996). Its inhibition results in an early block in the DN1 stage of development (Marco W. Schilham et al., 1998; Weerkamp et al., 2006). *Tcf7* is initially turned on by Notch signaling, but does not require Notch signaling for its continued expression (Germar et al., 2011). Tcf7 is unique in that it remains the only transcription factor that can initially drive T-cell development without Notch signaling (Weber et al., 2011). Notch signaling, Myb, Gata3 and PU.1 will be the main focus of Chapter 3 and so they will be discussed in detail below.

Notch Signaling

Notch signaling has been recognized as one of the earliest requirements for the initiation of T-cell development as well as a powerful antagonist for B-cell development. Notch1 deficient mice fail to generate T cells with a block is detected at the earliest stages of T-cell

development (Radtke et al., 1999). Radtke et al. reported that all other blood-cell lineages besides T-cell were generated and discovered the aberrant development of B-cells in the thymus. In an opposite experiment, Pui et al. induced expression of Notch signaling in bone marrow cells and detected the ectopic development of T-cells in the bone marrow and an absence of B-cells (Pui et al., 1999). T-cell dependence on Notch signaling for development and survival occurs early and continues until β-selection (Maillard et al., 2006b; Yang et al., 2010; Gonzalez-Garcia et al., 2012).

Notch signaling is an evolutionarily conserved signaling pathway that involves the interaction of a transmembrane Notch receptor on one cell and a Notch ligand on another cell. There are 4 mammalian Notch receptors (Notch1–4) and 5 Notch ligands (Delta1, Delta3, Delta4, Jagged1, and Jagged2). Upon ligand binding to the Notch receptor several cleavage events occur leading to the release of the intracellular domain of the Notch receptor (NICD- Notch intercellular domain) into the cytoplasm. The NICD is then translocated to the nucleus where it binds to the transcription factor CSL (CBF1/RBPjκ/Su(H)/Lag-1). CSL is normally a repressor of transcription, but NICD binding recruits co-activators, such as Mastermind, and CSL is converted to an activator of transcription. For a detailed review of the canonical Notch signaling pathway see Kopan et al. (Kopan and Ilagan, 2009). Notch1 is the main Notch receptor expressed on T-cells and Delta4 is the main ligand expressed in the thymic cells supporting T-cell development (Koch et al., 2008; Fiorini et al., 2009).

Notch1 is expressed in the earliest T-cell progenitors and its expression continues to be increased until β-selection when the levels of Notch1 are dramatically decreased (Taghon T, 2006; Yashiro-Ohtani et al., 2009). The regulation of the Notch1 receptor in

pro-T cells is not completely known, but there is evidence that it might be regulated by the E-protein, E2A. E2A knockout mice showed decreased levels of Notch1 as well as lower levels of some Notch target genes (Ikawa et al., 2006). Other experiments showed that Notch1 expression was at least partially controlled by Notch1 and E2A binding to the Notch1 regulatory region (Yashiro-Ohtani et al., 2009).

After the importance of Notch signaling for T-cell development became clear new methods of generating T-cell in-vitro have emerged (Schmitt and Zúñiga-Pflücker, 2006). Currently the most popular method for encouraging T-cell development outside of the mouse is the use of the stromal bone marrow OP9 system. These bone marrow stromal cells were originally used to support B-cell and myeloid cell development in-vitro. To support T-cell development in-vitro OP9 cells were engineered to express the Notch ligand, Delta1, which interact with the Notch1 receptor in pro-T-cells (Schmitt and Zuniga-Pflucker, 2002). For many of the studies presented here culturing pro-T cells in the presence or absence of Notch signaling consisted of culturing on OP9Dl1 (OP9-Delta-like1) or OP9-control cells. Other Notch signaling manipulations used in the studies presented here included using a chemical inhibitor of Notch receptor cleavage, GSI (γ-secretase inhibitor) to inhibit Notch signaling. Additionally, we increased Notch signaling by transducing cells with the intracellular portion of Notch (ICN) and knocked down Notch signaling using a dominant negative (dn) form of the Notch transcriptional cofactor Mastermind like (MAML) (Maillard et al., 2004).

Myb

Myb is a myb family transcription factor that has been implicated in driving differentiation, growth and survival of blood cells including erythroid, lymphoid and (Greig et al., 2008) myeloid progenitors (Lieu and Reddy, 2012). The role of *Myb* during T-cell development was initially difficult to study because *Myb* knockout mice died in response to severe anemia between days 13 and 15 of embryonic development (Mucenski et al., 1991); too early to study effects on T-cell development. With the advent of more sophisticated transgenic mice models *Myb* was shown to be essential for differentiation past the DN3 stage of T-cell development (III et al., 1999; Lieu et al., 2004). During early T-cell development *Myb* is already expressed at moderately high levels in DN1 cells and is up-regulated at DN2 after which its level of expression remains fairly constant (Tydell et al., 2007). The function of Myb during the earliest DN stages is not well understood, but Myb has been implicated in the regulation of genes expressed later in T-cell development like *Ptcra*, *Cd4*, and *Rag2* (Siu et al., 1992; WANG et al., 2000; Reizis and Leder, 2001). *Myb* itself was shown to exhibit positive autoregulation (Nicolaides et al., 1991).

The relationship between Myb and Ets family transcription factors, like PU.1, is complicated and cell specific. EMSA studies done in myeloid precursor cells found that PU.1 bound DNA near the *Myb* promoter and this suggested the repression of *Myb* by PU.1 (Bellon et al., 1997). Myb ChIPseq studies in a myeloid progenitor cell line showed that Myb can repress myeloid genes such as Sfpi1 PU.1 (*Sfpi1*), *CEBPβ*, and *JunB* (Zhao et al., 2011). Alternatively, it has been shown that PU.1, Cebpα and Myb collaborate to turn on neutrophil genes in human cells (Lennartsson et al., 2005). During early T-cell development Myb expression remains consistent while PU.1 expression is initially high

before it is shut off (Fig. 2). Other positive and negative interactions of Myb with Ets family members have also been described, but these types of studies during early T-cell development have not been pursued (O and KH, 1999; Geng and Vedeckis, 2005).

Gata3

Gata3 is a GATA family transcription factor that was mostly thought to be only involved in T-cell development. Recently the importance of Gata3 was discovered in the development of a newly described innate lymphoid cell (Natural Killer cell related) (Mjosberg et al., 2012) as well as in hematopoietic stem cell maintenance and cell cycle regulation (Ku et al., 2012). Gata3 expression is not required for B-cell or myeloid development while Gata3 knockout studies in ES cells showed cells were unable to enter the earliest DN stages of T-cell development (C.N. et al., 1996). Gata3 also plays a pivotal role in the later development of Th2 CD4+ T-cells (Zheng and Flavell, 1997) and for the choice between the generation of CD4 vs. CD8 single positive T cells (Hernández-Hoyos et al., 2003; Pai et al., 2003).

Gata3 is initially expressed at lower levels in the DN1 stage but then its expression increases and remains constant throughout the rest of DN T-cell development (Anderson et al., 2002a; Tydell et al., 2007). High levels of Gata3 are inhibitory to T-cell development and were shown drive the down-regulation of important T-cell genes such as *Ptcra* and *Il7ra* (Anderson et al., 2002a). Furthermore over-expression of Gata3 can divert some early T-cells to mast cells (Taghon et al., 2007). Transcriptional regulation of Gata3 during early T- cell development remains poorly understood, but there is some evidence for the regulation of *Gata3* by Notch signaling. Although Notch signaling has been shown to induce the expression of *Gata3* in early T-cell progenitors (Taghon et al., 2005), the link

between the two has been widely studied in later T-cell development (Th2 cell development) (Amsen et al., 2007; Fang et al., 2007). That genes regulated by Gata3 are still not completely known, but are actively being studied. Identification of Gata3 target genes was initially done using gene expression profiles of Gata3 knockdown and over-expression studies (Anderson et al., 2002a), but a more direct approach was recently carried out by the use of Gata3 ChIPseq analysis of isolated DN and DP thymocytes (Wei et al., 2011; Zhang et al., 2012). Gata3 was bound to several regulatory regions of T-cell genes including *Tcf7, Cd3d*, and *Zbtb7b* (Thpok). It is also worth mentioning that Zhang et al. characterized Gata3 DNA binding as being cell stage specific even when the expression of Gata3 itself remained constant across the stages that were analyzed.

There are several important interactions reported between Gata3 and two other transcription factors: Myb and PU.1. Gata3 was described as a Myb target gene in mature CD4+ T-cells (Maurice et al., 2007) and was later shown to be required for the MAP kinase driven regulation of Gata3 during CD4+ cell development (Gimferrer et al., 2011a). The effects of Myb over-expression on *Gata3* in earlier stages of T-cell development have not been extensively studied. Conversely, Gata3 over-expression in pro-T cells has been shown to have no effects on *Myb* expression (Anderson et al., 2002a). Gata proteins have been shown to repress PU.1 and block the ability of PU.1 to bind its co-activator c-Jun in in-vitro studies done with epithelial cells (Zhang et al., 1999). Data collected in pro-T cells showed that Gata3 over-expression drives the down regulation of PU.1 mRNA levels (Anderson et al., 2002a; Taghon et al., 2007). It is still unclear how this occurs since Gata3 binding sites near PU.1 (*Sfpi1*) regulatory regions have yet to be detected.

PU.1

PU.1 is an ETS family transcription factor expressed in hematopoietic stem cells (Iwasaki et al., 2005), multipotent progenitors (Arinobu et al., 2007; Wontakal et al., 2011), myeloid cells (Ghani et al., 2011), B-cells (Houston et al., 2007) and T-cell progenitors (Tydell et al., 2007). PU.1 was found to be essential for the earliest stages of T-cell development (Spain et al., 1999; Back et al., 2005; Dakic et al., 2005). The effects driven by the absence of PU.1 during early T-cell development are difficult to study since it leads to a severe reduction of lymphoid progenitors and subsequently to the absence of T-cells in these mice. PU.1 is expressed at high levels in DN1 cells and begins to be sharply down regulated from the DN1 to the DN2 stages and is no longer expressed in DN3 cells (David-Fung et al., 2006). PU.1 regulates the expression of the IL7 receptor and Flt3, which are involved in the proliferation and differentiation of T-cells, and also drives the expression of genes important for cellular communication (DeKoter et al., 2002; Turkistany and DeKoter, 2011). Studies to find more PU.1 target genes that support the early stages of T-cell development and clearly define the need for PU.1 in these cells are currently underway. The regulation of the PU.1 during early T-cell development remains unclear, but there is some evidence that Runx1 may be involved in the silencing of PU.1 (Huang et al., 2008; Zarnegar et al., 2010). PU.1 has been reported to positively autoregulate its expression in B-cells and myeloid cells (Okuno et al., 2005; Leddin et al., 2011). Ikaros has been shown to negatively regulate PU.1 in B-cells, but activate PU.1 in myeloid cells (Zarnegar and Rothenberg, 2012). Runx1 also represses PU.1 in T-cells while playing a critical role to activate PU.1 in hematopoietic precursors and myeloid cells (Rosenbauer et al., 2006; Huang et al., 2008; Zarnegar et al., 2010).

The ability of PU.1 to mediate transcriptional regulation has been shown to depend on the availability of co-activators such as c-Jun (Behre et al., 1999) or other transcription factors that active synergistically with PU.1 such as C/EBPa (Oelgeschläger et al., 1996; Smith et al., 1996; Jina et al., 2011). PU.1 has also been shown to mediate its inhibitory effects by direct binding to other transcription factors; for example, binding to Gata1 during erythroid development (Zhang et al., 2000). PU.1 transcriptional activity has been shown to be blocked via physical interaction with other transcription factors like Gfi1 (Dahl et al., 2007). Posttranslational modifications of the PU.1 protein by phosphorylation are also known to regulate its activity (Pongubala et al., 1993; Wang et al., 2003).

The expression pattern of PU.1 in thymocytes corresponds to their ability to develop into myeloid cells (Fig.2). We believe PU.1 plays a role in the natural progression of pro-T cells from a multi-potent state to a committed state. PU.1 has the ability to encourage multipotent pro-T cells to adopt a T-lineage identity in the presence of Notch signaling, but in the absence of Notch signaling encourage the adoption of a myeloid identity. In the following section we will discuss relevant studies and results exploring PU.1 and Notch signaling interactions during pro-T cell lineage fate decisions.

Diversion of pro-T cells to a myeloid fate by PU.1 and their protection by Notch signaling

In the previous section we discussed knockdown studies of PU.1 and how this led to an absence of T-cells (Spain et al., 1999; Back et al., 2005; Nutt et al., 2005). Here will discuss over-expression studies of PU.1. Over-expression of PU.1 in early T-cells is detrimental for normal development and results in a severe block at the DN3 stage (Anderson et al., 2002b). Over-expression of PU.1 in T-cell precursors can also reprogram

multipotent cells in to a macrophage or dendritic cells even at a stage when they would have normally been committed (Anderson et al., 2002b; Dionne et al., 2005; Lefebvre et al., 2005; Laiosa et al., 2006b). PU.1 is normally expressed in fetal thymocytes at high levels and yet this does not block the generation of T-cells in-vivo. The reason for this T-cell lineage fidelity in an environment with high PU.1 was found to be exposure to Notch signaling (Franco et al., 2006; Laiosa et al., 2006b). In these studies diversion was characterized as the induction of the myeloid PU.1 target gene, Mac1, on cells expressing the T-cell marker, Thy1. Dionne et al. showed that sorted DN3 cells that became Mac1+ when transduced with PU.1 underwent morphological changes resembling myeloid cells. These results suggested that Mac1 up-regulation could be used as a marker for a global change in gene expression resulting in the inhibition of the T-cell program and the initiation of a myeloid program. The PU.1-driven diversion response of these cells was described as all-or-none because the cells either up-regulated Mac1 or they did not with no cells expressing intermediate levels of Mac1. Gene expression analysis of sorted Thy1+ cells transduced with PU.1 showed that PU.1 inhibited the expression of many genes important for T-cell development including: *Myb*, *Tcf7*, E-proteins *HEBalt* and *E2A*, *Gfi1*, and the Notch target gene *Hes1*. Notch signaling was able to protect these genes from PU.1-driven inhibition with varying efficiency (Franco et al., 2006). Notch signaling was also able to inhibit the up-regulation of lineage-inappropriate genes like the E-protein inhibitor *Id2*. These early studies were able to describe phenotypic and some gene expression changes that occurred with PU.1 over-expression in the presence or absence of Notch signaling, but they did not identify the critical factors that either aided in the diversion or protection of these cells.

An early T-cell line was used in early studies to model the diversion of pro-T cells to the myeloid fate in response to PU.1 (Dionne et al., 2005). The Scid.adh.2c2 cell line is a subclone of the Scid.adh cell line. The Scid.adh.2c2 cell line resembles a DN3 stage cell and therefore does not express PU.1. Scid.adh cells were derived from a spontaneous thymic lymphoma and activate Notch signaling through a spontaneous, ligand-independent pathway (Carleton et al., 1999b). In other words, these cells do not require contact with a Notch ligand to receive Notch signals. Upon PU.1 transduction Scid.adh.2c2 cells display the same all-or-none diversion response as primary thymocytes. The gene expression analysis of Mac1+ (diverted) Scid.adh.2c2 cells showed that Mac1 up-regulation was accompanied by a vast reduction in expression of T-cell genes, similar to the responses seen in the primary cell samples. These included the transcription factor genes *Myb*, *HEBalt*/*can*, *Gata3* and the Notch target gene *Ptcra*. The Scid.adh.2c2 cells were also able to up-regulate the E-protein inhibitor, *Id2* in cells that expressed cell-surface Mac1. These early studies also showed that stimulation with phorbol ester (PMA) enhanced the diversion of PU.1+ Scid.adh.2c2 cells and this suggested that some down-stream signaling pathway activated by PMA could be involved in the PU.1-driven diversion response. The specific signaling pathway involved was not identified.

Scid.adh.2c2 cell studies showed that PU.1 caused changes in the transcription factor network of thymocytes and that the Scid.adh.2c2 system was a good model to study these changes (Anderson et al., 2002b; Dionne et al., 2005). These studies however, did not indentify specific factors that were involved in the diversion or protection aspects of the lymphoid vs. myeloid choice. It remained to be seen which factors were repressed by Notch signaling that were important for PU.1-mediated diversion and/or which factors

PU.1 inhibited in the absence of Notch signaling that were needed to maintain T-lineage fidelity. Other unresolved aspects included questions about posttranslational modifications of the PU.1 protein mediated by Notch signaling. The requirements of the timing and duration of Notch signaling required to protect the cells against PU.1-mediated diversion were also unknown.

Focus of Thesis

Development from a pro-T cell to a committed T-cell requires the acquisition of T-cell properties and the repression of alternative lineages. Remarkably it was shown that the balance between two factors, activated Notch and PU.1, could determine the developmental fate of these cells. This same phenomenon is seen during other developmental programs such as in red blood cell development with PU.1 and Gata-1 (Cantor and Orkin, 2002). Since Notch signaling activation depends on the cells environment its effects on PU.1 activity are therefore most likely mediated via intracellular collaborators. Once it was discovered that Notch signaling was a crucial factor in the decision to remain lymphoid or divert to myeloid, it became apparent that the Scid.adh.2c2 cells should be tested for a similar response. The intent of using the Scid.adh.2c2 cell line was to have a ready supply of easily manipulated cells to help decipher changes in the transcription factor network occurring during the lymphoid vs. myeloid decision.

This thesis will focus on using both fetal thymocytes and Scid.adh.2c2 cells to study changes in the transcription factor network in the presence of PU.1 and high or low levels of Notch signaling. In Chapter 2 data is presented showing that chemical inhibition of Notch signaling in PU.1 transduced Scid.adh.2c2 cells mimicked the diversion response seen in fetal thymocytes. I also discuss gene expression changes that occur with the up-

regulation of Mac1 and another myeloid marker CD11c. We show that Mac1 up-regulation marks "full diversion" in Scid.adh.2c2 cells and that the down-regulation of *Gata3*, *Myb*, and *Tcf7* does not occur with CD11c up-regulation, but does occur with the up-regulation of Mac1. Preliminary data using myeloid cytokines and MAP kinase inhibitors to modify PU.1-driven diversion is also presented. The data suggest that myeloid cytokines do not change the developmental outcome of 2-day PU.1 transduction experiments in Scid.adh.2c2 cells or fetal thymocytes. There is some evidence that the P38 MAP kinase pathway is involved in protecting Scid.adh.2c2 cells from PU.1-mediated Mac1 up-regulation.

In Chapter 3 we focus on using Scid.adh.2c2 cells to identify transcription factors involved in the lymphoid or myeloid lineage choice in response to PU.1 in the presence or absence of Notch signaling. In both fetal thymocytes and Scid.adh.2c2 cells, we tested changes to the PU.1 protein levels in the presence or absence of Notch signaling and found that Notch signaling does not cause the degradation or modification of PU.1 protein. We summarize gene expression results of fetal thymocytes transduced with PU.1 and cultured in the presence or absence of Notch signaling overnight. We found that several stem or progenitor type genes were up-regulated in response to PU.1 and that this was not always blocked by the presence of Notch signaling. This was also seen in Scid.adh.2c2 samples where the co-transduction of PU.1 and the intracellular portion of the Notch receptor (ICN) did not inhibit *Bcl11a* or *Lyl1* up-regulation. We also summarize gene expression data of Mac1+ cells and cells expressing PU.1 with high or low levels of Notch signaling. This data was used to choose candidate genes for double transduction experiments with PU.1 (Fig.3). *Myb* had been described as a gene that is protected by Notch signaling when PU.1

is expressed at high levels (Franco et al., 2006). We found the same was true in our fetal thymocyte and Scid.adh.2c2 samples, and so this made Myb an interesting gene to study in relationship to lineage decisions. In Chapter 3 we present evidence that co-expression of Myb with PU.1 can inhibit Mac1 up-regulation in Scid.adh.2c2 cells. Gene expression analysis of these cells showed that *Gata3* was enhanced in the PU.1 and Myb co-transduced samples compared to samples with only PU.1. We show that GATA3 protein levels are severely inhibited in PU.1+Mac1+ cells and that both Myb and Notch signaling can block this inhibition. Additional evidence for the importance of *Gata3* in protection of the cells was acquired in experiments showing increased Mac1 up-regulation with PU.1 and Gata3 knockdown. This data marks *Gata3* as a major player in the lymphomyeloid lineage decision. We additionally describe a mechanism by which PU.1 can inhibit Notch target genes via the inhibition of E-proteins.

The final chapter of this thesis is a discussion of possible interpretations of the data and includes proposals for future experiments.

This thesis also contains an appendix describing preliminary experiments in Scid.adh.2c2 cells involving the transcription factors Bcl11b, Lyl1, and PU.1. In these experiments we wanted to ask if Lyl1 or Bcl11b were involved in enhancing or blocking the PU.1-driven diversion of Scid.adh.2c2 cells. The data shown here suggests they are not. We also wanted to explore interactions between these factors during early T-cell development and found that Bcl11b may be inhibiting the ability of PU.1 to turn on progenitor genes. If this is true, then Bcl11b could be driving T-cell development forward by inhibiting genes expressed during a stem/progenitor cell program.

Figures:

Lymphoid	T-cell NK cell B cell Dendritic cell Innate lymphocyte
Myeloid	Macrophage Dendritic cell Basophil Eosinophil Neutrophil Monocyte
Erythroid-megakaryocyte	Erythrocyte Megakaryocyte Platelets

Table 1: Classification of blood cells. All blood cells are generated from hematopoietic stem cells and are often classified as belonging to a lymphoid, myeloid, or erythroid/megakaryocyte lineages. T-cells are defined as lymphoid while macrophages are included in the myeloid lineage. Different types of dendritic cells can be classified as myeloid DC or lymphoid DC (Katsura, 2002).

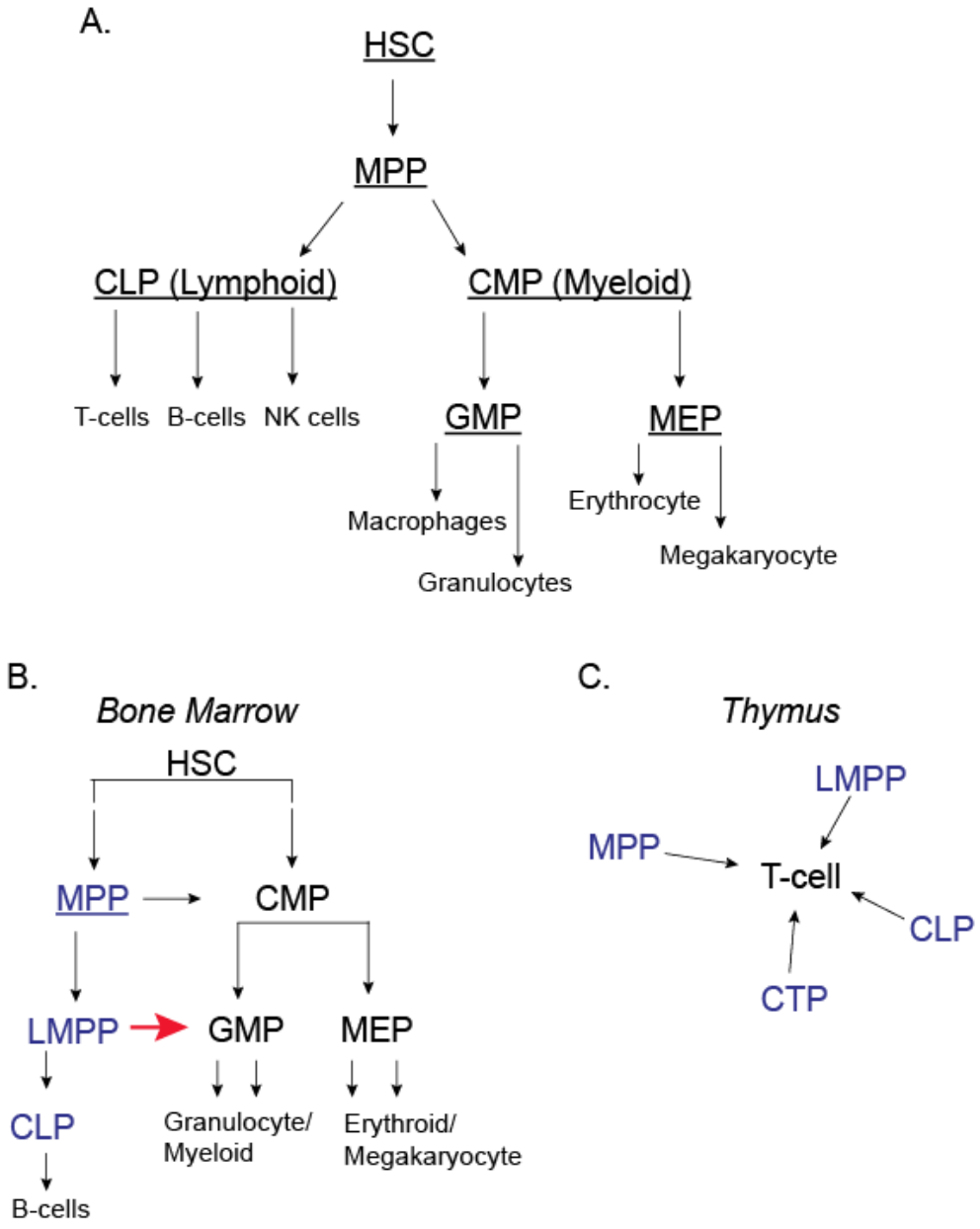

Figure 1. Models of Hematopoiesis. (A) This diagram depicts the 'classic' model of blood-cell development where HSC give rise to MPPs that generate lymphoid restricted CLP and myeloid restricted CMP cells. CLPs and CMPs then give rise to progenitors that will ultimately differentiate into mature cells. **(B)** The 'revised' model of hematopoiesis counters that although MPPs can give rise

to CMP, they can also generate LMPP which have the ability to generate myeloid cells. This model argues for the existence of a progenitor cell (LMPP) occurring later than the MPP that no longer has erythroid/megakaryocyte potential, but retains myeloid and lymphoid potentials. **(C)** This diagram shows the proposed progenitors that could migrate to the thymus and generate T-cells. The exact progenitor is unknown and in fact the thymus may be seeded by more than one progenitor. (Bhandoola et al., 2007; Lai and Kondo, 2008; Rothenberg, 2011)

Abbreviations: HSC-hematopoietic stem cell, MPP-multipotent progenitor, LMPP-lymphoid specified multipotent progenitor, CLP-common lymphoid progenitor, CMP-common myeloid progenitor, GMP-granulocyte monocyte progenitors, MEP-megakaryocyte erythrocyte progenitors

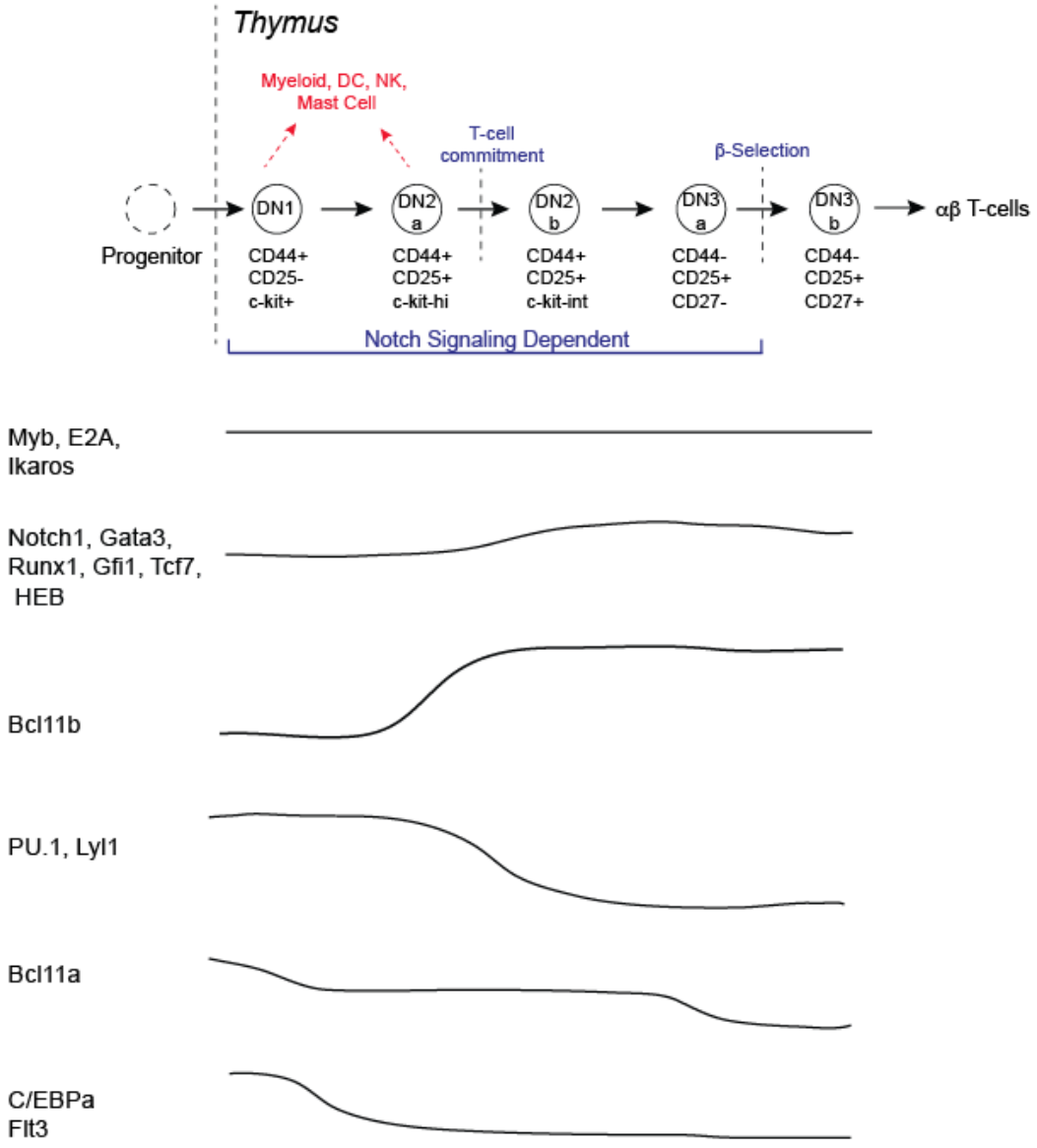

Figure 2. Diagram of early T-cell development and expression patterns of key transcription factors. T-cell progenitors migrate to the thymus where pro-T cells go through several DN (double negative stages- CD4-CD8-) that can be classified by the combinatorial expression of cell-surface receptors. DN1 and DN2a cells are able to give rise to alternative lineage cells if cultured under permissive conditions. Pro-T cells are dependent on Notch signaling from the DN1 to DN3a stages of development. The

expression patterns of several transcription factors expressed during T-cell development are depicted here. (Taghon T, 2006; Rothenberg et al., 2010; Yui et al., 2010).

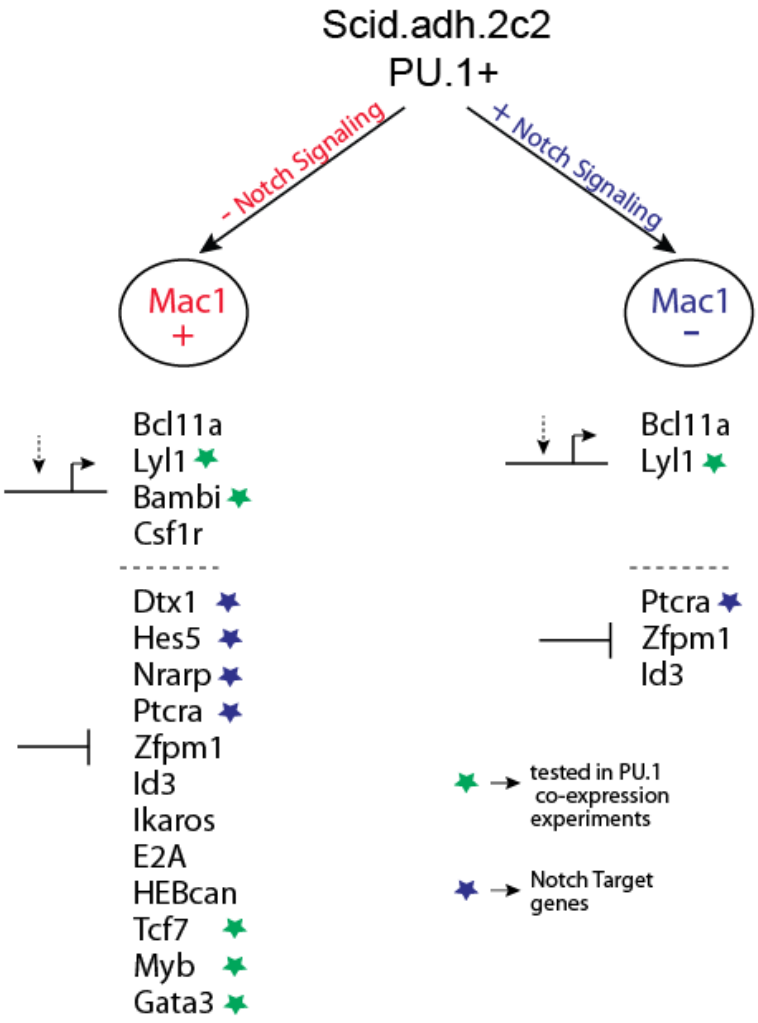

Figure 3. Snapshot of gene expression changes in Scid.adh.2c2 cells expressing PU.1 in the presence or absence of Notch signaling. The progenitor cell genes *Bcl11a* and *Lyl1* were up-regulated in response to PU.1 in cells with either high or low Notch signaling levels. *Tcf7*, *Myb*, *Gata3*, and several other T-cell genes were down-regulated in cells with PU.1 and low levels of Notch signaling. Not all genes, such as *Id3* and *Zfpm1*, could be rescued from PU.1-driven inhibition by exposure to high levels of Notch. These results of these experiments helped identify candidate genes that were used in PU.1 co-expression experiments in Scid.adh.2c2 cells (green stars).

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Chapter 2:

Characterization of the Scid.adh.2c2 cell line for the study of lymphomyeloid lineage decisions during early T-cell development.

Abstract

Cell lines have long been used as a tool to study developmental questions. Their highly proliferative nature allows large numbers of cells to be generated for experiments and they are often more easily manipulated than primary cells. This is especially true for studying early T-cell development when precisely staged fetal derived cells are required for many experiments. PU.1 is a transcription factor important for and expressed during early T-cell development, but nonetheless has the ability to divert early T-cells to a myeloid fate when Notch signaling is absent. Scid.adh.2c2 cells have previously been used to study the diversion response to PU.1. Here we discuss experiments that establish the early T-cell line, Scid.adh.2c2, as a system to study myeloid vs. lymphoid lineage decisions involving PU.1 and Notch signaling during early T-cell development. We characterized some of the gene expression changes that accompany the up-regulation of the myeloid markers CD11c and Mac1 in PU.1 transduced Scid.adh.2c2 cells. We also show that, as in fetal thymocytes, the decision of Scid.adh.2c2 cells to divert or remain in the T-lineage is also dependent on Notch signaling. Finally, we explore the involvement of the MAP kinase pathway, myeloid cytokines, and some PU.1 co-activators in the PU.1-driven diversion response.

Introduction

The Ets family transcription factor, PU.1, is expressed during the earliest stages of T-cell development (Back et al., 2005; Nutt et al., 2005) and is also important for the development of other cell types such as myeloid cells (Ghani et al., 2011). Over-expression experiments of PU.1 in early T-cells showed that PU.1 can divert these cells from a T-lineage to a myeloid lineage when protective Notch signals are absent (Anderson

et al., 2002b; Franco et al., 2006). During normal development T-cell progenitors require Notch signaling for their development and survival (Yang et al., 2010) and therefore, the endogenous expression of PU.1 does not drive these cells to a myeloid fate. The experiments conducted to study these lymphomyeloid developmental decisions used exogenous retroviral expression of PU.1. However, severe anti-survival effects were seen with PU.1 over-expression in fetal thymocytes, even with the use of transgenic mice expressing the anti-apoptotic gene, Bcl2. Therefore, a subclone of a Scid.adh thymic lymphoma, Scid.adh.2c2, was used to study the diversion of pro-T-cells in response to PU.1 (Dionne et al., 2005).

Scid.adh.2c2 cells resemble a DN3 stage pro-T-cell and so do not express PU.1 (Carleton et al., 1999a). Like thymocytes, Scid.adh.2c2 cells carry out Notch signaling, but unlike primary cells they do not receive their Notch signaling via interaction of their Notch receptor with a Notch ligand from the environment. Scid.adh.2c2 cells instead display spontaneous ligand-independent activation of Notch signaling and as a result do not require co-culture with Notch ligand-expressing cells. Also like primary cells, the myeloid cell-surface protein, Mac1, can be detected on Scid.adh.2c2 cells after transduction with PU.1 (Dionne et al., 2005). After it was discovered that Notch signaling plays a role in protecting PU.1-trandscued cells from diversion (Franco et al., 2006; Laiosa et al., 2006b) we wanted to explore the possibility of using Scid.adh.2c2 cells to study the interactions between Notch signaling and PU.1, in the context of protection (high Notch) and diversion (low Notch) using a chemical inhibitor of Notch signaling. Here we show that knockdown of Notch signaling using the chemical Notch inhibitor, Gamma secretase inhibitor (GSI), increases the percentage of Mac1+ Scid.adh.2c2 cells after PU.1 transduction. Here we

also identify a CD11c+ intermediate Scid.adh.2c2 cell type whose gene expression gives us clues to the changes that must occur before diversion is complete (i.e., Mac1 is up-regulated).

Previous studies with Scid.adh.2c2 cells transduced with PU.1 showed that stimulation with PMA (phorbol ester) also caused an increase in the percentage of the cells that up-regulated Mac1. PMA stimulation leads to the activation of PKC (protein kinase C) that in turn can activate several downstream signaling pathways, including the MAP kinase pathway. Since the MAP kinase pathway activation of PU.1 co-activators is important during normal developmental programs (Behre et al., 1999), we chose to explore several MAPK inhibitors to test whether MAP kinase signaling was involved in enhancing PU.1-driven diversion. Surprisingly, we present preliminary data suggesting that the P38 and MEK MAP kinase pathways may be involved in protecting Scid.adh.2c2 cells from diversion.

To further characterize the Scid.adh.2c2 cell-line system for PU.1-mediated lineage choice studies, we explored the effects of myeloid supportive cytokines on PU.1-transduced Scid.adh.2c2 cells. We also analyzed the involvement of known PU.1 collaborators, c-Jun and C/EBPα. Ultimately, we found that neither myeloid cytokines nor the tested co-activators influenced PU.1-driven diversion in Scid.adh.2c2 cells.

Results

Notch signaling specifically protects against diversion to a macrophage cell fate

Scid.adh.2c2 cells and fetal thymocytes have both been used to study lineage choice decisions in an environment with high PU.1 (Anderson et al., 2002b; Dionne et al., 2005; Laiosa et al., 2006b). In these studies the up-regulation of the myeloid marker, and PU.1 target gene, Mac1 has been used to identify cells that have initiated a myeloid program in response to PU.1. Here we also wanted to assay for the ability of PU.1 to induce fetal thymocytes to express the dendritic cell surface protein, CD11c, which help distinguish between macrophages and dendritic cells. This can help us classify the protection of Notch signaling as being against a broad myeloid program or against a specific type of myeloid cell. To explore this, we transduced embryonic day 15.5 Bcl2tg fetal thymocytes with PU.1 or empty vector and cultured the cells for 48 hours in the presence or absence of Notch signaling using the OP9 cell co-culture system. OP9 cells are bone marrow stromal cells can be used to support T-cell development when they are transduced to express the Notch ligand, Delta-like1 (OP9-Dl1) (Schmitt and Zuniga-Pflucker, 2002). The samples were then analyzed for their cell surface expression of Mac1 and CD11c using flow cytometry. Few cells with either marker emerged from the empty vector control-transduced Bcl2tg fetal thymocytes cultured either in the presence or absence of Notch signaling (Fig.1). When transduced with PU.1 in the absence of Notch signaling, Mac1+CD11c- thymocytes represented the majority of cells that developed, and thus suggesting their classification as macrophages (Fig. 1). This Mac1+ CD11c- phenotype was suppressed in the presence of Notch signals (dropping from 70% to 10% of transduced cells), whereas CD11c+Mac1+ cells resembling “myeloid dendritic cells” developed best in the presence of Notch signaling (increased from 12% to 25%). A lower percentage of CD11c+ only cells were also generated, suggesting another subset of

dendritic cells. These data show that Notch signaling is able to specifically protect pro-T cells from initiating a macrophage developmental program, although high PU.1 may still support dendritic cell-like development even in the presence of Notch.

Similar results were seen in Scid.adh.2c2 cells doubly transduced with PU.1 and a construct expressing a constitutively active Notch1 (ICN1) (Maillard et al., 2004). Here, both Mac1 and CD11c could be detected 48 hours after transfection with only PU.1. Just like in the fetal thymocytes data, co-expression of PU.1 and ICN did not block CD11c up-regulation as successfully as Mac1 up-regulation (data shown in Ch. 3, Fig. 4A). This suggests again that macrophage cell development is more sensitive to inhibition by Notch signaling.

A CD11c+ intermediate in the pathway to full diversion

PU.1 transduced Scid.adh.2c2 cells can up-regulate myeloid markers Mac1 (CD11b) and CD11c (Ch 3: Fig. 4A). Flow cytometric analysis of these cells show that three populations are generated when the cells are transduced with PU.1 (Fig. 3A); a Mac1+ population, a CD11c+ population and a Mac1+CD11c+ population. We were interested in investigating the gene expression differences between these populations of cells. When PU.1-transduced cells were sorted into subsets based on CD11c and Mac1 expression after 2 days, the gene expression profiles in these samples indicated that CD11c+ Mac1- cells could be an intermediate state in diversion (Fig. 2). PU.1 target genes *Bambi* and *Bcl11a* were up-regulated over their very low baseline levels in all the PU.1-expressing samples, but *Csf1r* and *Mac1*, expressed highly in macrophages, were much more up-regulated in cells which were surface Mac1+ compared to CD11c+ only cells. While some T-cell genes were already inhibited in the cells that had turned on CD11c alone

(especially *Hes5, Rag1,* and some Notch target genes), most were further repressed in the Mac1+ cells, indicating a progressive erasure of T-lineage identity rather than a single-step inhibition. Notably, T-cell genes including *Tcf7, Myb, Gata3, Fog1*, and *Gfi1* were inhibited much more in Mac1+ and Mac1+CD11c+ cells than in CD11c+ only cells. *Myb, Gata3* and *Gfi1* especially were expressed at essentially normal levels in the CD11c+ population, despite evidence of significant Notch pathway impairment; they were sharply down-regulated only when Mac1 was also expressed.

The gene expression profile of Mac1+CD11c+ and Mac1+ cells are very similar, while the CD11c+ cells seem to be an intermediate cell type. We wanted to perform a tracking experiment to characterize the nature of Mac1 and CD11c up-regulation in Scid.adh.2c2 cells. Scid.adh.2c2 cells were infected with PU.1 and cultured for two days and then sorted into four subsets according to their expression of Mac1 and CD11c (Fig. 3A). Each of these populations was then put back into culture for an additional two days and then reanalyzed for their expression of Mac1 and CD11c. As shown in Fig. 3B, cells that were initially Mac1-CD11c- as well as cells that were initially Mac1- CD11c+ tended to progress toward a Mac1+CD11c+ terminal phenotype, although Mac1 and CD11c expression could be activated in either order. These PU.1-overexpressing cells may take different pathways, but most ultimately end up expressing both Mac1 and CD11c. Late addition of GSI to the cells, in the second culture period only, slightly reduced the number of cells that were able to resist turning on either Mac1 or CD11c, and slightly increased Mac1 up-regulation in cells that initially expressed CD11c+ alone. However, the changes associated with induction of CD11c+ appeared to precede Mac1 expression for many cells.

Cell-surface levels of CD25 can be used as an approximation of Notch signaling strength in Scid.adh.2c2 cells

CD25 is a known Notch target gene that is used as a marker to distinguish between the early stages of T-cell development (Maillard et al., 2006b). Scid.adh2c2 cells, which are used as a model for cells in the DN3 stage of development, also express this marker on their surface. CD25 down regulation in normal thymocyte development occurs in response to calcium and MAP kinase signaling during β-selection. A major response of this selection event in-vivo is the shut off of Notch signaling in these cells. Scid.adh.2c2 cells grow as a mixed population of CD25 high and CD25 intermediate cells. Our group found that PMA stimulation (which can trigger MAP kinase signaling) could induce the increased generation of CD25 low/intermediate cells (Dionne et al., 2005). It may also be the case that Scid.adh2c2 cells with higher levels of cell-surface CD25 also have higher levels of Notch signaling. To investigate this, we sorted CD25 high and CD25 intermediate cells and then lysed the cells for RNA extraction. The RNA was reverse transcribed and used for QPCR analysis. CD25 high cells expressed higher levels of Notch target genes *Ptcra*, *Lef1*, *Notch1*, *Notch3*, *Nrarp*, and *Dtx1* compared to CD25 intermediate cells (Fig. 4). A number of regulatory genes expressed during early T-cell development were tested, such as *Gata3*, *Tcf7*, *Myb*, and *Id2,* were expressed at similar levels in both the CD25 high and CD25 intermediate cells (Fig. 4). This data, and other gene expression data presented in Ch. 3 (Ch.3: Fig.4A), suggest that cell-surface CD25 levels can be used to approximate the level of Notch signaling activity in the cell.

A higher percentage of CD25 intermediate Scid.adh.2c2 cells up-regulate Mac1 in response to PU.1 expression compared to CD25 high cells.

Scid.adh.2c2 cells have been used to study the diversion of T-lineage cells to a myeloid fate (Dionne et al., 2005). In those studies it was demonstrated that these cells diverted in an all-or-none fashion after transduction with PU.1; meaning that the PU.1-expressing cells were either Mac1+ or Mac1-. Furthermore, when single cell clones of Scid.adh.2c2 cells were transduced with PU.1 and cultured for two days, in all cases both Mac1 positive and Mac1 negative cells were generated (Dionne et al., 2005). A single cell was able to give rise to two populations, one that remained in the T-lineage and another that diverted to a myeloid lineage.

Although we know that single PU.1-expressing Scid.adh.2c2 cells can give rise to these two populations, we wanted to investigate the possibility that the CD25 intermediate cells were more predisposed to diversion because of their lower levels of Notch signaling. To test this we sorted CD25 high and CD25 intermediate Scid.adh.2c2 cells and infected each with PU.1 and an empty vector control. The four samples were then cultured for 2 days. Using flow cytometry we looked for the up-regulation of the PU.1 target gene, Mac1, as a sign of diversion. As expected, both the CD25 high and CD25 intermediate empty vector controls did not up-regulate Mac1. Interestingly however, the sorted CD25 high cells re-generated a CD25 intermediate population and the sorted CD25 intermediate cells re-generated a CD25 high population. PU.1 was able to divert more cells to the myeloid lineage when expressed in the CD25 intermediate samples, although PU.1 was also able to drive diversion in the CD25 high cells. About 50% of the CD25 high cells up-regulated Mac1 compared to about 60% of the cells in the CD25 intermediate samples (Fig.

5). Although the Scid.adh.2c2 cells do not need to be predisposed to low levels of Notch signaling to divert, a larger percentage of the CD25 intermediate cells diverted. This suggests that the same interaction between PU.1 and Notch signaling seen in fetal thymocytes can be studied in the Scid.adh.2c2 cells.

Notch signaling manipulations in Scid.adh.2c2 cells

The results from the previous section suggest that higher levels of Notch signaling protect Scid.adh.2c2 cells from PU.1-driven diversion. We wanted to further investigate the effects of Notch signaling manipulation on the ability of Scid.adh.2c2 cells to divert. We first tried to culture the cells in an environment where they would be receiving increased Notch signaling using the OP9 co-culture system. Scid.adh.2c2 cells were infected with PU.1 or empty vector and then cultured on OP9-Dl1 to try to promote increased Notch signaling, on OP9-control for no external ligand mediated activation of Notch signaling, and a control sample without any stromal cells. After 2 days the cells were analyzed for their expression of the diversion marker, Mac1, using flow cytometry. We found that culture of Scid.adh.2c2 cells on OP9-dl1 stromal layers did not decrease the percentage of cells that diverted compared to the OP9-control and no stroma controls (data not shown). We later used retroviral transduction of intracellular Notch to increase Notch signaling and these results are described in Chapter 3.

To knock down Notch signaling we used the chemical Notch signaling inhibitor GSI (gamma secretase inhibitor). GSI interferes with Notch signaling by blocking the cleaving of the intracellular portion of the Notch receptor. In the presence of GSI, when the Notch receptor is engaged, the intracellular portion of Notch is not cleaved and therefore never reaches the nucleus where its transcriptional effects would be mediated. Firstly, we wanted

to test if GSI could be used to knockdown Notch signaling in Scid.adh.2c2 cells and if this inhibition of Notch signaling was reversible by washing the GSI out of the culture media. To accomplish this we cultured Scid.adh.2c2 cells with 0.5 μM GSI for 3 days and then monitored for changes in expression of the Notch target gene, CD25. After 3 days in culture with GSI about 97% of the cells had lowered CD25 cell-surface expression levels showing that Notch signaling was indeed lowered (Fig. 6A). To test the reversibility of the GSI we washed the cells and cultured them in GSI-free media for an additional 4 days and found that CD25 levels could be restored (Fig. 6B).

Since GSI can lower Notch signaling in Scid.adh.2c2 cells we wanted to know if GSI could enhance the up-regulation of a myeloid cell marker, CD11c, in the PU.1-expressing cells. To test this we transduced Scid.adh2c2 cells with PU.1 and empty vector and cultured the cells with no GSI, 0.5 μM GSI, and 1.0 μM GSI and monitored the cell-surface expression of CD25 and CD11c 2 and 4 days after culture. We found that by 2 days CD25 levels had significantly dropped in the empty vector control samples (Fig. 7). CD25 down regulation was not increased with a higher concentration of GSI or with a longer incubation time (Fig.7B vs. Fig. 7C). About 40% of the Scid.adh.2c2 cells expressing PU.1 with no added GSI up-regulated CD11c and this was increased to 55% the with the addition of both 0.5 μM and 1 μM GSI in both the 2-day and 4-day cultures (Fig. 7B, C). This suggests that Scid.adh.2c2 cells can be used to study PU.1 and Notch signaling interactions in relation to the lymphomyeloid lineage decision.

The previous results demonstrate that using 0.5 μM GSI in a 2-day culture with PU.1 expressing Scid.adh.2c2 cells is sufficient to mimic the conditions during which PU.1

diverts cells in the absence of Notch signaling. We wanted to investigate the possibility that GSI could cause a greater percentage of diverting cells if the cells were pretreated with GSI prior to infection with PU.1. To test this we pretreated Scid.adh.2c2 cells with 0.5 μM GSI for 2 days and cultured another group of cells with vehicle (DMSO) as a control. We then infected those samples with PU.1 and empty vector overnight. We split the pretreated and non-pretreated samples into cultures containing GSI or DMSO. After 2 days the cells were analyzed for their expression levels of CD25 and Mac1. The PU.1-expressing Scid.adh.2c2 cells that were not pretreated and then cultured in DMSO had about 54% of the cells up-regulate Mac1 compared to 62% of the GSI pretreated Scid.adh.2c2 cells cultured with DMSO (Fig. 8A vs. Fig. 8C). The PU.1+ cells that were not pretreated and were then cultured with 0.5 μM GSI had similar percentages of Mac1+ cells as the GSI pretreated samples cultured in 0.5 μM GSI after infection (80%) (Fig. 8B vs. Fig. 8D). If the cells were cultured without GSI for the 2 days after PU.1 expression, the pretreatment seemed to enhance the diversion response, possibly because the cells were still Notch inhibited. However, if GSI is to be used to inhibit Notch signaling, it is not necessary to pretreat the cells with GSI before PU.1 transduction.

Testing additional pathways that could be involved in the diversion/protection decision in Scid.adh2c2 cells expressing PU.1: Map kinases

In previous studies it was shown that the addition of phorbol ester and ionomycin (PMA/I) enhanced the diversion of PU.1 expressing Scid.adh.2c2 cells (Dionne et al., 2005). The exclusion of ionomycin from the cell culture did not change those results and PMA was therefore deemed to be sufficient to make PU.1-driven diversion more efficient.

The authors also described the down-regulation of CD25 in the empty vector samples, suggesting a decrease of Notch signaling in these cells. PMA can activate protein kinase C (PKC), which is known to be involved in several signal transduction pathways including MAP kinase signaling (Griner and Kazanietz, 2007; Rosse et al., 2010). MAP kinase signaling pathways have been linked to the phosphorylation of a powerful PU.1 co-activator, c-Jun (Behre et al., 1999) and we therefore focused on the MAP kinase pathway in follow up studies presented here. We used specific MAP kinase inhibitors to try to identify a MAP kinases involved in lowering the threshold of diversion in PU.1-expressing cells. If these kinases were involved in lowering the cells ability to resist diversion, then their inhibition would cause decreased diversion. We focused on 3 MAP pathway kinases that could be activated by the addition of PMA to the cell cultures: JNK, P38, and MEK.

The JNK inhibition studies were done on bulk Scid.adh.2c2 cells transduced with PU.1 and empty vector. The cells were cultured in 0 μM, 10 μM, 25 μM, and 50 μM of the MEK inhibitor U0126 for 2 days and then analyzed for the expression of CD25 and Mac1 using flow cytometry. The addition of 10 μM U0126 to the culture did not down-regulate CD25 levels in the empty vector control samples, while the 25 μM and 50 μM concentrations did reduce CD25 levels (Fig. 9). The PU.1 transduced samples did not seem to respond to the 10 μM and 25 μM concentration of the JNK inhibitor. However, the 50 μM sample had about half the percentage of cells up-regulate Mac1 compared to the control PU.1-expressing cells (Fig. 9). However, cell survival/proliferation were reduced in these samples and the Mac1+ cells that diverted in the presence of high MEK inhibitor seemed to have lower levels of Mac1. These results were deemed inconclusive because of

effects on cell survival. However, they could indicate a need for collaboration between PU.1 and MEK/ERK in promoting diversion.

For the P38 and JNK inhibition studies we transduced Scid.adh.2c2 cells with PU.1 or empty vector for 2 days. Mac1 and CD11c (another myeloid marker) specific antibodies were used to tag and remove diverted cells using magnetic bead columns (Fig. 10A and Fig. 11A). For the P38 inhibition studies with the chemical, SB203580, the Mac1/CD11c depleted Scid.adh.2c2 cells were cultured for an additional 2 days with the chemical vehicle DMSO, 0.5 μM GSI, 1 μM SB203580, or 10 μM SB203580 to evaluate the generation of additional CD11c/Mac1+ cells. As expected (see Ch.3), the addition of GSI increased the percentage of Mac1 compared to samples with no Notch manipulation (Fig. 10). The cells cultured with 1 μM and 10 μM P38 inhibitor also increased levels of Mac1 and CD11c up-regulation compared to controls with no inhibitor, but lower percentages than the cells cultured with GSI (Fig. 10B, *data not shown*). This response to P38 inhibition is the opposite of what we were expecting if P38 were involved in the increased diversion of the cells. These results suggest that signaling mediated via P38 is actually involved in protecting the cells from diversion.

The JNK inhibition studies were performed with PU.1 or empty vector Mac1/CD11c depleted Scid.adh.2c2 cells that were cultured in 5 μM or 15 μM of the JNK inhibitor, JNK II. Like the P38 studies, the PU.1-expressing Scid.adh.2c2 cells cultured in 0.5 μM GSI had increased percentages of Mac1 compared to control PU.1-expressing cells cultured in DMSO (Fig. 11B). The samples with 5 μM JNK II gave slightly increased percentages of Mac1 compared to untreated samples, while the 15 μM JNK II inhibitor sample had a

much higher percentage of Mac1+ cells (Fig. 11B). These results suggest that a pathway simulated by JNK is also involved in protecting PU.1-expressing Scid.adh.2c2 cells from diversion. These results with the MAP kinase inhibitors were unexpected and more experiments are required to help us understand them. These results suggest that the pathway(s) stimulated by PMA, which led to increased diversion were not dependent on the MAP kinases studied here (except for the possible exception of MEK/ERK).

Pro-myeloid cytokines do not enhance the up-regulation of Mac1 in Thy1+ PU.1-transduced fetal thymocytes or in Scid.adh.2c2 cells

Cytokine signaling is important during several stages of hematopoiesis. For early T-cell development, IL-7 is an important cytokine needed for survival (for review see (Ciofani and Zuniga-Pflucker, 2006)). However, it is important to maintain these cytokines, like IL-7 and Flt3L, at appropriate concentrations during development. For example, at lower levels IL-7 can enhance thymocyte survival, while at higher levels IL-7 can oppose their proliferation (El Kassar et al., 2004). For in-vitro culture systems of T-cells, such as the OP9-DL1 co-culture system, the concentration of cytokines can be easily controlled. It is common practice to add IL-7 and Flt3L to early T cell cultures with OP9-DL1 to encourage T-cell development. A similar practice is used when attempting to generate myeloid cells in-vitro. The macrophage colony-stimulating factor (MCSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) cytokines are important for survival during myeloid cell development (Karen L. Anderson et al., 1999). We wanted to investigate the possibility that pro-T-cells diverting to the myeloid pathway in response to high PU.1 required myeloid cytokines for diversion or survival after diversion.

To investigate the effects of pro-myeloid cytokines on the PU.1-driven diversion response, we transduced PU.1 and empty vector into E15.5 Bcl2tg fetal thymocytes and cultured them in the presence of absence of Notch signaling conditions with T-supportive cytokines or T- and myeloid-supportive cytokines. We then used flow cytometry to detect the expression of a T-cell marker (Thy1) and a myeloid marker (Mac1) after 2 days in culture. Thy1+Mac1+ cells would mark cells that entered the T-lineage pathway, but then began diversion to a myeloid pathway. Mac1+ cells could mark early pro-T-cells (DN1) cells that had not yet up-regulated Thy1 or myeloid progenitors present in the E15.5 fetal thymus. As expected, we found that initial exposure to Notch signaling was enough to protect PU.1-transduced fetal thymocytes from diverting in the presence of the T-supportive cytokines (Fig. 12A, condition 1). Culture of PU.1 transduced fetal thymocytes with myeloid cytokines in the presence of Notch signaling for 3 days did not increase the percentage of Thy1+Mac1+ cells (Fig. 12B). However, the percentage of Mac1 single positive cells did increase by about 10% with the inclusion of the myeloid cytokines. In the absence of Notch signaling the increase of Mac1 single positive cells was even bigger, from 20% to 54% with the inclusion of myeloid cytokines (Fig. 12B, condition 2). The percentage of the diverted, Thy1+Mac1+, cells did not increase with the addition of myeloid cytokines in the absence of Notch signaling. This suggests that myeloid cytokines are not yet required for the survival of diverted Thy1+Mac1+ thymocytes. Where we do see an effect, in the Mac1 single positive, the cells could be further along in myeloid development and therefore be more dependent on these cytokines signals for growth and development.

We were also interested in the effects of myeloid cytokines in PU.1-transduced Scid.adh.2c2 cells. We infected Scid.adh.2c2 cells with PU.1 and empty vector and then cultured the cells in the presence or absence of the myeloid cytokines MCSF and G-MCSF. We also manipulated Notch signaling in these samples by culturing the cells in GSI, to knockdown Notch signaling. We cultured the cells for 2 days and then analyzed them for the expression of Mac1 using flow cytometry. Like in fetal thymocytes, we did not detect an increase in the percentage of diverted PU.1+Mac1+ positive cells in either the GSI or vehicle control samples (Table 1). This suggests that at this early timepoint the appearance of diverting cells does not depend on myeloid cytokines for fetal thymocytes or Scid.adh.2c2 cells and hence these cytokines can be left out of cell cultures when doing 2-day PU.1-driven diversion studies.

PU.1 co-activators c-Jun and CEBP/a are not expressed in Scid.adh.2c2 cells

We have thus far discussed investigations of interactions of Scid.adh.2c2 cells with Notch signaling, MAP kinase signaling pathways, and myeloid cytokines. Here we describe PU.1's interactions with two well-defined PU.1 co-activators c-Jun and C/EBPα. C-Jun, a bZIP transcription factor, is typically found as part of the AP-1 transcription factor complex and has been shown to be important for the survival, proliferation, and development of myeloid cells (Friedman, 2007). C-jun was described as being able to bind to PU.1 and cooperatively regulate the expression of *Mcsfr* (Behre et al., 1999). Here we wanted to ask if c-Jun could also be involved in the lymphomyeloid lineage choice driven by PU.1 expression in the absence of Notch signaling. We transduced Scid.adh.2c2 cells with PU.1 and empty vectors and cultured the cells in the presence or absence of GSI for 2

days. Designing primers for qRT-PCR analysis is difficult for c-Jun since it is a small gene with only 1 exon, so we detected c-Jun protein levels using Western blot analysis with c-Jun-specific antibodies. We were able to detect c-Jun expression in the macrophage cell line, RAW246.7, but not in any of the Scid.adh.2c2 cell samples, whether they were transfected with PU.1 or not (Fig. 13). Therefore, its collaboration with PU.1 is not required for PU.1-driven gene expression changes in Scid.adh.2c2 cells.

C/EBPα is similarly important for myeloid development and its cooperation with PU.1 has been well documented. In myeloid cells PU.1 and C/EBPα have both been shown to regulate *Gmcsfr* (Hohaus et al., 1995) as well as other myeloid cytokine receptors such as Mcsfr (DE et al., 1996). However, we were not able to detect any C/EBPα up-regulation in Scid.adh.2c2 cells transduced with PU.1 using qRT-PCR primers (*data not shown*). It seems therefore, that PU.1 is mediating its pro-myeloid effects without its well-known collaborators, c-Jun and C/EBPα.

Discussion

The study of T-cell development is a difficult task to undertake considering the difficulty in generating enough material for experiments that are often sensitive to cell numbers. This problem is enhanced when genetic manipulations that effect cell survival and/or proliferation are forced upon the cells. The time that it takes to generate these cells through timed mating experiments is also troublesome when attempting to do several pilot experiments quickly. Previously, we had shown that the Scid.adh.2c2 cell line could be used to study the response of early T-cells to PU.1 over-expression. These cells were

characterized as being able to express myeloid markers and begin to shut down the T-cell program. Here we investigated using Scid.adh.2c2 cells for studying the interactions of PU.1 and Notch signaling in cells that divert vs. cells that do not divert. We were able to identify Mac1 up-regulation as a 'more diverted' stage in a population of cells that can express Mac1 alone, Mac1 and CD11c, and CD11c alone. This validates the use to Mac1 for sorting 'diverted' cells vs. the use of the dendritic cell marker, CD11c.

Scid.adh.2c2 cells have spontaneous ligand-independent Notch signaling and express the Notch target gene CD25 in an unstable way. We show that manipulating these cells with GSI can decrease the level of Notch signaling and increase the diversion response just like in primary cells. We fine-tuned the experimental conditions to study Scid.adh.2c2 cells in this context to 2 days in culture with 0.5 μM GSI and no added cytokines. We preferred 2 days in culture over 4 days because we know that PU.1 transduction has deleterious effects on cells growth and survival (Dionne et al., 2005) and we wanted to minimize unwanted side effects. We also found that pretreatment of the cells with GSI was not necessary for the cells to enhance Mac1 up-regulation in the presence of high PU.1 and we therefore add it at the time of transduction. These parameters were also useful in deciding how to set up experiments involving PU.1 co-infection with constitutively active Notch1 (ICN1) or the dominant negative inhibitor of Notch-dependent transcription, dnMAML (Maillard et al., 2004). We found that co-infection of PU.1 and either Notch construct gave similar results to a two-step transduction adding the Notch construct first and then PU.1 (*data not shown*). Using ICN and dnMAML also allowed us to control the unstable Notch signaling found in the Scid.adh.2c2 cells and eliminates the possibility of any cross inhibition of other signaling pathways by GSI. Gene expression changes in

Scid.adh.2c2 cells resulting from these two methods of Notch signaling manipulations (chemical inhibition vs. co-transfection) were mostly in agreement and thus strengthened our results.

Here we also reported additional data that helped characterize optimal experimental conditions for experiments exploring questions about PU.1-driven diversion. We discovered that although there are examples of cytokines having instructive roles in development (Panopoulos et al., 2002), the addition of myeloid cytokines did not affect the outcome of diversion in Thy1+Mac1+ cells or in PU.1-transduced Scid.adh.2c2 cells. *Mcsfr* mRNA can be detected after 2 days of PU.1 transduction in the absence of Notch signaling in fetal thymocytes and Scid.adh.2c2 cells (Ch. 3: Table S1 and Fig. 4B). However 2 days could be too early for these mixed lineage cells to depend on myeloid cytokines for their survival/development.

In this chapter we also discussed C/EBPα and c-Jun. We thought that Notch signaling could be interfering with PU.1 transcriptional activity by inhibiting these two well-known PU.1 co-activators. Since neither of these factors was ever detected at the early time points studied here, we believe that PU.1 must be mediating its transcriptional activity with the aid of other known collaborators (Yamamoto et al., 1999; Hu et al., 2011) or other unidentified collaborators.

We also identified P38 or MEK kinases as potentially involved in protecting pro-T-cells from diversion. Interactions between MAP kinases and both PU.1 and Notch signaling have been described. PU.1 protein phosphorylation has been shown to alter its transactivation activity (Hamdorf et al., 2011b). P38 kinase by definition can

phosphorylate other proteins and P38 kinase can indeed interact with PU.1. During bone development PU.1 has been implicated in a complex with P38 kinase (Sharma et al., 2007) and during early B-cell development the transactivational activity of PU.1 is increased via a P38 kinase dependent mechanism (Wang et al., 2003). Studies have also shown that PU.1 DNA-binding activity is reduced when MEK is inhibited (Miranda et al., 2005). However, we did not detect any differential phosphorylation patterns in Scid.adh.2c2 cells transduced with PU.1 (Ch.3: Fig. S1). These MAP kinases are therefore probably not interacting with PU.1 directly in the Scid.adh.2c2 cell system. Interactions with Notch signaling and these MAP kinases in Scid.adh.2c2 cells are also uncertain. We do know however that experiments using GSI to knockdown Notch signaling in a human carcinoma cell line do not affect the phosphorylation of JNK or P38 (SM et al., 2011). Also, in a muscle cell line Notch signaling has been proven to inhibit the activity of P38 by directly up-regulating an inhibitor of P38 (Kondoh et al., 2007) while in a pancreatic cancer cells MEK activity can promote the expression of Notch target genes (Tremblay et al., 2012). There is evidence that these MAP kinases can have either inhibitory or activating effects on PU.1 and/or Notch signaling. Yet it is true that their inhibition yields the same results in PU.1-transduced Scid.adh.2c2 cells. The roles of MAP kinases during the double negative (DN) stages of T-cell development have been described, but are still not well understood (Rincón, 2001). The preliminary data presented here may be very important, but many more experiments would be required to begin to understand the role/s of the MAP kinase pathway in maintaining T-cell fidelity in an environment with high PU.1.

In the subsequent chapter we will describe in detail the gene expression changes that occur in PU.1-transduced Scid.adh.2c2 cells in the presence and absence of Notch

signaling. We use this system to describe a transcription factor network involving PU.1, Myb, Gata3, and Notch signaling that partially defines a lymphomyeloid switch during early T-cell development.

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Materials and Methods

Mice

C57BL/6-Bcl2tg mice (B6.Cg-Tg(BCL2)25Wehi/J) were housed under specific pathogen-free conditions, bred in-house, and cared for by Caltech Animal Facility staff. Embryonic Day (E) 15.5 fetal thymocyte precursors were used. All animal work followed protocols approved by the Institutional Animal Care and Use Committee.

MAPK inhibition Studies

The starting culture for the MEK/ERK inhibition studies were bulk PU.1 or empty vector transduced Scid.adh2c2 cells that were then cultured with 10 μM, 25 μM, or 50 μM U0126 (Cell signaling) for 2 days. These cells where then analyzed for their cell surface expression of CD25, CD11c, and Mac1 with flow cytometry.

The starting cultures for the P38 and JNK inhibition studies were PU.1 and empty vector transduced Scid.adh2c2 cells that were given 2 days to up-regulate Mac1. The Mac1 positive cells were separated from the Mac1 negative cells by positive selection using magnetic columns (Miltenyi Biotech). The cells were incubated with biotin-labeled-antibody specific for Mac1 and CD11c (eBioscience) and then passed through a streptavidin-coated magnetic bead column coated (MACS) and separated on a MACS magnet (Miltenyi Biotech). The Mac1 negative cells were allowed to pass through the column and these cells were then cultured with 1 uM or 10 uM of the P38 inhibitor SB203580 (Promega), or 5 μM or 15 μM of the JNK inhibitor (JNK II inhibitor from Calbiochem). After 2 days the cells were analyzed for their expression of CD25, Mac1, and CD11c using flow cytometry.

Notch inhibition studies using GSI

For Notch signaling inhibition experiments using γ secretase inhibitors, InSolution™ γ-Secretase Inhibitor X (EMD Millipore) was added at 1 μM and 0.5 μM where indicated.Scid.adh2c2 cells were cultured for the specified amount of time and then analyzed using flow cytometry.

Cell Culture

Scid adh.2C2 and adh.6D4 cells were cultured in RPMI media supplemented with 10% fetal bovine serum (Sigma), sodium pyruvate, non-essential amino acids, penicillin/streptomycin/glutamine (Gibco), and beta-mercaptoethanol. Cells were incubated at 5% CO2 in 37°C.

Fetal thymocytes were cultured on OP9-Delta-like1 or OP9-control stromal layers in alpha-MEM medium supplemented with 20% fetal bovine serum (Sigma), penicillin/streptomycine/glutamine, beta-mercaptoethanol, 5 ng/ml of IL-7, and 5 ng/ml Flt3 ligand (cytokines from Peprotech). OP9 cells were plated two days before seeding with thymocytes, in 24-well or 96-well plates. Cells were harvested by pipetting and OP9 cells were partially excluded during transfers by passage through a nylon mesh.

Cell Staining and Sorting/Flow Cytometry

Fitc, PE, APC, and PerCPCy5.5-conjugated antibodies from eBioscience (San Diego, CA) or Cell Signaling (Danvers, MA) (CD25, Mac1/CD11b, CD11c) were used for cell surface staining. Fc receptors were blocked before staining with incubation with 2.4G2 supernatant for 20 min on ice. Cells surfaced staining was achieved in CBSS+0.5% BSA incubated on ice for 30 min. Viability staining was done with 7-AMINO-ACTINOMYCIN D (Invitrogen) staining by adding it to cell samples prior to sorting or analysis.

Cells were sorted using a BD FACS Aria (Becton Dickinson) and analyzed using a FACSCalibur (Becton Dickinson Immunocytometry Systems) or MACSQuant (Miltenyi) and FlowJo software (Tree Star, Ashland, OR).

RNA extraction and qRT-PCR

cDNA was prepared from total RNA using RNeasy extraction kits (Qiagen) and reverse transcribed using random hexamer primers and SuperscriptIII (Invitrogen).

cDNA samples were analyzed on a ABI Prism 7900HT Sequence Detector (ABI, Mountain View, CA) using SyberGreenER mix (Invitrogen) and gene specific primers (Operon). Samples were normalized to Actinb expression and results were calculated using the ΔCt method.

Retroviral Infection

Non-tissue culture treated plates (Corning) were incubated with Retronectin (Takara) at 40–50 ng/mL overnight at 4°C. Retronectin was removed and viral supernatant added and spun at 2000 g for 2 hours at 32°C. Viral supernatant was removed and cells were added in their preferred media at a concentration of $1x10^6$ cells/mL. Cells were then incubated on the plates at 37C in 5% CO_2 for 4 hours or overnight. After incubation the cells were harvested from the wells and cultured according to their cell type.

Western Blots

Cell extracts were mixed with 4x Laemmli sample buffer, boiled and run on an SDS-PAGE gel. Proteins were transferred to a PVDF Immobilin membrane (Millipore) using the wet transfer method (BioRad). The blots were blocked with 5% milk in TBS-T (Tris-buffered saline, 0.5% Tween-20) for 1 hour at 4°C and then incubated overnight with primary antibody at a 1:1000 dilution. Blots were washed with TBS-T and incubated with secondary antibody at 1:2000 for 1 hour at 4°C and then washed again with TBS-T. The blot was incubated with substrate (SuperSignal Pierce) for one minute. The blots were then

exposed to film for various lengths of time. Sp1 antibody was from Santa Cruz Biotechnology (sc-59), and c-Jun antibody from Santa Cruz (sc-1694).

Figures:

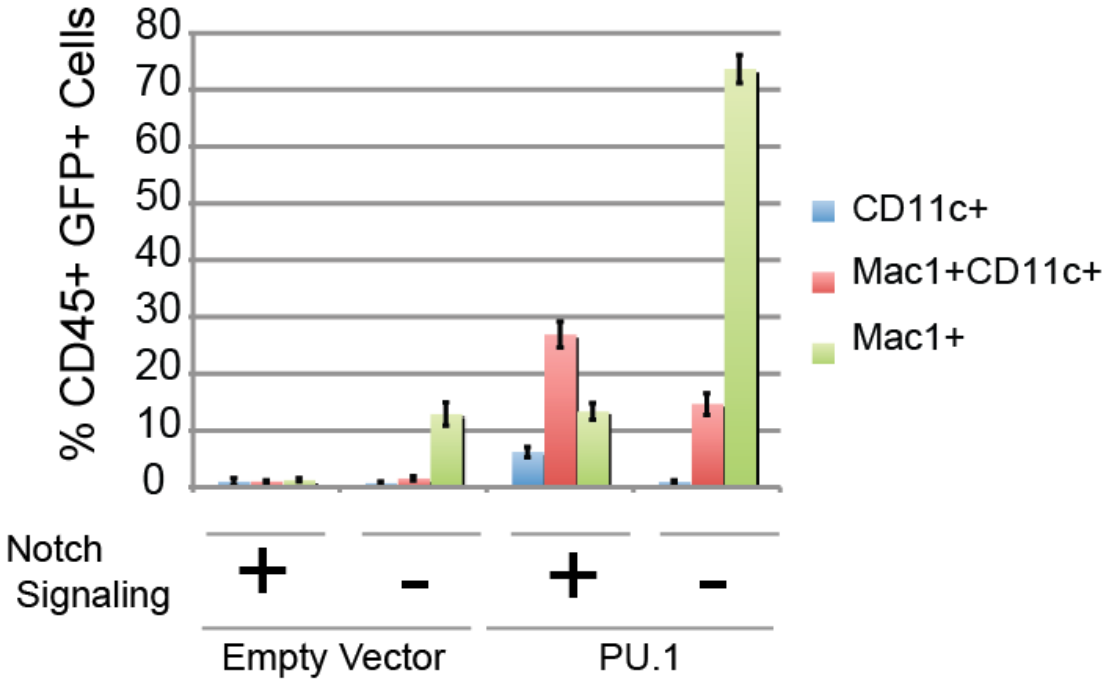

Figure 1. Notch signaling protects against Mac1 up-regulation in fetal thymocytes. E15.5 Bcl2tg fetal thymocytes were infected with PU.1-GFP or empty vector for 4 hours and then transferred to OP9-DL1 or OP9-Control for 2 days. The CD45+GFP+ cells were analyzed for their expression of Mac1 and CD11c using flow cytometry.

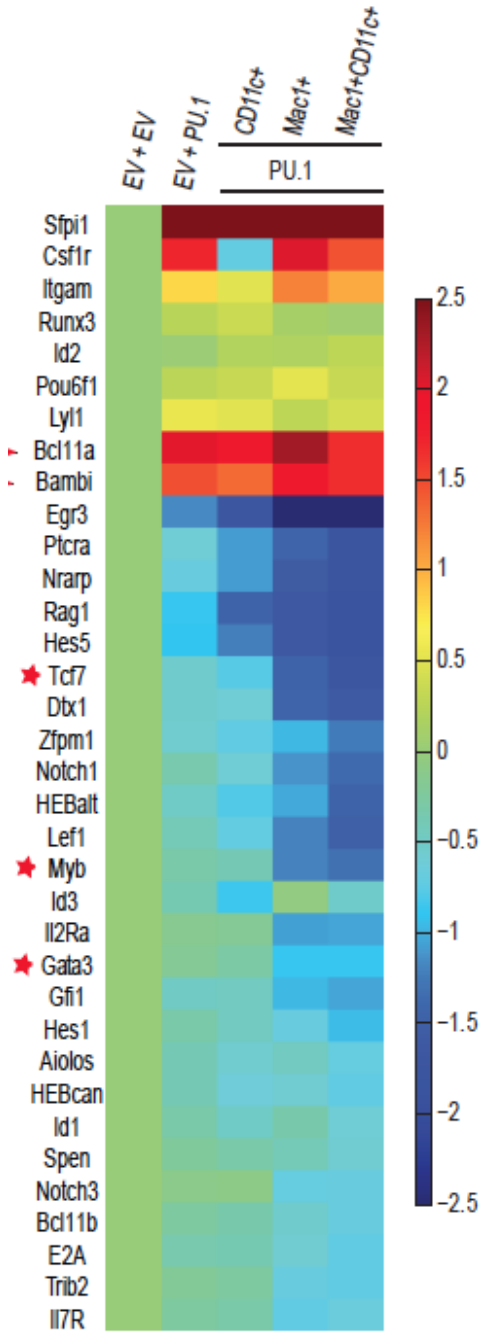

Figure 2. Mac1+ and Mac1+CD11c+ Scid.adh.2c2 cell gene expression patterns are similar, while CD11c+ cells may be an intermediate cell state before 'full' diversion. Heatmap of gene expression in Scid.adh.2c2 cells sorted according to their expression of Mac1 and CD11c 2 days after transduction with PU.1.

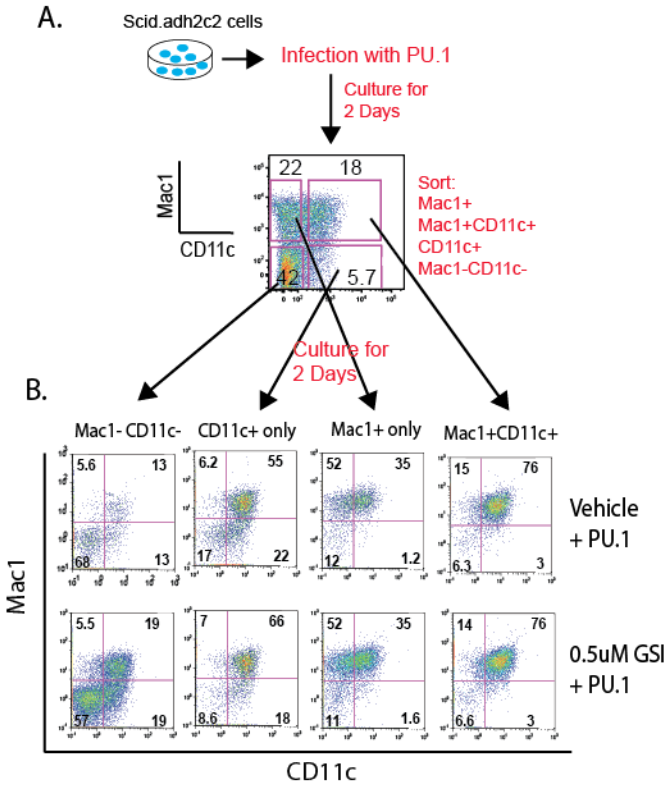

Figure 3. Scid.adh.2c2 cells can express either Mac1 or CD11c first on their way to the terminal phenotype: Mac1+CD11c+. GSI enhances the up-regulation of Mac1 in sorted CD11c+ cells.

(A) Scid.adh.2c2 cells were transduced with PU.1 and cultured for 2 days. The transduced cells were sorted according to their expression of Mac1 and CD11c and (B) then cultured for an additional 2 days in the presence or absence of 0.5 μM GSI. The cells were then reanalyzed for their expression of Mac1 and CD11c using flow cytometry.

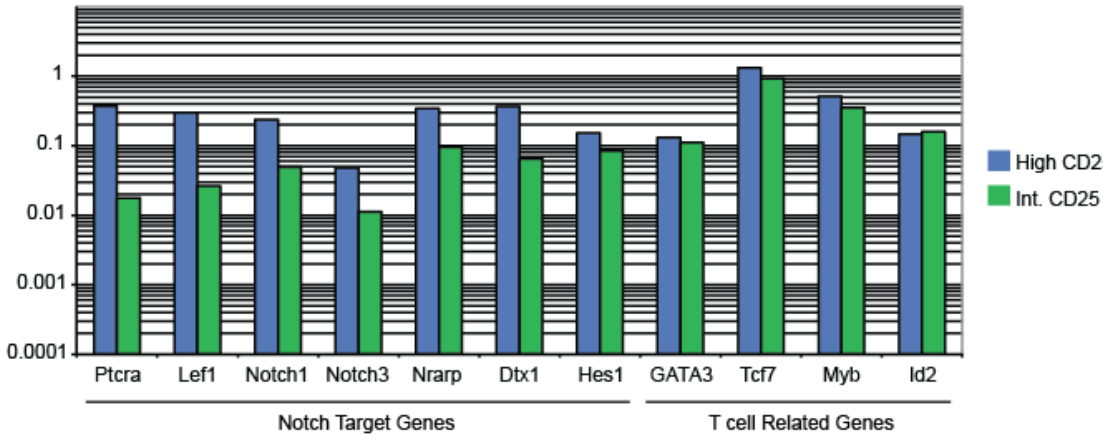

Figure 4. CD25 levels can be used to approximate the strength of Notch signaling in Scid.adh.2c2 cells.

Scid.adh.2c2 cells were sorted to isolate cells expressing intermediate or high levels of CD25. cDNA was obtained from the samples and Q-RTPCR analysis was performed (data from one sorted sample is plotted above). CD25 high cells had higher expression levels of Notch targets compared to CD25 intermediate cells. The CD25 high and CD25 intermediate cells did not differentially express non-Notch target genes important during early T-cell development.

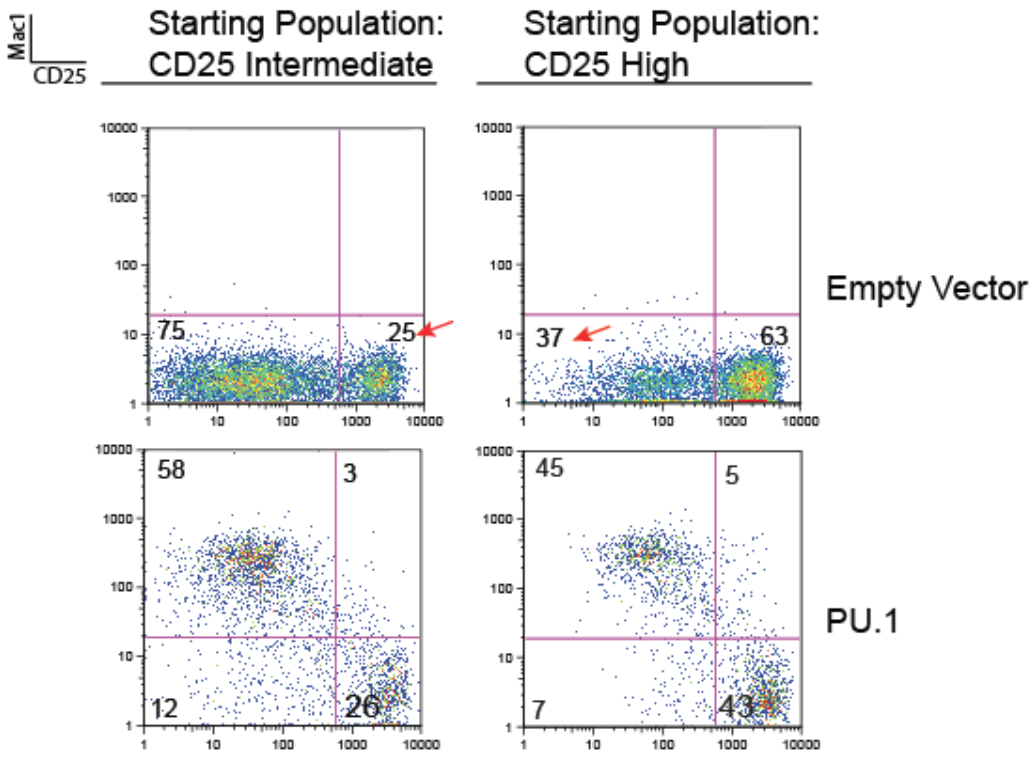

Figure 5. CD25 high and CD25 intermediate Scid.adh.2c2 cells can both up-regulate Mac1 in response to PU.1 expression.

CD25 high and CD25 intermediate Scid.adh.2c2 cells were infected with PU.1 or empty vector and cultured for 2 days. Cell surface CD25 and Mac1 was done using flow cytometry. Neither empty vector samples up-regulated Mac1. The CD25 high empty vector samples generated a population of CD25 intermediate cells, while the CD25 intermediate cells generated a CD25 high population. About 60% of the CD25 intermediate PU.1 transduced Scid.adh2c2 cells were Mac1 positive while about 50% of the CD25 high cells were Mac1 positive.

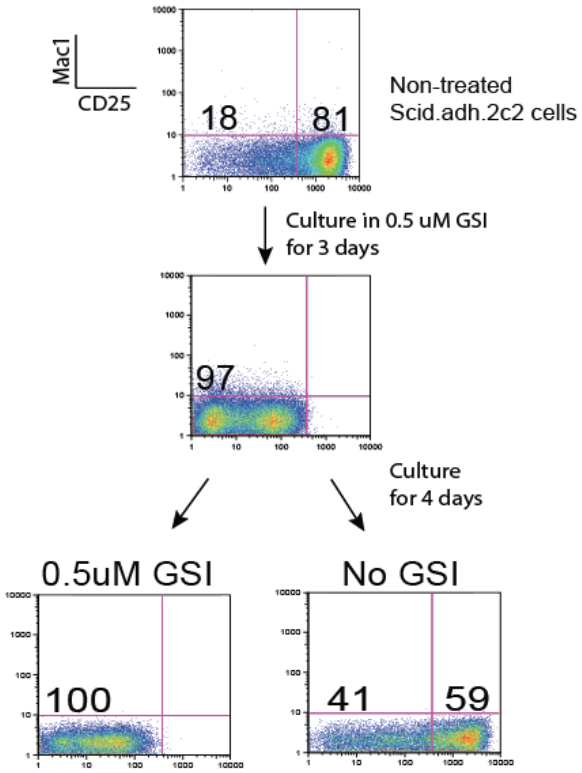

Figure 6. Gamma secretase inhibitor could be used to knockdown Notch signaling in Scid.adh.2c2 cells and this inhibition is reversible.

(A) Scid.adh.2c2 cells were cultured with 0.5 μM GSI for 3 days and then analyzed for their expression of CD25 and Mac1 using flow cytometry. (B) After 3 days of culture with GSI, Scid.adh.2c2 cells were washed and cultured with or without GSI for an additional 4 days. CD25 and Mac1 levels were again measured using flow cytometry. Cells cultured without GSI were able to up-regulate CD25 levels.

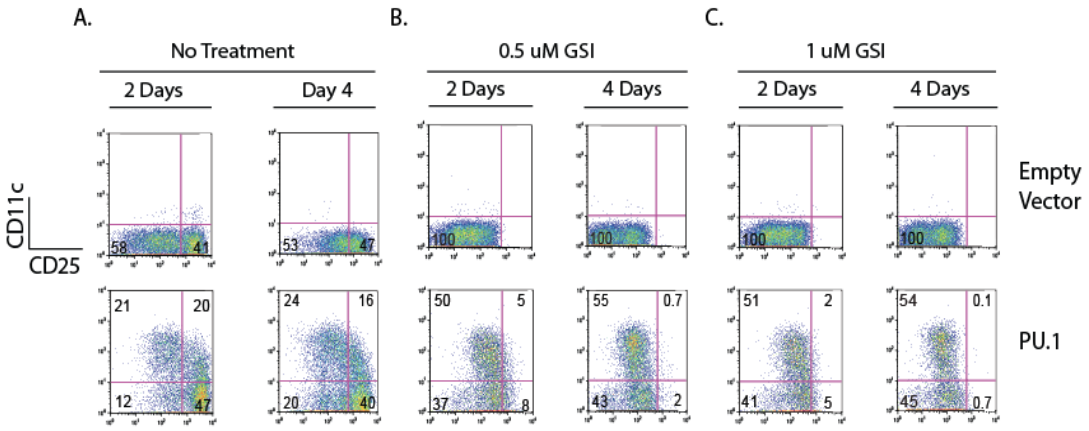

Figure 7. Gamma secretase inhibitor can increase the percentage of PU.1-expressing Scid.adh.2c2 cells that up-regulate CD11c.

(A) Scid.adh.2c2 cells were transduced with PU.1 or empty vector and cultured for 2 or 4 days. About 40% of the PU.1 positive cells up-regulated Mac1 after 2 and 4 days. (B) Scid.adh2c2 cells were transduced with PU.1 or empty vector and cultured in 0.5 μM GSI for 2 days or 4 days. The 2-day and 4-day samples showed an increase in CD11c up-regulation compared to the control cells in (A) with no GSI. (C) The addition of 1 μM GSI to the culture media did not increase the percentage of the cells that diverted compared to the 0.5 μM GSI samples.

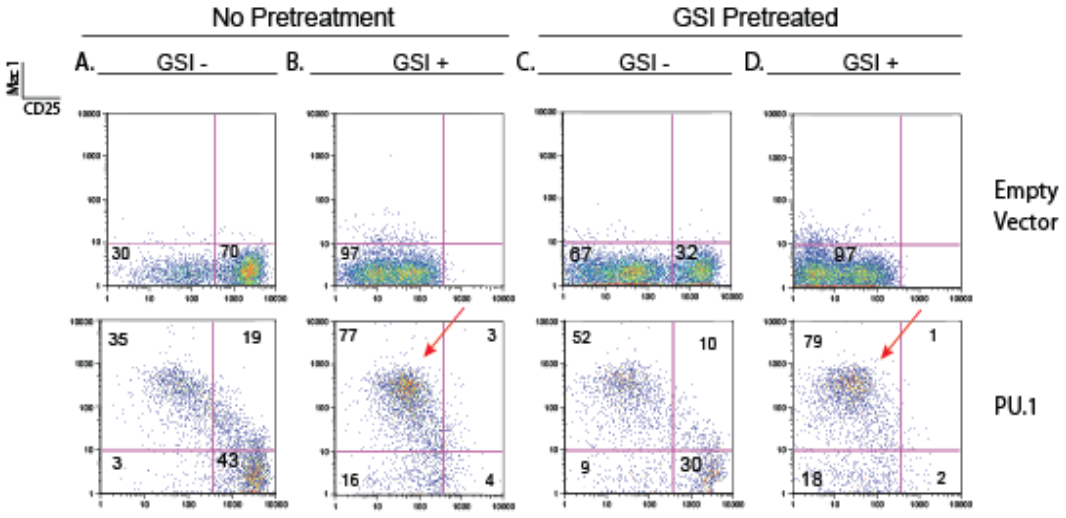

Figure 8. Pretreatment of Scid.adh.2c2 cells with 0.5 μM GSI before PU.1 expression did not cause more up-regulation of Mac1 compared to samples where GSI was added after infection.

(A) Scid.adh.2c2 cells were transduced with PU.1 or empty vector and cultured for 2 days without GSI (B) or with 0.5 μM GSI. (C) Scid.adh.2c2 cells pretreated with GSI and then transduced with PU.1 or empty vector and cultured for 2 days in the absence of GSI or (D) the presence of GSI. All samples were analyzed for the expression of CD25 and Mac1 using flow cytometry.

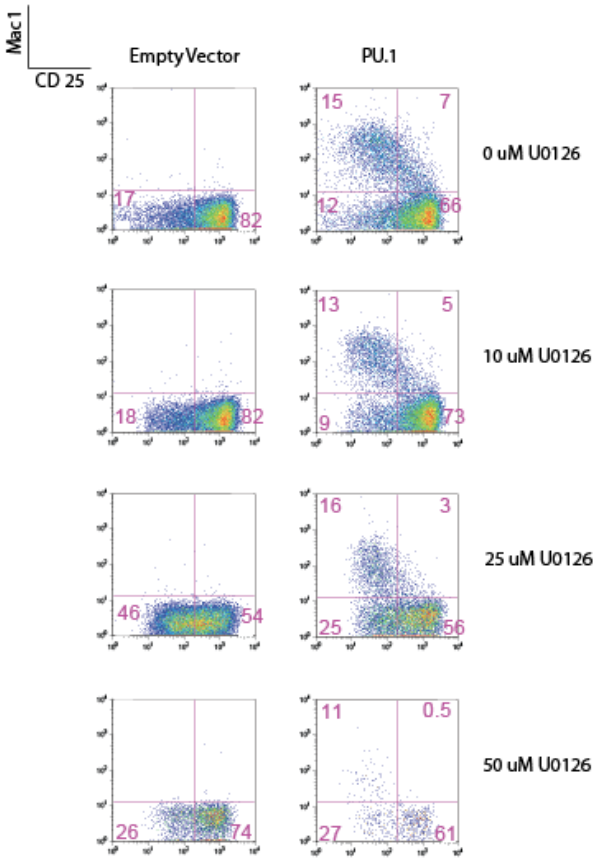

Figure 9. Inhibition of MEK/ERK did not decrease the percentage of Mac1+ cells in PU.1-transduced Scid.adh.2c2 cells at lower concentrations.

(A) Scid.adh.2c2 cells with transduced with PU.1 or empty vector and cultured in the presence of no inhibitor, 10 μM U0126, 25 μM U0126, or 50 μ U0126 for 2 days. After culture the cells were analyzed using flow cytometry for their expression of Mac1 and CD25. 7AAD was used as a viability marker.

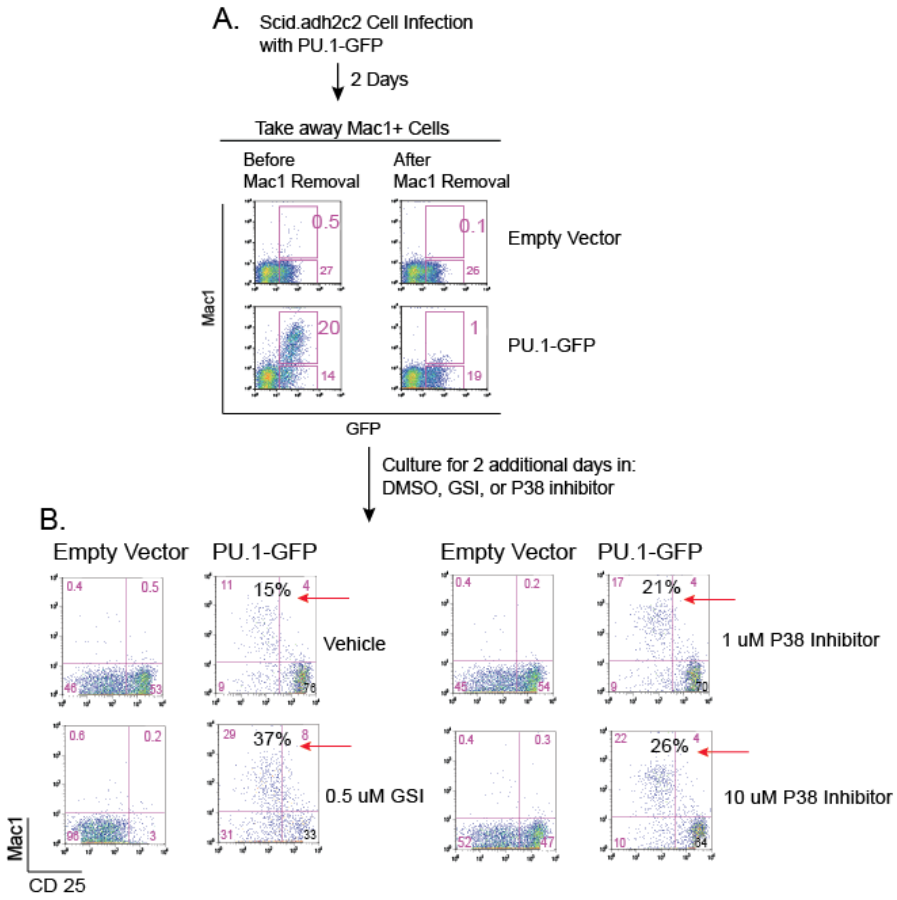

Figure 10. Inhibition of the P38 pathway enhances the up-regulation of Mac1 in PU.1-transduced Scid.adh.2c2 cells, but not as much as GSI.

(A) Scid.adh.2c2 cells were transduced with PU.1 or empty vector and cultured for 2 days. Mac1 and CD11c-biotin conjugated cells were using in conjunction with streptavidin-coated magnetic beads to remove cells expressing Mac1 and/or CD11c. The flow cytometry profile of the cells before and after depletion shows that for the PU.1-transduced samples Mac1+ cells were removed and some PU.1-transduced (GFP+) cells remained. (B) The depleted cells were cultured for an additional 2 days in 0.5 μM GSI, 1 μM P38 inhibitor, 10 μM P38 inhibitor, or in vehicle control. We then checked the expression of levels of CD25 and Mac1 using flow cytometry.

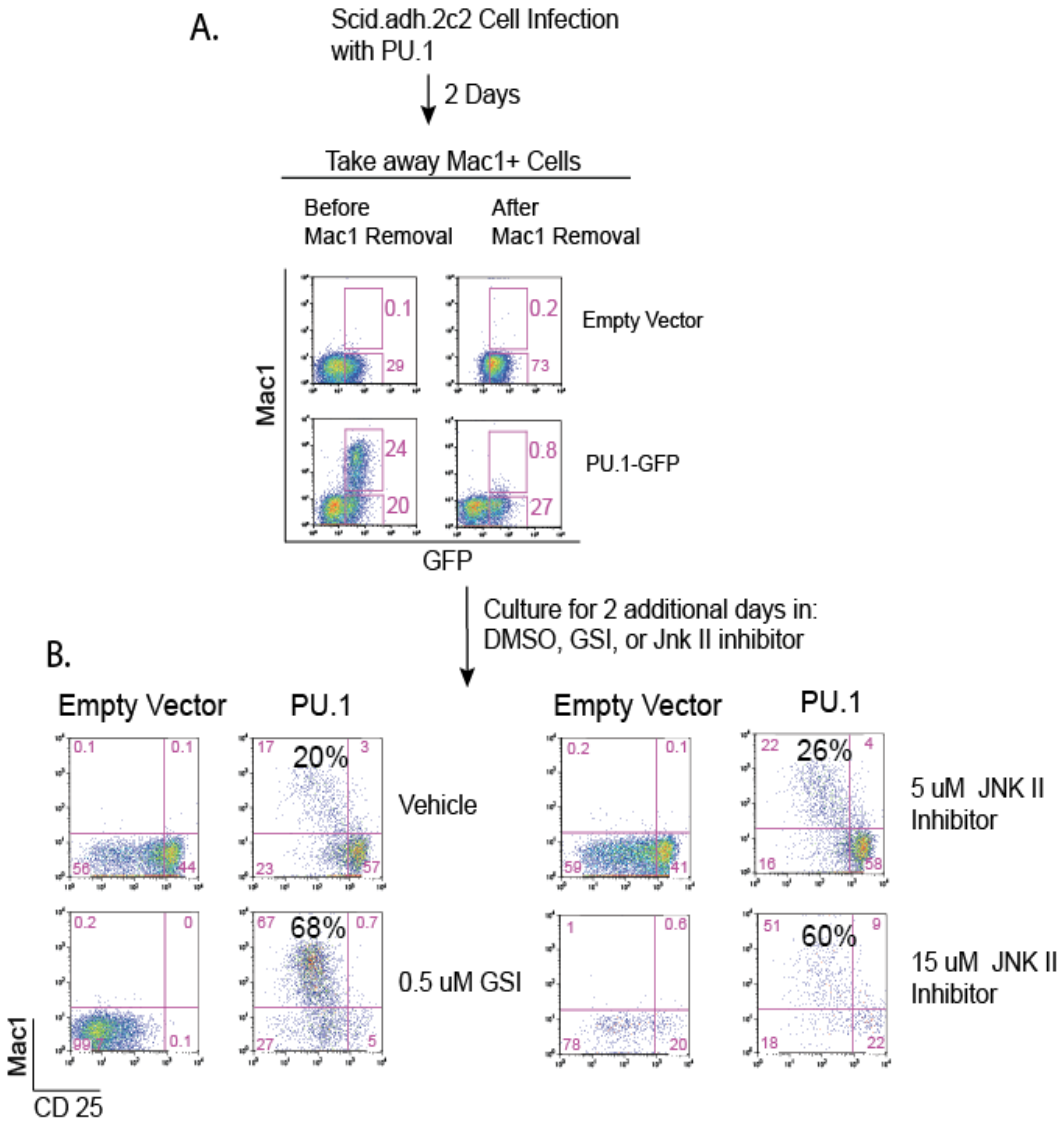

Figure 11. Inhibition of JNK enhances the up-regulation of Mac1 in PU.1-transduced Scid.adh.2c2 cells almost as well as GSI.

(A) Scid.adh.2c2 cells were prepared as in Fig.7A (B) The depleted cells were cultured for an additional 2 days in 0.5 μM GSI, 5 μM MEK inhibitor, 15 μM MEK inhibitor, or in vehicle control. We then analyzed for the expression of levels of CD25 and Mac1 using flow cytometry.

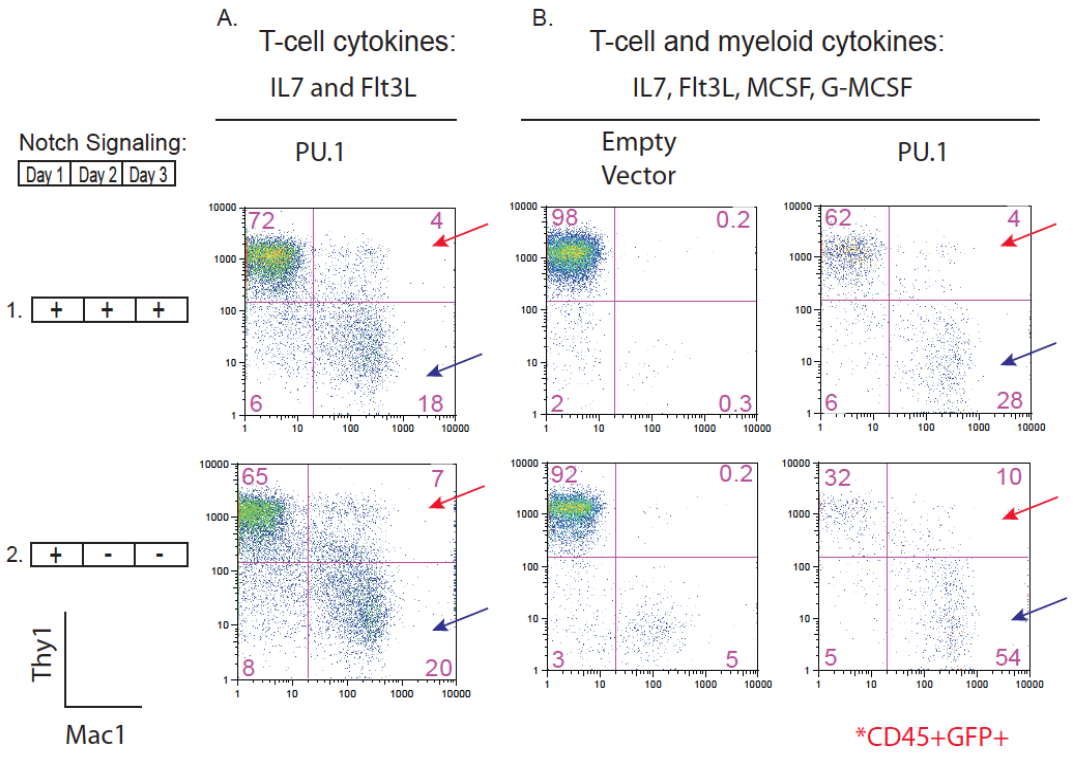

Figure 12. Myeloid cytokines do not increase the percentage of Mac1+Thy1+ PU.1-transduced fetal thymocytes.

(A) E15.5 Bcl2tg fetal thymocytes were transduced with PU.1 or empty vector and then cultured in the presence of Notch signaling for 3 days or in the presence of Notch signaling for 1 day and then switch to an environment with no Notch signaling for 2 days. The 3-day culture was supplemented with T-lineage supportive cytokines, IL-7 and Flt3L. (B) E15.5 Bcl2tg fetal thymocytes were cultured in the presence of both T-supportive and myeloid supportive cytokines (MCSF and G-MCSF) in the two different Notch signaling conditions. Samples from (A) and (B) were both analyzed for their expression of the myeloid marker (Mac1) and the T-cell marker (Thy1) using flow cytometry.

Sample	% Mac1+
PU.1 + Vehicle	56
+ Vehicle + MCSF	60
+ Vehicle + G-MCSF	61
+ Vehicle + G-MCSF + MCSF	61
+ GSI	69
+ GSI + MCSF	72
+ GSI + G-MCSF	72
+ GSI + G-MCSF + MCSF	70
Empty Vector + Vehicle	0.2
+ Vehicle + MCSF	0.2
+ Vehicle + G-MCSF	0.2
+ Vehicle + G-MCSF + MCSF	0.2
+ GSI	0.2
+ GSI + MCSF	0.2
+ GSI + G-MCSF	0.1
+ GSI + G-MCSF + MCSF	0.1

Table 1: Myeloid cytokines do no enhance diversion in PU.1-transduced Scid.adh.2c2 cells.

Scid.adh.2c2 cells were transduced with PU.1 or empty vector and cultured with different combinations of the myeloid cytokines MCSF and G-MCSF in the presence or absence of GSI for 2 days. The cells were then analyzed for their expression of Mac1 using flow cytometry.

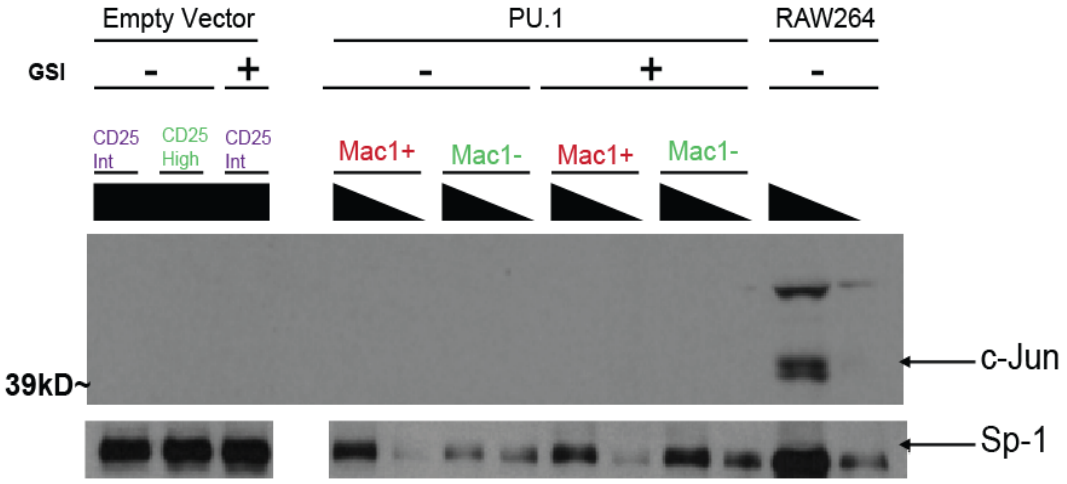

Figure 13: Scid.ahd.2c2 cells do not express the PU.1 co-activator c-Jun.

Scid.adh.2c2 cells were infected with PU.1 or empty vector for 2 days in the presence or absence of GSI. The cells were then sorted according to their expression levels of Mac1. The cells were lysed and ran on an SDS-page gel at 1x and 0.2x concentrations and transferred onto a nitrocellulose membrane. A Western blot analysis was performed with c-Jun specific antibodies to detect c-Jun protein expression. A macrophage cell line, RAW246.7 was used as a positive control for the detection of c-Jun protein and the ubiquitous transcription factor SP1 was used as a loading control.

Chapter 3:

Architecture of a lymphomyeloid developmental switch controlled by PU.1, Notch, and GATA-3

Marissa Morales Del Real and Ellen V. Rothenberg

Abstract

Hematopoiesis is a classic system to study developmental potentials and to investigate gene regulatory networks that control choices among alternate lineages. T-cell progenitors seeding the thymus retain several lineage potentials. The transcription factor PU.1 is involved in the decision to become a T-cell or a myeloid cell and the developmental outcome of expressing PU.1 is dependent on exposure to Notch signaling. PU.1-expressing T-cell progenitors without Notch signaling often adopt a myeloid program while those exposed to Notch signals remain in a T-lineage pathway. Here we show that Notch signaling does not alter PU.1 transcriptional activity by degradation or alteration of PU.1 protein. Instead, Notch signaling protects against the down-regulation of T-cell factors so that a T-cell transcriptional network is maintained. Using an early T-cell line we describe two branches of this network. The first involves inhibition of E-proteins by PU.1 and a resulting inhibition of Notch signaling target genes. Effects of E-protein inhibition can be reversed by exposure to Notch signaling. The second network involves PU.1's ability to inhibit important T-cell transcription factor genes like *Myb*, *Tcf7*, and *Gata3* in the absence of Notch signaling. We show that maintenance of GATA3 protein levels by Myb and Notch signaling is linked to the ability to retain T-cell identity in response to PU.1.

Introduction

T-cell development depends on proper expression of an intricate transcription factor network and signaling from the environment. T cells develop from multipotent progenitors that migrate from the bone marrow to the thymus, where they become dependent upon Notch signaling for their development and survival (Yang et al., 2010). At the early DN stages, pro-T cells retain lineage plasticity until the DN2b stage where they become committed pre-T cells. The ETS family transcription factor PU.1 is important during early T-cell development (Back et al., 2005; Nutt et al., 2005), and highly expressed initially, but repressed during commitment (Fig. 1A). This pattern must be maintained for development to succeed. In early T-cell stages PU.1 drives expression of cytokine receptors such as IL7R and Flt3 and genes important for cell communication (Turkistany and DeKoter, 2011). However, it is also required for the development and function of other cell types including hematopoietic stem cells (Iwasaki et al., 2005), multipotent progenitors (Wontakal et al., 2011), myeloid cells (Ghani et al., 2011), and B cells (Houston et al., 2007). Forced over-expression of PU.1 can divert these early T-cells to a myeloid lineage (Anderson et al., 2002b; Dionne et al., 2005; Lefebvre et al., 2005; Laiosa et al., 2006c). However, in the context of T-cell development the progenitors are normally protected from diversion, even while expressing high levels of PU.1, by their exposure to Notch signaling from the environment (Franco et al., 2006; Laiosa et al., 2006c)(Fig. 1A).

Tracking effects on several dozen genes, the interaction between PU.1 and Notch can have dichotomous effects on early T-progenitors, with cells partitioning between those maintaining a T-cell gene expression pattern and those shifting toward a myeloid pattern (Dionne et al., 2005; Franco et al., 2006). This suggests competition between two self-

reinforcing network states. However, the actual gene network underlying this choice has been obscure.

Here we explore the mechanisms that mediate the regulatory competition between PU.1 and Notch signals, using primary mouse fetal thymocytes and a clonal pro-T cell line system to dissect the regulatory impacts of PU.1 and Notch signaling. We show that Notch signaling does not inactivate PU.1 protein but re-channels its transcriptional effects. However, PU.1 and Notch signaling are involved in a mutually inhibitory network as PU.1 can repress Notch targets. Our results further reveal two branches of the T-cell gene network that collaborate against PU.1-mediated diversion: one involving basic helix-loop-helix E proteins in a tight positive feedback linkage with Notch, and a separate branch for GATA-3 and the *Gata3*-activating factor Myb. We show that PU.1 undermines GATA-3 expression, foreshadowing diversion in individual cells. The two T-lineage protective pathways converge as Myb and Notch signaling each enable GATA-3 expression to be maintained in the face of high-level PU.1.

Materials and Methods

Mice

C57BL/6-Bcl2tg mice (B6.Cg-Tg(BCL2)25Wehi/J) were housed under specific pathogen-free conditions, bred in-house, and cared for by Caltech Animal Facility staff. Embryonic Day (E) 14.5 or 15.5 fetal thymocytes were used. All animal work followed protocols approved by the Institutional Animal Care and Use Committee.

Cell Culture

Scid.adh.2C2 and Scid.adh.6D4 cells were cultured in RPMI1640 with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), sodium pyruvate, non-essential amino acids, penicillin/streptomycin/glutamine (Gibco/Life technologies/Invitrogen, Grand Island, NY), and 50 μM β-mercaptoethanol. Cells were incubated at 5% CO2 and 37°C.

For Notch signaling inhibition γ-secretase inhibitors, InSolution™ γ-Secretase Inhibitor X (EMD Millipore, Billerica, MA) were added at 0.5 μM.

Fetal thymocytes were cultured on OP9-Delta-like1 (OP9-DL1) or OP9-control stroma in α-MEM supplemented with 20% fetal bovine serum, penicillin/streptomycine/glutamine, 50 μM β-mercaptoethanol, 5 ng/ml IL-7, 5 ng/ml Flt3 ligand (cytokines from Peprotech, Rocky Hill, NJ).

Cell Staining, Flow Cytometry, and Sorting

FITC, PE, APC, APCe780, Pacific Blue, and PerCPCy5.5-conjugated antibodies from eBioscience (San Diego, CA) or Cell Signaling (Danvers, MA) (against CD25, CD44, CD45, Mac1/CD11b, CD11c, Thy1, NGFR, and human CD8) were used for cell surface staining. Fc receptors were blocked before staining with 2.4G2 anti-FcR. PU.1 intracellular staining using the BD cytofix/cytoperm kit (Becton-Dickinson Immunocytometry Systems, San Jose, CA) was done using PU.1 (9G7) Rabbit mAb (Alexa Fluor® 647 Conjugate) antibody (Cell Signaling Technology, Danvers, MA) and rabbit mAb IgG (#2985 Cell Signaling) as an isotype control. GATA3 intracellular staining using Foxp3 Staining Buffer Kit (eBioscience 00-5523-00) was done with Alexa Fluor647 mouse anti-GATA3 (BD Biosciences Pharmingen, La Jolla, CA, cat:560068) and Alexa Fluor647 mouse IgG1 κ as an isotype control (BD Biosciences).

Cells were sorted using a BDIS FACS Aria IIu or iCyt Mission Technology Reflections cell sorter and analyzed using a FACSCalibur (BDIS) or MACSQuant (Miltenyi Biotec, Auburn, CA) and FlowJo software (Tree Star, Ashland, OR).

RNA extraction and quantitative realtime RT-PCR

cDNA was prepared from total RNA using RNeasy extraction kits (Qiagen) and reverse transcribed using random primers and SuperscriptIII (Invitrogen).

Specific gene expression in cDNA samples was measured by qRT-PCR (ABI Prism 7900HT Sequence Detector; ABI, Mountain View, CA) using SyberGreenER mix (Invitrogen). Results were calculated (ΔCt method) and normalized to *Actinb* levels. For actual values see Tables S1A,B (Fig. 2) and Tables S2A-E (Figs. 3–7). Primers used for qRT-PCR were described previously (David-Fung et al., 2009; Li et al., 2010a; Yui et al., 2010), or are listed in Table S3. All cross an intron except one set detecting *Bcl11b* exon 4.

Heatmap Generation

Heatmaps were generated using a Matlab (MathWorks, Natick, MA) script written by Dr. HaoYuan Kueh (available upon request). Briefly, values are $\log_{10}$-transformed averages of expression levels determined by qRT-PCR from 2–4 independent experiments: n=2–3 (Fig. 2C,D), n=4 (Figs. 3C, 4B), n=2 (Figs. 5B, 6D, 7E). Levels for each gene in different samples are presented relative to the level in the control sample (empty vector transduced=1.0). The color scale ranges from ~ 10^{-2} to 10^{2} times this reference value, as indicated. Ordering of genes was by hierarchical clustering (median method, Matlab).

Cloning/Sub-cloning

Gfi1, *Id2*, *Bambi*, and *Cebpa* coding sequences were purchased from Genscript and subcloned into retroviral vectors: LZRS or MIGR1 with a GFP marker, or derivatives with an NGFR marker. Standard cloning employed New England Biolabs restriction enzymes and buffers. For retroviral packaging, Phoenix-Eco cells were transfected with long-term puromycin selection for LZRS-based vectors, whereas 293T cells were transiently cotransfected with pCL-Eco plasmid for MIGR1-based vectors. *Tcf7* in a retroviral vector with a Vex reporter and ICN1 and dnMAML in MIGR1 were kind gifts of Avinash Bhandoola and Warren Pear, respectively (University of Pennsylvania). *Gata3*shRNA in the Banshee retroviral vector was donated by Gabriela Hernandez-Hoyos.

Retroviral Infection

Non-tissue culture treated plates (Corning) were incubated with Retronectin (Takara) at 40–50 ng/mL overnight at 4°C. Retronectin was removed and viral supernatant added and spun at 2000 g for 2 hours at 32°C. Unbound virus was removed and cells added in their preferred medium at $1x10^6$ cells/mL, then incubated at 37C in 5% or 7% CO_2 for 4 hours or overnight.

Western Blots

Cell extracts in Laemmli sample buffer were boiled for SDS-PAGE. Proteins were transferred to PVDF Immobilin (Millipore) by wet transfer (BioRad). Blots were blocked with 5% milk in TBS-T (Tris-buffered saline, 0.5% Tween-20) for 1 hour at 4°C, then incubated overnight with Sp1 (sc-59) or PU.1 (sc-352) antibody (Santa Cruz Biotechnology, 1:1000 dilution), washed with TBS-T, incubated with secondary antibody

(1:2000) for 1 hour at 4°C. They were washed again with TBS-T before incubation with substrate (SuperSignal, Pierce; 1 minute) for film detection.

Results

Notch signaling protects against diversion at early and late time points after PU.1 over-expression

In the early T-cell stages when PU.1 is active, it provides cells with access to developmental alternatives and is therefore a risk to T-lineage fidelity. We showed previously that thymocytes can be protected from PU.1-mediated lineage diversion if they receive Notch signals (Franco et al., 2006), as they would in the normal thymus in vivo. However, the mechanism through which Notch signaling counteracts the activity of PU.1 has been obscure.

To investigate the critical time interval in which Notch signaling affects thymocyte responses to PU.1, we forced fetal thymocytes to express PU.1 by retroviral transduction and exposed them to differing Notch signaling conditions for 3 days, using switch cultures based on co-culture with OP9-DL1 or OP9-control stromal cells. Bcl2-transgenic (Bcl2tg) thymocytes were used to enhance recovery of cells after regulatory perturbation (Franco et al., 2006; Taghon et al., 2007). OP9-control stroma supports B cell, natural killer cell, and myeloid development, but when transfected to express the Notch ligand Delta-like1 (DL1), OP9-DL1 cells support T-cell development (Schmitt and Zuniga-Pflucker, 2002). Thus, thymocytes were infected with empty vector or PU.1-expressing retrovirus in a 4-hour incubation, cultured with OP9-DL1 or OP9-control stroma for a day, and then either returned to the same Notch signaling condition or switched to the opposite condition for 2

more days (Fig.1B). Thy1, itself Notch-insensitive, was used to identify cells that had entered the T-pathway (Taghon et al., 2007), as Mac1 (CD11b; encoded by *Itgam*) marked entrance to the myeloid pathway (Dionne et al., 2005). As these markers are normally mutually exclusive, activation of Mac1 on Thy1+ cells identifies T-lineage cells beginning myeloid diversion. Later, these become Mac1+ Thy1-.

On OP9-control stroma in the absence of Notch signals, the empty vector-transduced thymocyte population was revealed to include some cells with natural myeloid potential, but few were $Thy1^{+}$, i.e., derived from T-lineage precursors (Fig. 1B2: 13% total Mac1+ vs. 2% Thy1+ Mac1+). Fetal thymocytes transduced with PU.1 generated far more Mac1+ cells than thymocytes transduced with empty vector under all conditions (Fig. 1B): if cultured in the absence of Notch signals, most became Mac1+, > 50% derived from Thy1+ cells. As expected, the samples cultured in the presence of Notch signaling on OP9-DLl cells throughout the 3-day culture contained a far smaller percentage of Thy1+Mac1+ diverted cells as well as fewer Mac1+ cells overall than those cultured on OP9-control. Notch signals restored for the last two days after an initial day of deprivation also reduced diversion, as these samples mimicked the conditions we had used previously (Franco et al., 2006). Notably, however, samples that were initially cultured on OP9-DL1 for only a day and then shifted to OP9-control were also protected, almost as strongly as in continuous presence of DL1 (Fig. 1B3). Thus, Notch signaling through the onset of PU.1 over-expression could establish a regulatory state making Thy1+ fetal thymocytes relatively resistant to diversion.

Pro-T cells with high levels of PU.1 protein are able to resist Mac1 up-regulation in the presence of Notch Signaling

A possible mechanism for protection of pro-T cells from PU.1 mediated diversion could be to inactivate PU.1 protein. PU.1 phosphorylation can affect its DNA binding (Seshire et al., 2011) and transactivation domain engagement (Hamdorf et al., 2011a). Notch signaling can regulate protein phosphorylation (Vo et al., 2011) and trigger protein degradation by promoting ubiquitination (Lim et al., 2011). To test directly whether Notch-Delta signaling resulted in changes in PU.1 protein levels, fetal thymocytes were infected with PU.1 or empty vector and cultured on either OP9-DL1 or OP9-control for 2 days, and then stained for both intracellular PU.1 and cell-surface Mac1. The intermediate and high levels of PU.1 protein in transduced cells matched the levels of endogenous PU.1 in those control thymocytes that revealed natural myeloid potential when Notch signals were removed (Fig. 1C1, empty vector, OP9-control).

The distributions of PU.1 protein in PU.1-transduced cells were not globally altered by the presence or absence of Notch signals. However, the response to a given level of intracellular PU.1 depended strongly on Notch signaling, as cells made all-or-none choices between remaining Mac1- and diverting to high Mac1+ states (Fig. 1C1, 3). High, intermediate, and low levels of intracellular PU.1 protein all drove > 90% of cells to become Mac1+ in the absence of Notch ligand (Fig. 1C2). In the presence of Notch ligand, Mac1 could still be induced at the highest levels of PU.1 protein, and Notch signaling did not affect the levels of Mac1 expressed (Fig. 1C3). However, the high, intermediate, and low PU.1 level cells each generated substantially lower Mac1+ percentages in the presence of Notch signaling. Importantly, cells that now resisted Mac1 up-regulation (Fig. 1C2, 3) expressed the same levels of PU.1 protein that promoted Mac1 expression when Notch signals were absent. Thus, Notch signaling can sharply raise the dose-dependent threshold

for PU.1 to induce expression of Mac1, without affecting accumulation of PU.1 protein itself.

PU.1 protein is intact in the presence of Notch signaling

Similarly, when PU.1 was introduced into a pro-T-cell-like cell line, Scid.adh.2C2, Western blotting measurements showed that Notch signaling affected the PU.1 dose threshold for the cells to divert to a Mac1+ state (Fig. S1). However, qualitative PU.1 electrophoretic mobility patterns were the same in diverted and diversion-resistant cells, whether Notch signaling was active or inhibited, offering no evidence for differential phosphorylation or ubiquitination (Fig. S1). This suggests that PU.1 itself remains biochemically competent in the presence of Notch signaling.

Notch signaling effects on initial changes in gene expression in fetal thymocyte responses to high-level PU.1

Because even transient exposure to Notch signaling could protect PU.1-overexpressing thymocytes from diversion, Notch signaling might alter the earliest responses to PU.1. Previous studies had shown that Notch signals protect important T-cell genes from repression 40–48 hours after PU.1 transduction (Franco et al., 2006). Those analyses were potentially skewed toward diversion, however, because the cells were initially deprived of Notch signals during > 16 hr transduction. Also, survival effects could obscure gene-specific regulation, for thymocytes naturally increase Notch-dependence as they progress from DN2 to DN3, when many T-cell genes are induced (Yui et al., 2010). Therefore, we infected fetal thymocytes with PU.1 or empty vector for only 4 hours, then cultured them with or without Notch signaling for 16 hrs before sorting for RNA analysis, separately isolating transduced DN2 and DN3 cells (Fig. 2,Table S1A,B).

The genes analyzed showed 4 different patterns of response to PU.1 and Notch-DL1 interaction, as illustrated by representative bar graphs of expression for individual genes (Fig. 2A,B) and summary heatmaps of PU.1 effects on DN2 and DN3 cell gene expression (Fig. 2C & D). The normal developmental expression patterns of key genes are also shown (Fig. 2E). One group of genes was up-regulated efficiently by PU.1 over-expression, whether Notch signaling was present or absent (e.g., Fig. 2A, C, D). This group included stem and progenitor cell-associated genes *Lyl1*, *Bcl11a*, and *Hhex*, and the myeloid gene *Fes1*. The effectiveness of PU.1 was partly constrained by these genes' natural limits of expression from DN2 to DN3 (Zhang et al., 2012)(Fig. 2E): e.g., effects on *Hhex* and *Lmo2* were seen in DN2 cells but not significantly in DN3 cells (Table S1C). Only select genes, e.g., *Lmo2* (Fig. 2A) and *Mac1 (Itgam)*, were inhibited from responding to PU.1 by Notch-DL1 interaction. Thus, PU.1 can indeed act positively on many target genes even in the presence of Notch signaling.

T-lineage-specifically expressed genes showed three patterns of response (Fig. 2B–D). Some were down-regulated by PU.1 whether or not Notch signaling was present. These included *Ets1* and the critical T-cell regulatory gene *Tcf7*, a gene that is initially induced by Notch (Germar et al., 2011; Weber et al., 2011) but is not acutely dependent on Notch signaling for its maintenance. Another pattern was defined by Notch target genes (e.g., *Deltex1*, *Hes1*, *HEBalt*, and *Nrarp*), which depended on Notch signals even in control cells: e.g., *Deltex1*. A third group consisted of genes that were down-regulated by PU.1, but much more severely if Notch signaling was absent. These included genes important for T-cell development including *Myb*, *Fog1* (*Zfpm1*), and *Gfi1*. However, in general the Notch

target genes were also PU.1-inhibited, and additively affected by Notch deprivation and PU.1 (Fig. 2B–D: *Nrarp*, *HEBalt*, *Hes1*).

Though we anticipated Notch to influence PU.1 effects, these results suggest that PU.1 in early T-cells antagonizes responses to Notch as well. Thus PU.1+ cells may demand higher-intensity Notch signaling to maintain expression of directly and indirectly Notch-regulated genes.

A clonal early T-cell line can be used to study Notch signaling protection against diversion of pro-T-cells

The lasting protective effects of Notch signaling in early pro-T cells and its impact on early responses to PU.1 over-expression imply that these early-affected genes may be involved in deciding between protection and diversion in cells with high PU.1 expression. Testing these genes for epistatic or synergistic effects by co-transfection would be difficult in fetal thymocytes. Therefore, we utilized a previously described early T-cell line (Dionne et al., 2005) devoid of intrinsic myeloid potential, which is much more permissive for co-transduction experiments.

Scid.adh.2C2 cells, DN3-like cells that do not express endogenous PU.1, were previously used to demonstrate the all-or-none diversion response of early T cells after PU.1 over-expression (Dionne et al., 2005). Scid.adh.2C2 cells were cloned from a cell line, Scid.adh, derived from a spontaneous pro-T cell tumor (Carleton et al., 1999b), and show spontaneous, ligand-independent Notch pathway activation. We tested whether the Scid.adh.2C2 response to PU.1 was also subject to Notch-dependent protection. Notch signaling in these cells was inhibited by γ-secretase inhibitor (GSI), as shown by the down-

regulation of the Notch-dependent marker, CD25 (Fig. 3A "empty vector", 0.5 μM GSI). Cells survived well with or without Notch signaling. Scid.adh.2C2 cells transduced with PU.1 up-regulated Mac1 in a fraction of the population, and the percentage of cells becoming Mac1+ increased with the addition of GSI (Fig. 3A). Interestingly, another Scid.adh subclone that was unable to divert in response to PU.1 alone (6D4)(Dionne et al., 2005) also showed strong diversion when Notch signaling was inhibited (Fig. S2). Thus, Notch signaling limits the response to PU.1 in these Scid.adh-derived clonal cell lines just as in primary thymocytes.

Although many PU.1 over-expressing Scid.adh.2C2 cells up-regulated Mac1, a population of Mac1-CD25+ cells still remained. CD25 is encoded by a Notch target gene, *Il2ra* (Maillard et al., 2006a), and expression levels of other Notch target genes correlate with CD25 levels (M.M.D.R., data not shown). Individual Scid.adh.2C2 cells that remain Mac1-negative might simply express insufficient PU.1 to divert, or they might resist because of higher Notch signaling, suggested by their high CD25 expression. To distinguish these possibilities, we transduced Scid.adh.2C2 cells with PU.1 for 2 days, sorted the apparently diversion-resistant PU.1+Mac1-CD25+ cells, then cultured them for two more days with or without GSI and assessed whether they remained Mac1-negative (Fig. 3B). Some cells in the vehicle control samples did up-regulate Mac1 after 2 days, but the cells cultured in GSI generated a much higher percentage of Mac1+ cells (Fig. 3B). Thus Scid.adh.2C2 cells expressing levels of PU.1 that are barely adequate for diversion can be efficiently diverted when endogenous Notch signaling is blocked.

Diversion depends on PU.1-mediated inhibition of Notch signaling in Scid.adh.2C2 cells

Although inhibition of Notch signaling facilitated diversion, the final molecular phenotype of the diverted cells was the same with or without Notch inhibition, and the features of this response largely matched those of fetal thymocytes. Fig. 3C–D (values in Table S2A) summarizes gene expression patterns in cells that were transduced with PU.1 or empty vector and cultured for 2 days with GSI or control vehicle, then sorted to separate Mac1+ diverted cells from cells remaining Mac1-. A set of Notch-dependent target genes was detectably inhibited by GSI, both in the absence of PU.1 and in PU.1-transduced cells (Fig. 3D1). Also, PU.1 turned on one set of genes that were neither dependent on Notch signaling nor on Notch inhibition (Fig. 3D2). These were activated in Mac1+ and Mac1- PU.1-expressing cells alike, showing that PU.1 is active in all these cellular contexts. However, the induction of Mac1 by PU.1 heralded a global gene expression shift. Macrophage-associated genes like *Csf1r* and Mac1 (*Itgam*) were up-regulated by PU.1 selectively in the cells becoming Mac1+ (Fig. 3D3). As in fetal thymocytes, PU.1 also inhibited T-cell genes (Fig. 3D4–6). Unlike activation, repression primarily occurred in Mac1+ cells, not in cells remaining Mac1- (Fig. 3D5,6), implying that these genes are only repressed when the regulatory threshold for diversion has been crossed. Notably, cells becoming Mac1+ in response to PU.1 alone maximally down-regulated the Notch target genes, with or without GSI (Fig. 3D6). Thus, forced PU.1 expression can initiate a mechanism leading to severe Notch pathway inhibition in Scid.adh.2C2 cells, and this event is tightly correlated with diversion.

Dissection of PU.1-dependent gene expression effects in the presence and absence of Notch signaling

To dissect the mechanism of Notch pathway interaction with PU.1, we used Scid.adh.2C2 cells for co-transduction experiments to combine PU.1 with constitutively active Notch1 (ICN1) or the dominant negative inhibitor of Notch-dependent transcription, dnMAML (Maillard et al., 2004). Doubly transduced cells were sorted based on their co-expression of both viral vectors after 2 days. When ICN1 was co-expressed with PU.1, most of the cells remained CD25+ and did not up-regulate Mac1. This protection depended on Notch-dependent transcription, since the addition of dnMAML with PU.1 not only extinguished CD25 expression but also caused most of the cells to up-regulate Mac1 (Fig. 4A). However, PU.1 could still induce gene expression changes in Scid.adh.2C2 cells, including expression of the dendritic-cell marker CD11c, even in the presence of ICN1 (Fig. 4A).

The ability to manipulate Notch signaling independently of PU.1, while maintaining viability, enabled us to ask how much of the "PU.1" effect on T-cell gene expression depended on its Notch inhibition effects (Fig. 4B). Doubly-transduced cells (cf. Fig. 4A) were sorted for RNA analysis (PU.1+ ICN1+ cells were < 12% Mac1+;PU.1+ dnMAML+ cells were > 70% Mac1+). As expected, PU.1 with dnMAML mimicked the full range of the diverted phenotype. However, separate regulatory components were distinguished with dnMAML alone, and when PU.1 expression was combined with ICN1 (Fig. 4B, Table S2B). Forced expression of ICN1 could protect classic Notch target genes even in the presence of PU.1 (Table S2B, "response group" 5), and these genes could be up-regulated by ICN1 alone (Table S2B2), implying an additive effect. However, three additional relationships emerged.

First, ICN1 could not protect all T-cell genes from PU.1 (Table S2B6). Thus, PU.1 represses these genes through a mechanism that depends on something besides Notch inhibition. Second, some PU.1-dependent genes were actually enhanced in expression by ICN1, implying distinct gene-specific rules for interaction (Table S2B4). Third, importantly, T-cell regulatory genes including *Myb*, *Tcf7*, and to a lesser extent *Gata3* were only down-regulated by the combination of PU.1 with loss of Notch signaling (Table S2B7): they were minimally affected by ICN1, dnMAML, or PU.1 alone. dnMAML alone was highly effective at blocking Notch target gene expression (Table S2B1), and yet it had absolutely no effect on *Myb, Tcf7, Gata3* or *Fog1*. However, in a CD11c+ Mac1- intermediate stage leading to diversion, *Myb, Tcf7* and *Gata3* also remained less affected (Fig. S3). Thus, to complete diversion (Fig. 3C), PU.1 must shut off these genes by another mechanism, beyond antagonism of Notch, even though Notch signaling maintains the inputs that protect their expression.

Id2 co-infection with PU.1 increases diversion to Mac1+ cells via inhibition of the Notch pathway in Scid.adh.2C2 cells

The data thus far indicate that diversion to a Mac1+ state is linked with PU.1-dependent repression of at least two distinct groups of T-cell genes. Of these, Notch-dependent target genes like those inhibited by dnMAML (Table S2B1) represent one component but others like *Myb*, *Gata3*, *Gfi1* and *Tcf7* represent a separate, possibly rate-limiting component. These genes encode among the most important transcription factors known for T-cell development (Rothenberg et al., 2008) and may themselves play a role in maintaining T-cell identity.

We reasoned that extinction of the T-cell program must occur only when the T-cell gene(s) that resist(s) diversion was finally turned off or neutralized. This resistance factor might be TCF-1 (encoded by *Tcf7*), Myb, Gfi1, or GATA-3, but it might also be basic helix-loop-helix E protein (E2A, HEB=Tcf12) activity, which reportedly controls both T-cell differentiation genes like *Rag1* and other T-cell regulatory genes (Ikawa et al., 2006; Schwartz et al., 2006). Indeed, PU.1 could neutralize E proteins: in Mac1+ diverted cells, the E protein antagonist Id2 is up-regulated, and this up-regulation is blocked by Notch signaling. Although this response is weak on its own, PU.1 over-expression also reduces expression of the E proteins E2A, HEB(canonical) and HEBalt, in Scid.adh.2C2 cells and fetal thymocytes alike (Franco et al., 2006).

To test whether E protein activity could set the threshold for diversion in response to PU.1, we co-expressed Id2 with PU.1 in Scid.adh.2C2 cells. In fact, Id2 and PU.1 together reproducibly increased the percentage of cells becoming Mac1+ as compared to PU.1 alone (Fig. 5A). This distinguished Id2 from two other regulators we tested as alternative candidates for collaborators with PU.1. Both the well-known myeloid factor C/EBPα and the PU.1-induced factor Bambi failed to increase the percentage of PU.1-transduced Scid.adh.2C2 cells becoming Mac1+, although C/EBPα did reduce CD25 expression (Fig. S4A and data not shown). Id2 over-expression alone also decreased CD25 levels, although it did not up-regulate Mac1. This suggested that the Id2 effect might involve inhibition of Notch signaling. E proteins have been shown to be rate-limiting positive regulators of *Notch1* (Yashiro-Ohtani et al., 2009), as well as positive contributors to the expression of some Notch target genes (Ikawa et al., 2006) such as *Ptcra*.

Gene expression analysis confirmed that Notch target genes are down-regulated maximally in samples with Id2 alone, as well as in samples co-expressing PU.1 and Id2 (Fig. 5B, Table S2C1). If Id2 over-expression affects the same pathway as Notch inhibition, then forced Notch signaling in PU.1 and Id2 co-expressing samples might be epistatic to Id2. In a triple-transduction experiment, Scid.adh.2C2 cells were infected with PU.1, Id2 and ICN1. Cell surface staining of these cells after 2 days showed that the effect of Id2 to enhance diversion to Mac1+ cells was indeed canceled out when Notch signaling was enforced by the addition of ICN1 (Fig. 5A, bottom). Thus, E protein antagonism does play a role in diversion, and induction of Id2 and repression of E2A and HEB likely provide one part of the mechanism through which PU.1 inhibits Notch activation in a positive feedback to promote a myeloid fate.

However, Id2 alone had minimal effect on *Gfi1, Myb* or *Tcf7* expression (Table S2C4,5). Furthermore, as reported in earlier E2A knockdown studies (Wei and Kee, 2007), we detected an up-regulation of *Gata3* with Id2 alone (Table S2C2), an effect reversed when PU.1 was present and quite different from the phenotype of diverted cells. Therefore, the mechanism through which these T-lineage regulatory target genes are inhibited by PU.1 to complete diversion is not simply by blocking E protein activity, any more than it is simply by blocking Notch activity.

Myb protects against PU.1-driven diversion

Myb and *Tcf7* were consistently down-regulated in response to PU.1 during diversion, and were prominent candidates as diversion "barriers" because the cells do not turn on Mac1 until these two genes are down-regulated (Figs. 3C, S3). *Myb* is already expressed strongly during the first stage of T-cell development (DN1), increasing slightly in the DN2 and DN3

stages (Tydell et al., 2007) (Fig. 2E). To test whether forced expression of *Myb* could block PU.1's ability to up-regulate Mac1, we infected Scid.adh.2C2 cells with retroviral Myb for 24 hours, then superinfected them with PU.1 and cultured the cells for an additional 48 hours (Fig. 6A). Despite < 3x increased Myb, co-expression of Myb with PU.1 modestly but reproducibly decreased the percentage of Mac1+ cells (Fig. 6B,C).

Gene expression analysis (Fig. 6D, Table S2D) showed that Myb did not inhibit PU.1 from up-regulating targets like *Bcl11a*. Importantly, protection by Myb did not seem to be mediated primarily through Notch signaling either, as Myb did not prevent PU.1 repression of Notch target genes (Table S2D3). However, *Gfi1*, *Tcf7*, *Gata3,* and *HEBalt* were expressed at higher levels in cells with Myb and PU.1 compared to those with PU.1 alone (Table S2D2,4). This group of protected genes was tested in turn for protection against PU.1-mediated diversion, but they did not perform as well as Myb. TCF-1 (*Tcf7*) was a high priority candidate; however, as many cells co-expressing TCF-1 and PU.1 were Mac1+ as cells expressing PU.1 and an empty vector (Fig. S4B). Coexpression of Gfi1 or HEBalt with PU.1 also did not block induction of Mac1 (Fig. S4C,D). In fact, Gfi1 actually exacerbated the diversion response in the Scid.adh.2C2 cells, and in fetal thymocytes, when co-expressed with PU.1 (Fig. S4C and *data not shown*). Thus, although incomplete, the protective effect of Myb against diversion was specific, implicating Myb as well as Notch signaling as separate control points for resistance to diversion.

A specific effect of PU.1 on GATA3 protein: Myb protects Gata3 protein levels

The gene that was most affected by Myb over-expression, one that Myb rendered most resistant to PU.1, was *Gata3*. Indeed, Myb-transduced cells expressed higher levels of *Gata3* RNA than controls with or without PU.1, raising the question of whether GATA3

could help to resist diversion. GATA3 is essential and rate-limiting for T-lineage development and specifically down-regulated in Mac1+ cells (Fig. 3C). It was lower priority to test for control of pro-T-cell lineage fidelity only because the magnitudes of PU.1 and Notch effects on *Gata3* RNA were weak. To test for GATA3 effects more sensitively at the single-cell level, we performed intracellular staining of the GATA3 protein in Scid.adh.2C2 cells with and without over-expressed PU.1 (Fig. 6E). In fact, PU.1 over-expression markedly down-regulated GATA3 protein levels in one subset of the transduced cells, even while it slightly up-regulated GATA3 protein, relative to controls, in another subset. This split had the same all-or-none quality as the diversion response itself. GATA3 down-regulation was seen at a much greater frequency in cells expressing high levels of PU.1 (Fig. 6E, red), in which GATA3 levels were five-to-ten fold reduced. Those cells that down-regulated GATA3 protein were also the ones that up regulated Mac1 (Fig. 6F).

Myb may positively regulate *Gata3* in later T-cell development (Maurice et al., 2007; Gimferrer et al., 2011b). To test whether *Myb* could also maintain GATA3 despite PU.1 over-expression, we compared GATA3 protein levels in Scid.adh.2C2 cells co-expressing PU.1 and *Myb* with GATA3 in cells co-expressing PU.1 and an empty vector (Fig. 6G). Cells co-expressing PU.1 with an empty vector showed lowered GATA3 protein levels, but GATA3 was rescued to normal levels in cells co-expressing PU.1 and *Myb* (Fig. 6G). Guaranteed expression of Myb thus seems to protect Scid.adh.2C2 cells from the PU.1-driven mechanism that down-regulates GATA3 protein.

Gata3 as a gatekeeper: Gata3 knockdown in PU.1 expressing cells enhances Mac1 up-regulation

To investigate whether GATA3 down-regulation was simply a marker or actually caused differences in the ability of PU.1 to divert the cells, we used shRNA to reduce GATA3 expression in PU.1-transduced cells and measured the impact on diversion. Scid.adh.2C2 cells were first infected with a construct expressing a short hairpin RNA against *Gata3* (Hernandez-Hoyos and Alberola-Ila, 2005); then after 24 hours the cells were infected with a PU.1-expressing vector and cultured for 48 hours more (Fig. 7) before analysis. The *Gata3*-shRNA alone knocked down GATA3 protein to levels that were comparable to the lowest GATA3 protein levels in PU.1 expressing cells (Fig. 7A). Unexpectedly, the Scid.adh.2C2 cells co-expressing *Gata3* shRNA together with PU.1 had even lower levels of GATA3 protein, some with 20 fold reduction compared to unperturbed cells. The impact of this GATA3 reduction was to make PU.1-expressing cells more susceptible to diversion. As shown in Fig. 7B, the fraction of cells remaining Mac1- CD11c- was halved, while increased percentages of cells acquired these myeloid markers.

Reduced GATA3 protein by itself had little effect on gene expression in the Scid.adh.2C2 cells, but the combination of PU.1 expression and GATA3 knockdown had a powerful effect on gene expression (Fig. 7E, Table S2E). GATA3 knockdown did not generally cause further up-regulation of genes induced by PU.1, and it did not exacerbate PU.1-mediated repression of several Notch targets (Table S2E3). However, we found that multiple T-cell genes that are down-regulated by PU.1 were further down-regulated in cells with PU.1 and lowered GATA3 (Table S2E4). *Notch1* and *Notch3* themselves were affected. Loss of GATA3 thus sensitizes cells to the effects of PU.1, with potency comparable to Notch inhibition.

Notch and GATA3 pathway interlinkage: Notch signaling makes GATA3 resistant to PU.1

These results imply that GATA3 down-regulation can complement the inhibition of Notch responses by PU.1 and make cells susceptible to diversion. However, our earlier results indicate that direct manipulations of Notch signaling were also sufficient to regulate PU.1-driven diversion, despite little detectable effect of Notch inhibition on *Gata3* RNA. In fact, dnMAML alone could slightly elevate *Gata3* RNA (Fig. 4B). To revisit whether there is any convergence between these two regulatory mechanisms for protecting T-cell identity, we tested whether manipulations of Notch signaling in the context of PU.1 activity might have clearer effects on GATA3 protein.

By themselves, transduction with dnMAML or ICN1 had virtually no effect on GATA3 protein levels in Scid.adh.2C2 cells (Fig. S5). However, when PU.1 transduction was combined with dnMAML or ICN1, the effect on GATA3 was dramatic (Fig. 7C). Cells co-expressing PU.1 + ICN1 uniformly expressed GATA3 at the highest level. In contrast, cells co-expressing PU.1 + dnMAML shifted almost completely to the low level of GATA3, normally seen only in cells with the highest expression of PU.1. Thus Notch signaling affects not only Notch target gene expression but also the mechanism for GATA3 stabilization, with later impact on GATA3 targets.

Kinetically, the impact of Notch inhibition on GATA3 levels could precede appearance of the diverted phenotype (Fig. 7D). Mac1 expression is not evident on PU.1-transduced Scid.adh.2C2 cells until 48 hours (Fig. 7D, right panel). However, the combination of PU.1 + dnMAML began to down-regulate GATA3 protein in the whole population of transduced Scid.adh.2C2 cells by 24 hours, falling lower by 48 hours. Thus any decrease in Notch signaling undermined the resistance of GATA3 in the cells to

inhibition by PU.1, precipitating the positive-feedback cascade that eventually silences genes dependent on GATA3 and Notch signaling alike.

Discussion

T-lineage specification of blood-cell precursors is promoted by Notch interaction with Delta expressed in the thymic microenvironment. However, throughout multiple cell cycles in this environment, the differentiating precursors continue to express transcription factors like PU.1 that are associated with multipotentiality. Their access to other fates is revealed if removed from the thymus. How does the thymus predictably manage to impose a T-cell fate on virtually all these cells, despite their intrinsic delay of commitment? Our results reveal the architecture of a regulatory gene network switch circuit through which environmental Notch signaling interacts with PU.1 to determine T-cell, myeloid, or progenitor-cell status (Fig. 8).

Two branches of this network are positively regulated by PU.1. One involves up-regulation of myeloid genes like *Itgam* and *Csf1r*, while another involves expression of stem-cell or progenitor-cell genes like *Bcl11a*, *Lyl1,* and possibly also *Bambi*. Environmental Notch signaling blocks activation of *Itgam* and *Csf1r,* but not PU.1-dependent activation generally. Concomitantly, there are two network branches through which PU.1 can negatively regulate the T-cell differentiation program. Extinction of T-lineage regulatory gene expression is most tightly correlated with a switch to myeloid fate. One branch involves the ability of PU.1 to attenuate transcriptional responses to Notch signaling: PU.1 raises the threshold of Notch signaling needed for expression of Notch target genes. This occurs in part through inhibition of an E protein—Notch

positive feedback circuit. In parallel, however, we show that PU.1 also antagonizes expression of a second set of T-cell regulatory genes, including *Myb* and its activation target *Gata3*. These seem crucial to sustain *Gfi1*, *Zfpm1* (Fog1), and *Tcf7* expression in the presence of PU.1. All these genes can also be protected against PU.1 by Notch signals, but are not otherwise Notch-dependent, implying that the protective effect of Notch on this gene set is indirect, e.g., via maintenance of GATA3. Myeloid-lymphoid lineage choice is thus a bifurcation between opposing feed-forward network circuits, one dominated by PU.1, and the other by Notch signals, which protect both Notch-E protein targets and Myb-GATA3 targets.

Our results suggest that the balance may be tipped from resistance to diversion by initial weakening of either protective mechanism in PU.1-expressing cells. Reduction either of GATA3 or of Notch signaling can sensitize the cells to diversion, and Notch signaling not only protects GATA3 but also protects its positive regulator, Myb, from inhibition by PU.1. However, it is notable that when PU.1 and Notch signals "balance", T-cell regulatory gene expression can be maintained, along with expression of specific progenitor-associated PU.1 target genes. This is exactly the situation in early T-cell precursors before lineage commitment (Fig. 1A). Our results with *Bcl11a*, *Lyl1*, and possibly also *Hhex* and *Bambi* regulation, all naturally expressed in early thymocytes, thus open the way for PU.1 to play a stage-specific positive role for early T cells.

Our results are drawn from both primary fetal thymocytes and a DN3-like clonal cell line, and the relationships are similar if not completely identical. Scid.adh.2C2 cells do not perfectly match the gene expression states of the primary cells, and as magnitudes of

specific gene expression responses to PU.1 change with normal developmental progression, they also differ between the cell line and the primary cells. These probably reflect differences in basal Notch transduction machinery, E protein activity, and GATA3 expression between these cell types (M. M. Del Real, J. A. Zhang and E.V.R, unpublished results). *Tcf7* is less protected by Notch signaling in the primary cells than in Scid.adh.2C2 cells, whereas genes like *Ptcra* are more protected. However, these are not the PU.1 repression targets that appear to set the threshold against diversion. Instead, the key components of the network core architecture shown in Fig. 8 are consistent with results in both types of cells.

PU.1 opposition to GATA3 recalls the PU.1:GATA1 opposition that underlies erythroid/myeloid fate determination, which is based in part on protein-protein interaction (rev. by (Cantor and Orkin, 2002; Laiosa et al., 2006a)). Here, GATA3 appears important for *Zfpm1* maintenance against PU.1, like GATA1 in erythroid development. ChIP-seq analysis shows that *Zfpm1*, *Gfi1*, *Myb*, and *Tcf7* are all linked with GATA3 binding sites in early T cells (Zhang et al., 2012)(*Bambi, Bcl11a, Itgam, Id2* are not), suggesting that high PU.1 may primarily inhibit these T-cell genes by blocking positive GATA3 inputs. However, GATA3-PU.1 antagonism itself is more conditional. Although PU.1 reduces GATA3 protein when Notch signaling is inhibited, PU.1 slightly up-regulates GATA3 when Notch signals are active. PU.1 binds multiple sites around *Gata3* in early T cells (Zhang et al., 2012), potentially contributing to both effects. PU.1 can be repressed by high-level GATA3 (Taghon et al., 2007), but the genomic sites through which GATA1 silences PU.1 expression (Chou et al., 2009) are not bound by GATA3 in early T cells (Zhang et al., 2012). Furthermore, although reduced GATA3 makes cells more diversion-

sensitive, increased GATA3 cannot bypass the need for Notch signaling to make cells diversion-resistant (M. M. Del Real and E.V.R, unpublished results). This suggests that PU.1-GATA3 relationships are probably asymmetric.

The relationship between PU.1 and Notch signaling provides a discrete, microenvironmental threshold-setter for lymphoid precursor fate determination. In normal thymocytes though not in Scid.adh.2C2 cells, the signals actually received depend on environmental density of Notch ligands. Within the pro-T cells, signaling not only requires E proteins to maintain Notch1 expression but also a positive feedback loop with E protein activity, for expression of both Id2 and Id3 E protein antagonists increases when Notch signaling is reduced. The molecular mechanism through which PU.1 inhibits Notch-dependent transcription still requires more investigation. However, our results show that the expression of PU.1 in the earliest T-cell precursors itself becomes a sensor that determines what level of Notch signal from the environment will suffice to promote entry and forward progression along the T-cell pathway.

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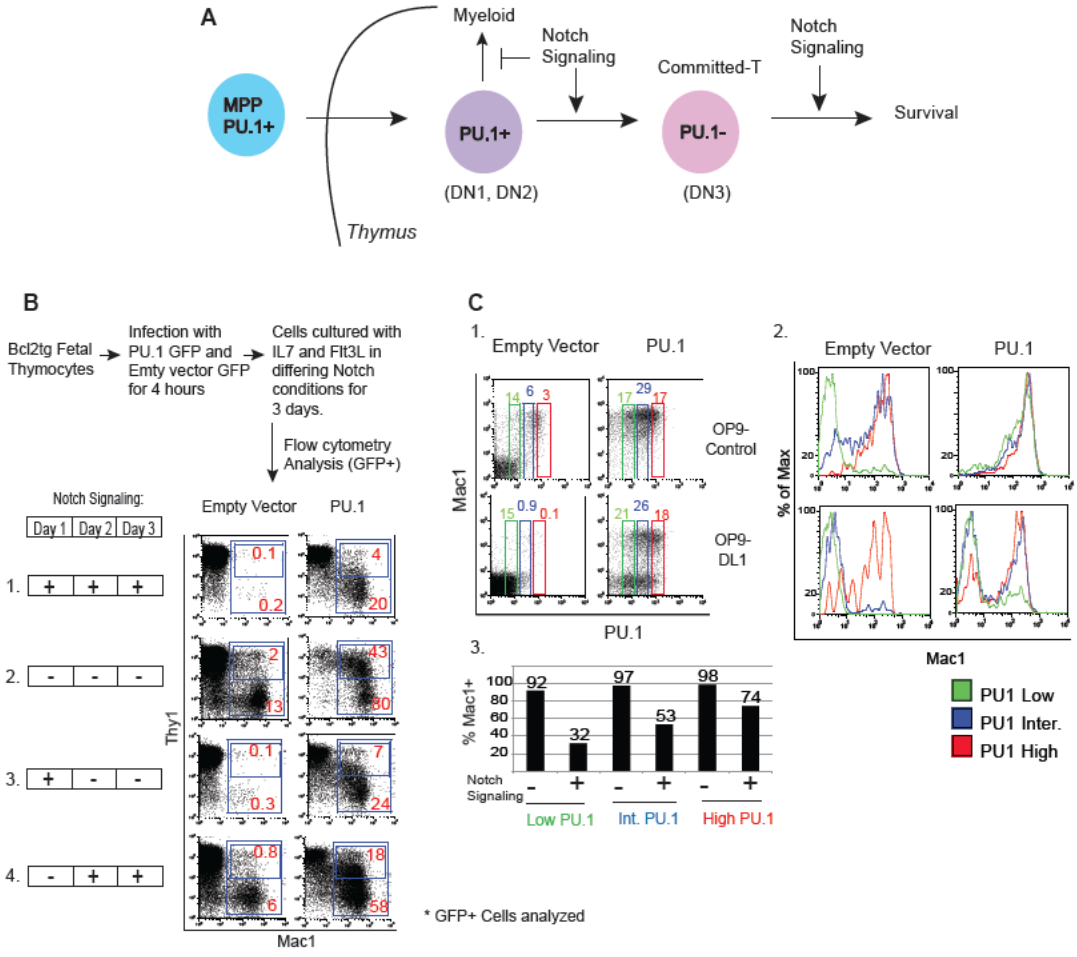


Figure 1. Continuous Notch signaling is not required to protect fetal thymocytes from diversion. Notch signaling can protect cells with high PU.1 protein levels.

(A) Diagram of PU.1 and Notch signaling interactions during early T-cell development. (B) E15.5 fetal thymocytes transduced with PU.1 and empty vector were cultured in different Notch signaling conditions for 3 days with IL7 and Flt3 ligand. The transduced cells were analyzed for the expression of the T-cell marker Thy1 and the myeloid marker Mac1. (C) E15.5 thymocytes were transduced with PU.1 or an empty vector. The percentage of Mac1+ cells in samples expressing high, intermediate and low levels of PU.1 protein were obtained using flow cytometry.

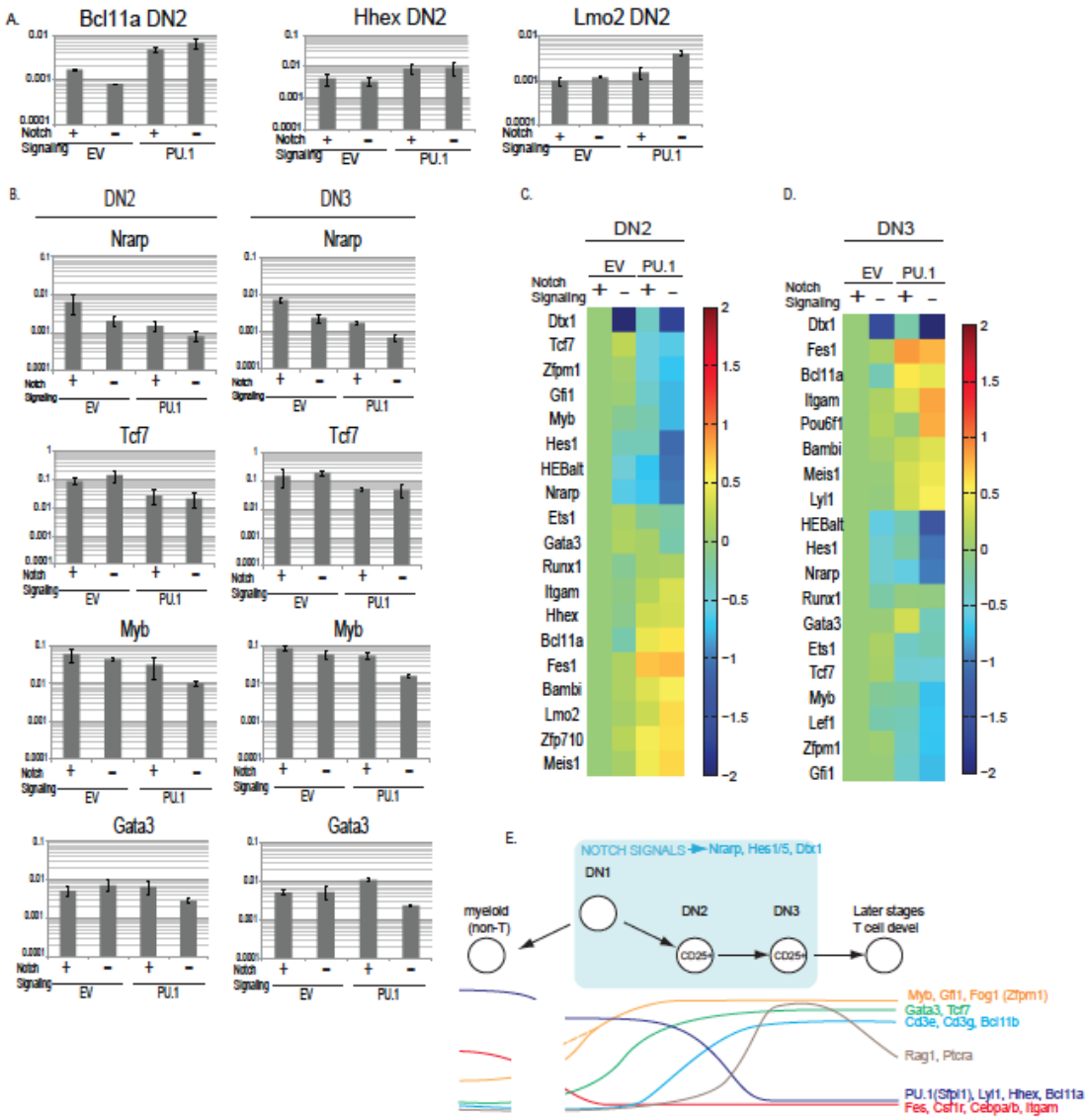
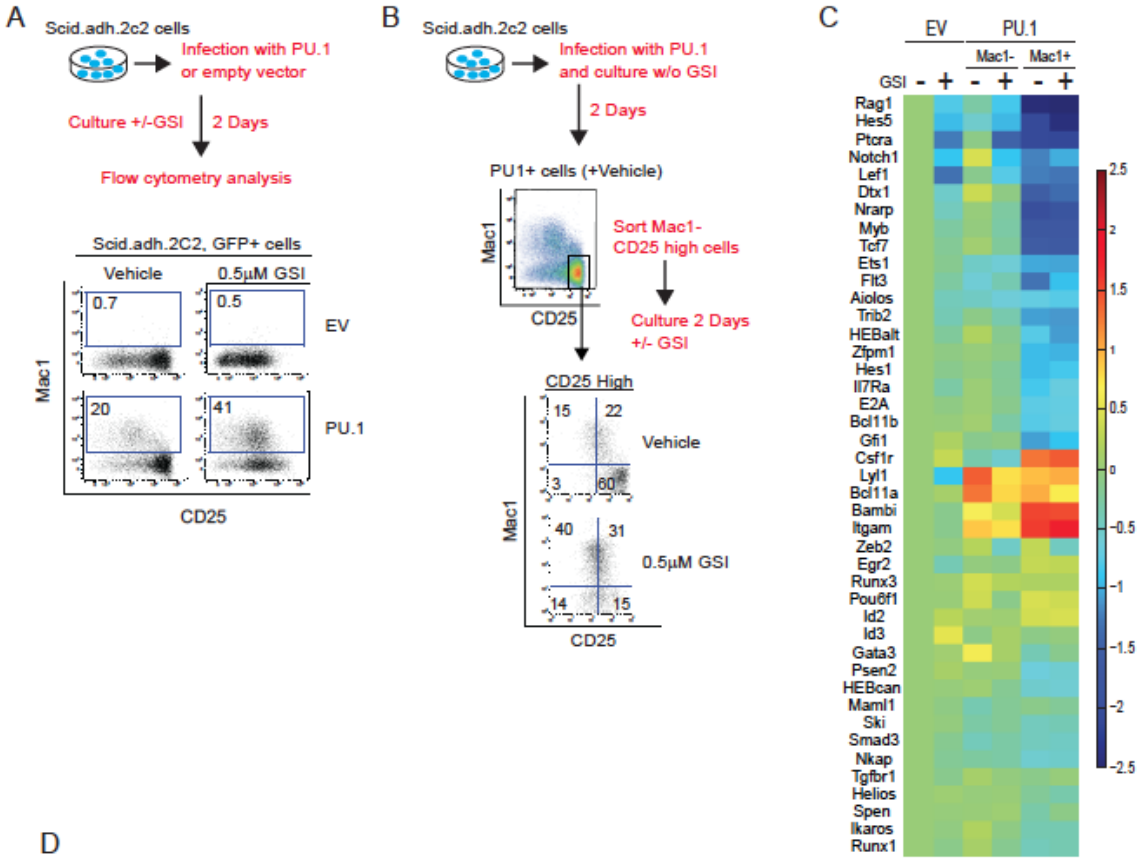


Figure 2. Gene expression profile of fetal thymocytes in response to high-levels of PU.1 in short-term cultures

E15.5 fetal thymocytes were infected with PU.1-GFP or empty vector-GFP and transferred to OP9-DL1 or OP9-control cells overnight. DN2 and DN3 GFP+ cells were sorted and gene changes were detected using qRT-PCR. (A) Genes up-regulated with PU.1. (B) Genes down-regulated in DN2 and DN3 cells with PU.1. (C and D) Heatmap of gene expression obtained by qRT-PCR in DN2 and DN3 fetal thymocytes expressing PU.1 for

16 hours in the presence or absence of Notch signaling. (E) Diagram of early T-cell regulatory gene expression patterns.



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Response Group	Genes
1. Notch dependent	*Rag1, Hes5, Ptcra, Notch1, Lef1, Dtx1, Nrarp*
2. PU.1 activated	*Lyl1, Bcl11a, Bambi, Itgam, Zeb2, Egr2, Runx3, Pou6f1, Id2*
3. PU.1 activated only (more) in Mac1+ diverted cells	*Csf1r, Egr2, Id2 (Bambi, Itgam)*
4. Downregulated by PU.1	*Ets1*, Aiolos*, Flt3**
5. Downregulated by PU.1 only (more) in diverted cells	*Myb, Tcf7, Trib2, HEBalt, Zfpm1, Hes1, Il7ra, Tcfe2a, Bcl11b, Gfi1, Gata3, HEBcan, Psen2, (Ets1), (Flt3)*
6. Downregulated in diverted cells and in Notch-inhibited cells	*Rag1, Hes5, Ptcra, Notch1, Lef1, Dtx1, Nrarp*

Figure 3. Scid.adh.2C2 cells can be used to study PU.1 and Notch signaling

interactions.

(A) Scid.adh.2C2 cells expressing PU.1 or empty vector were cultured with or without GSI for 48 hours. Mac1 and CD25 expression levels were measured using flow cytometry. (B) PU.1+Mac1- Scid.adh.2C2 cells were cultured in the presence or absence of GSI for 2 days. Mac1 and CD25 expression levels were measured using flow cytometry. (C) Heatmap of gene expression in Scid.adh.2c2 cells expressing PU.1 or empty vector with or without GSI for 2 days and sorted according to Mac1 expression.

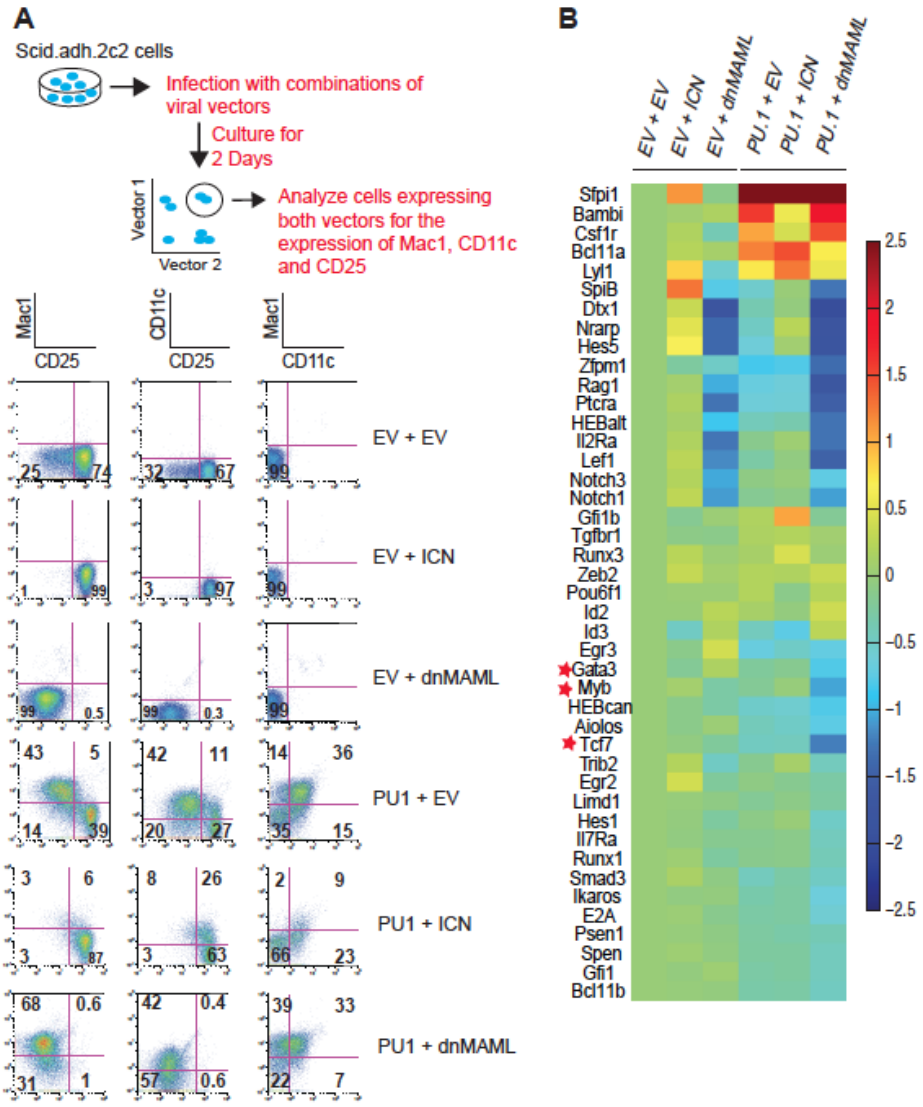


Figure 4. PU.1-dependent gene expression effects in controlled Notch signaling conditions using Scid.adh.2C2 cells

(A) Diagram of experimental set up and flow cytometric analysis of CD25, Mac1, and CD11c expression. (B) Heatmap of gene expression in sorted Scid.adh.2C2 cells expressing PU.1 with dnMAML, ICN1 or empty vector obtained from qRT-PCR.

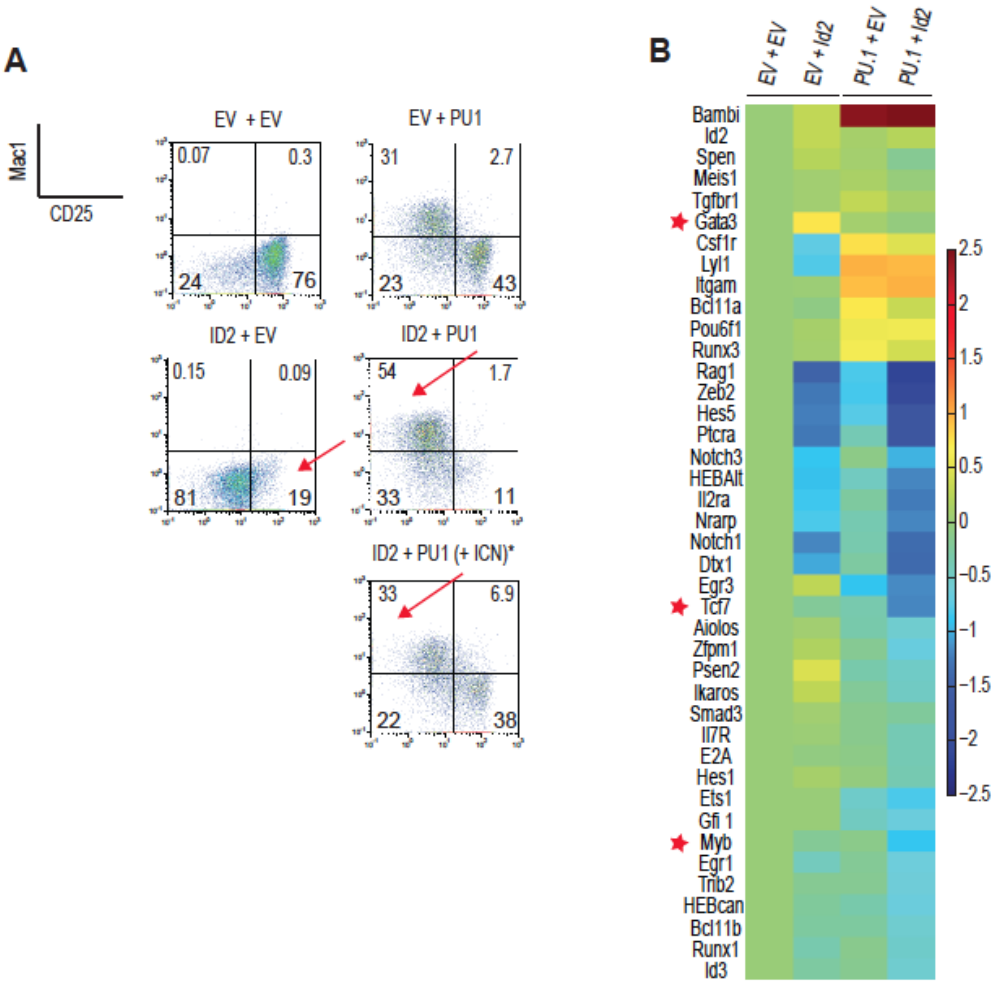


Figure 5. E-protein inhibition is a mechanism for reducing Notch signaling, but does not account for all PU.1-mediated effects.

(A) Scid.adh.2c2 cells expressing PU.1 and ID2 or an empty vector and PU.1, ID2 and ICN were cultured for 2 days and then analyzed for their expression of Mac1 and CD25 using flow cytometry. (B) Heatmap of Scid.adh.2C2 gene expression in sorted cells co-expressing ID2 and PU.1 obtained by qRT-PCR.

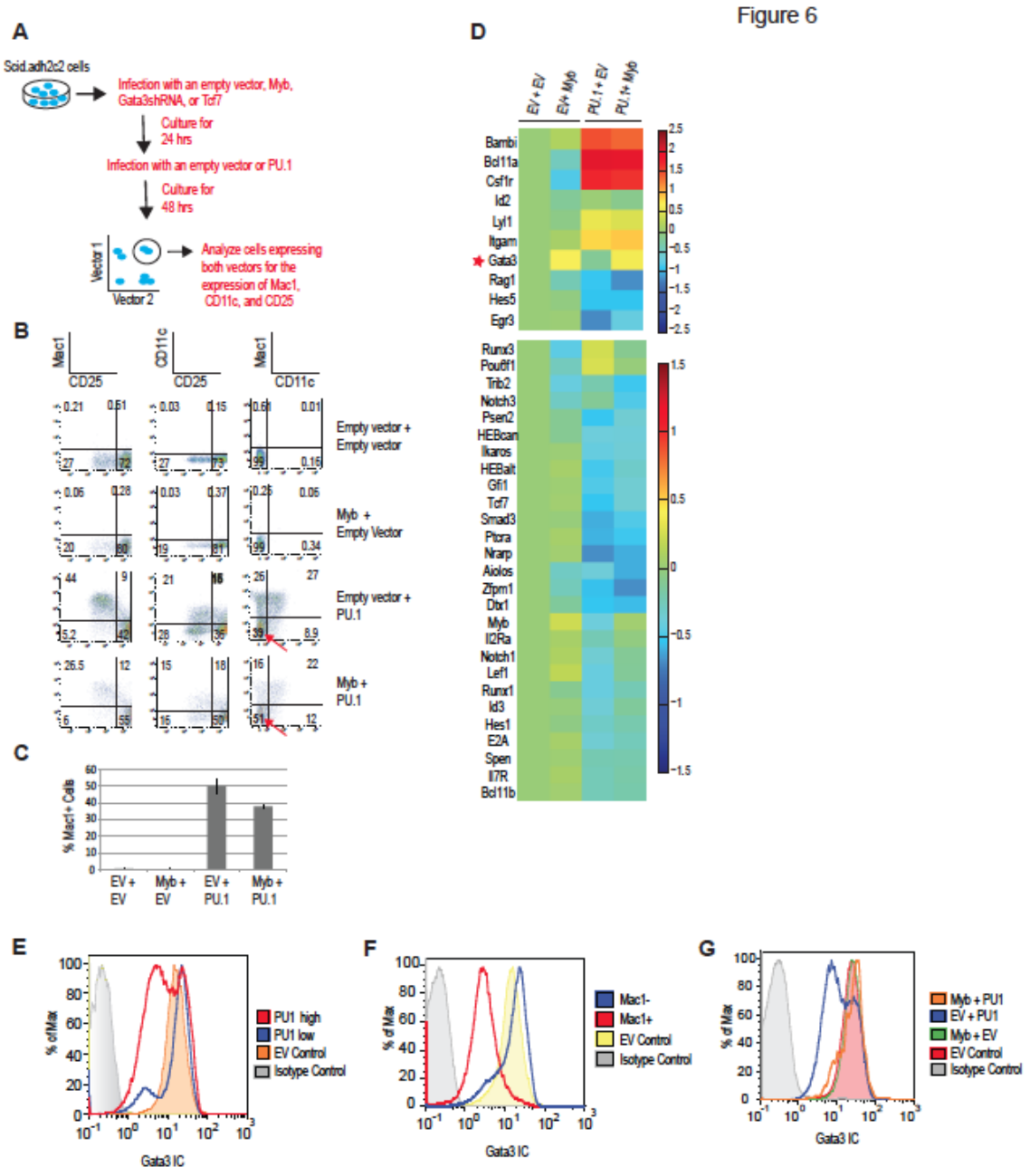


Figure 6. Co-expression of Myb and PU.1 in Scid.adh.2C2 cells reduced the percentage of Mac1+ cells. This is mediated in part by the protection of Gata3.

(A) Diagram of experimental set-up. (B and C) Mac1, CD25 and CD11c flow cytometric analysis of scid.adh.2c2 cells expressing PU.1 and Myb for 2 days. (D) Heatmap of gene expression analysis of Scid.adh.2c2 cells. (E) Gata3 intracellular staining of Scid.adh.2c2 cells expressing PU.1. (F) Gata3 intracellular staining of Mac1+ and Mac1- PU.1-expressing Scid.adh.2c2 cells. (G) GATA3 protein levels in Scid.adh.2c2 cells expressing a combination of PU.1, Myb and empty vector.

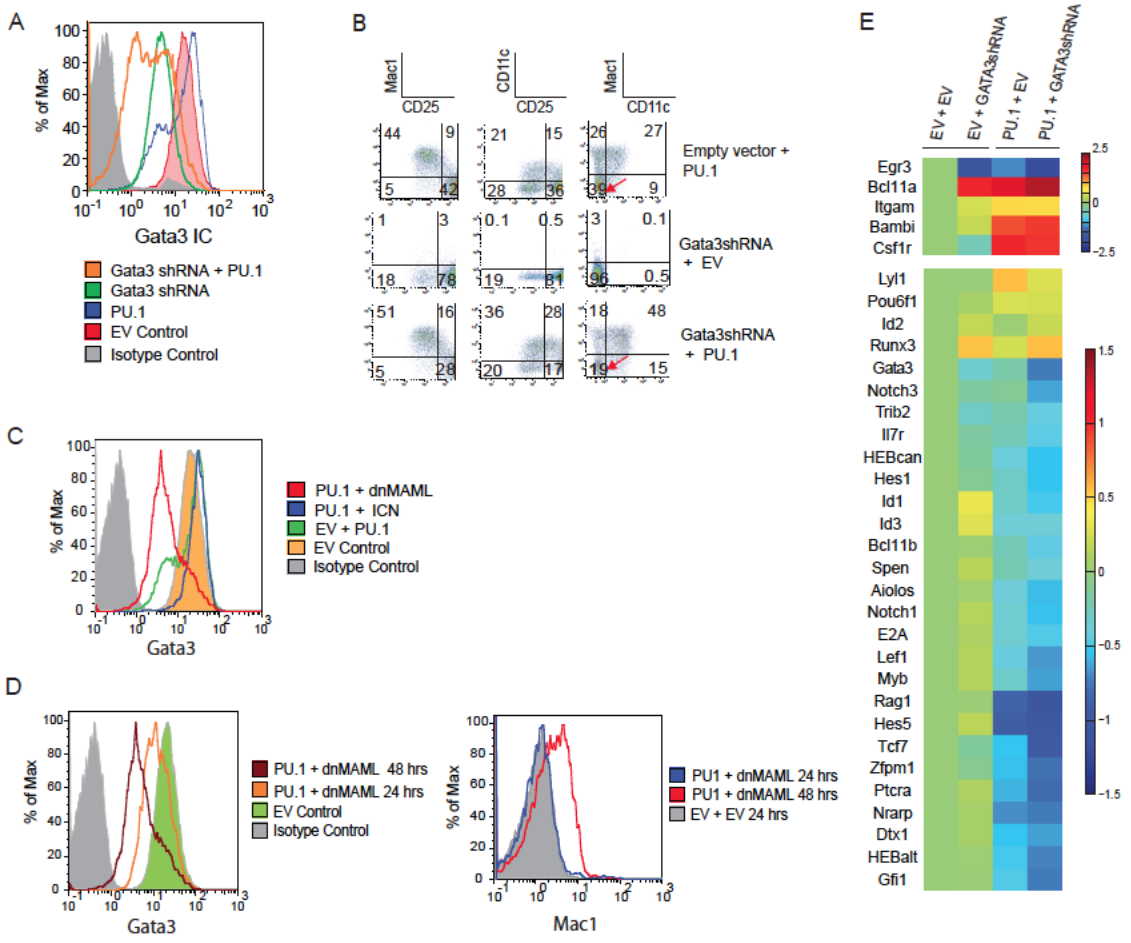


Figure 7. Inhibition of GATA3 protein enhances PU.1-driven Mac1 up-regulation in Scid.adh.2C2 cells. Notch signaling blocks PU.1-driven GATA3 protein inhibition. (A) GATA3 intracellular staining of samples expressing PU.1 and Gata3shRNA after 3 days. (B) Experimental set up as in Fig.6A. Cells were analyzed for their expression of CD25, Mac1, and CD11c. (C) GATA3 protein levels in cells expressing PU.1 and ICN, dnMAML, or empty vector for 2 days. (D) GATA3 protein levels in Scid.adh.2C2 cells co-expressing PU.1 and dnMAML for 24 and 48 hours (left panel). Mac1 expression in the same cells at 24 and 48 hours (right panel). (E) Heatmap of gene expression in Scid.adh.2C2 cells expressing a combination of Gata3 shRNA, PU.1 and empty vectors for 3 days.

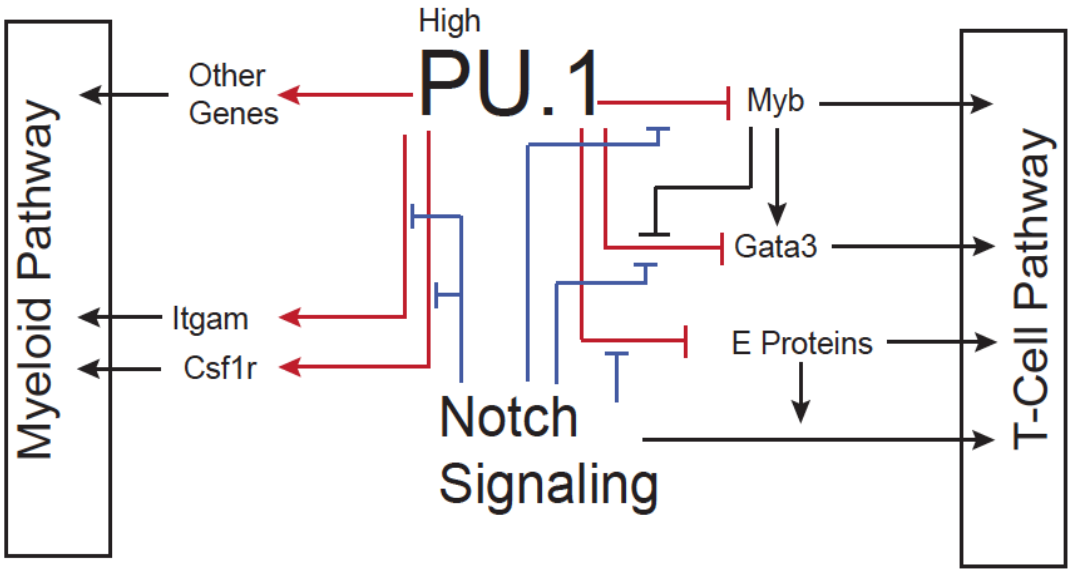
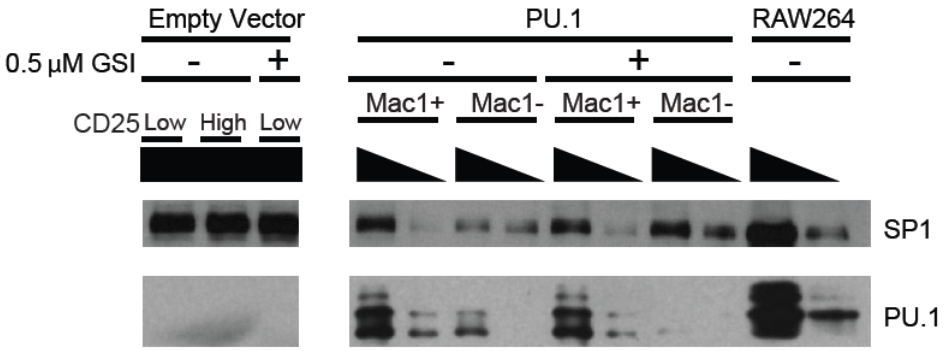


Figure 8. Interactions between PU.1, Notch signaling and regulatory genes that partially define a lymphomyeloid switch during early T-cell development.

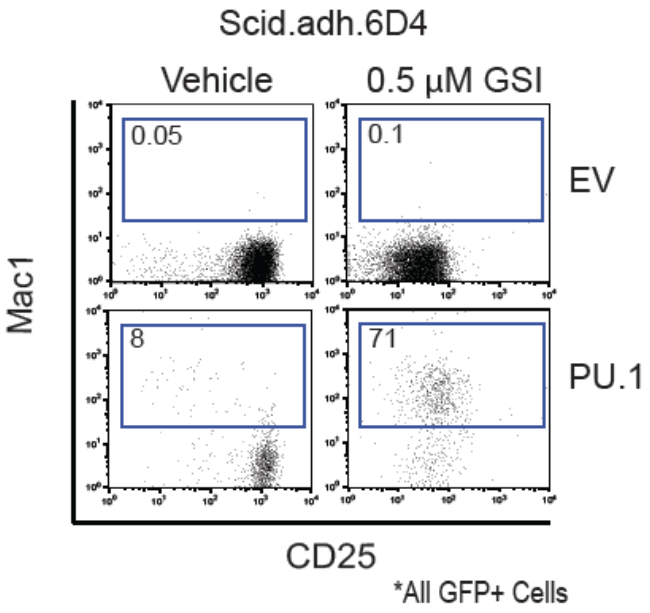
SUPPLEMENTAL MATERIAL: M. M. Del Real & E. V. Rothenberg

Fig.S1



Supplementary Figure 1. Inhibition of Notch signaling in Scid.adh2c2 cells lowered the PU.1 dose threshold for the cells to divert to a Mac1+ state, but did not alter PU.1 protein patterns. Scid.adh.2c2 cells were infected with PU.1 or empty vector and cultured in the presence or absence of γ secretase inhibitor (GSI) for 48 hours. The cells were then sorted according to their expression of Mac1 and lysed for Western blot analysis, each sample assayed at 1x and 0.2x concentration with the decreasing concentrations indicated by wedge symbols over the lanes. The Western blot was probed with anti-PU.1 antibody and antibody against the ubiquitous transcription factor SP1 as a loading control. The RAW264.7 macrophage cell line was used as a positive control for normal myeloid levels of PU.1 relative to SP1. The figure shows that cells with detectable PU.1 levels can remain Mac1- in the absence of GSI but not in the presence of GSI. Note that the hierarchy of PU.1 band strengths, representing different post-translational modifications, is different in the myeloid cells from that in the transfected Scid.adh.2c2 cells. However, Mac1- and Mac1+ Scid.adh.2c2 cells have the same band patterns, in the presence or absence of GSI.

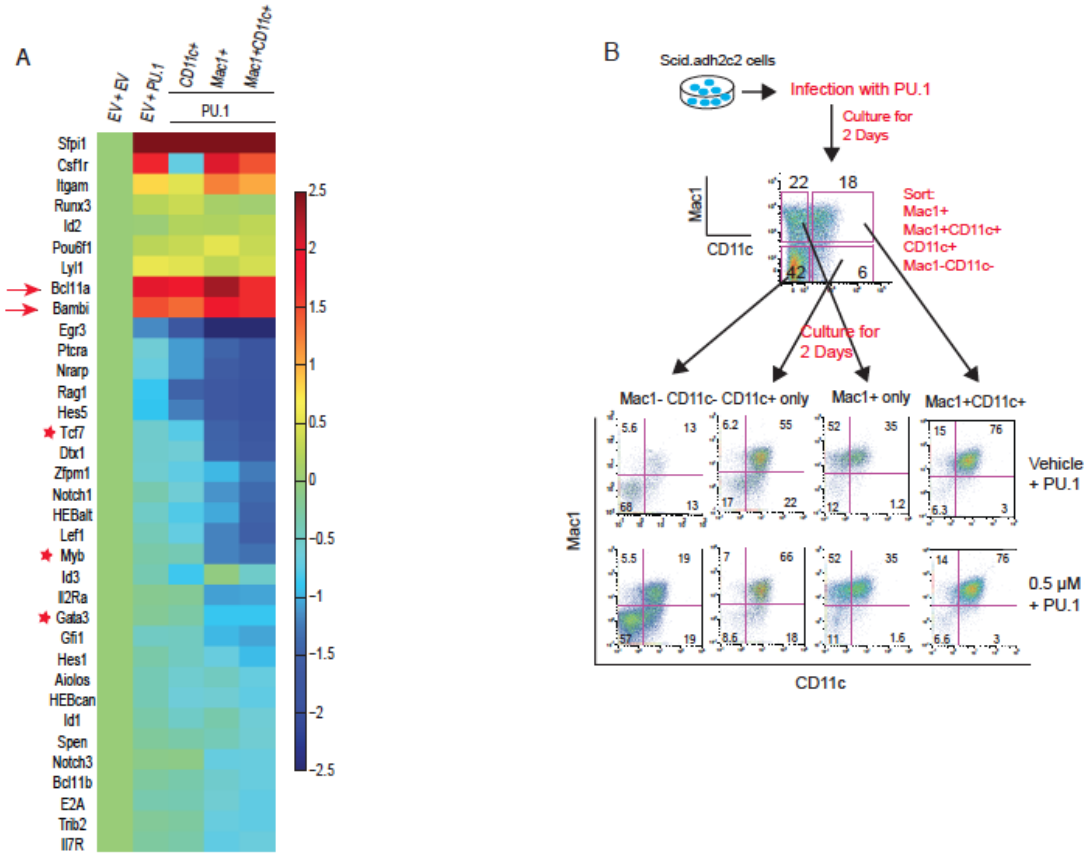
Fig.S2



Supplementary Figure 2. A diversion-resistant subclone of Scid.adh cells becomes susceptible to diversion by PU.1 if endogenous Notch signaling is inhibited.

Scid.adh.6D4 cells were transduced with empty vector or PU.1, and then cultured in the presence or absence of 0.5 μM GSI for two days as in Fig. 3A. Analysis shows the lack of Mac1 up-regulation in these cells in response to PU.1 alone, but the efficient induction of Mac1 by PU.1 when Notch signaling is inhibited. These cells experience reduced viability in response to forced expression of PU.1 (Dionne et al., 2005), but the treatment with GSI is not more toxic to them than the effects of PU.1 alone.

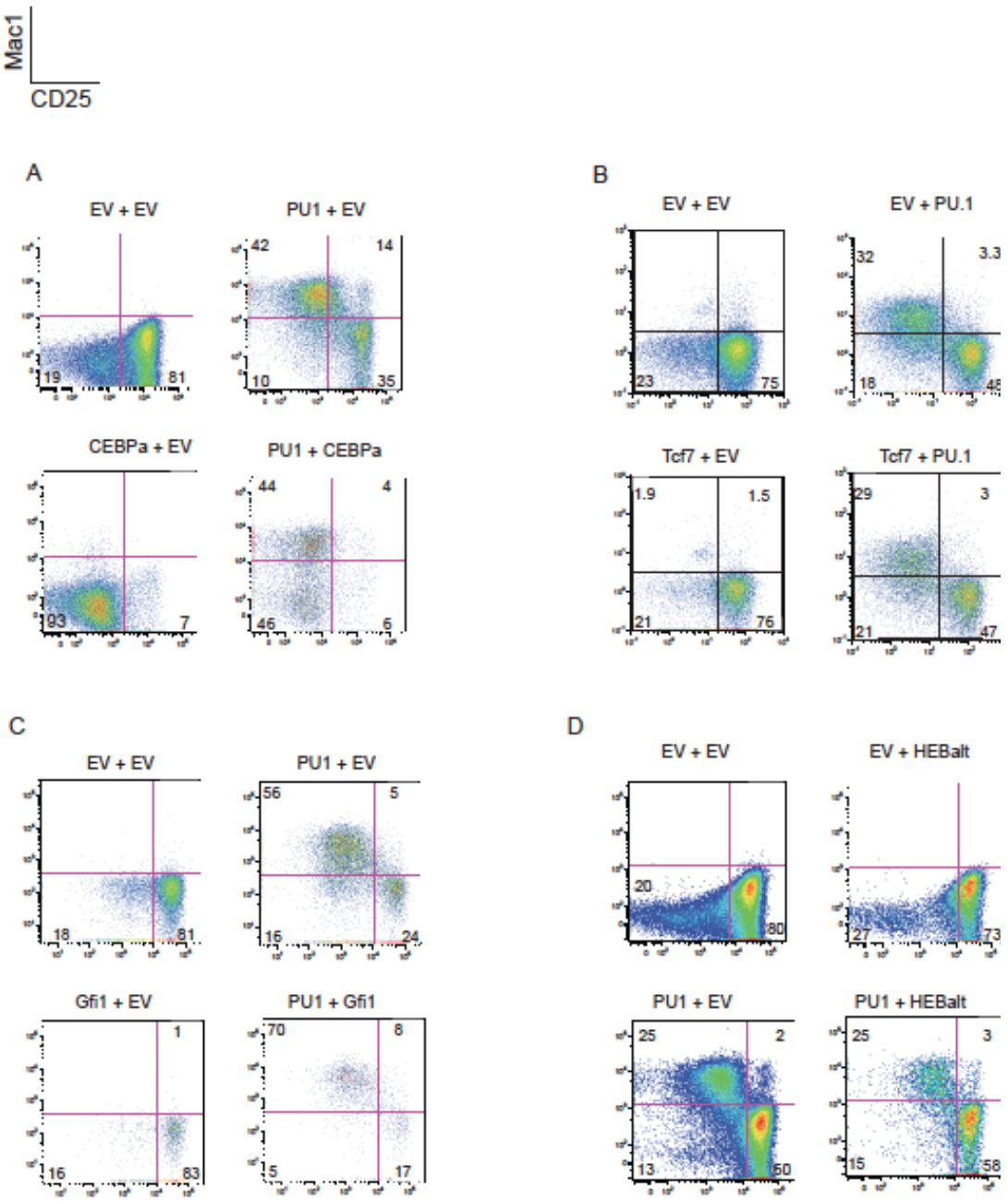
Fig.S3



Supplementary Figure 3. Mac1 up-regulation in Scid.adh.2c2 cells accompanies a more severe repression of T cell genes such as *Tcf7, Myb and Gata3* when compared to the up regulation of CD11c. (A) Scid.adh.2c2 cells were infected with PU.1 or empty vector and cultured for 48 hours. The cells were then sorted according to their expression of the viral vector and the expression of Mac1 and/or CD11c. QRT-PCR analysis was performed on the samples. As shown in Fig. 4A, the up-regulation of these two markers is not completely coordinate, and Notch signals appear more effective at blocking Mac1 expression than at blocking CD11c expression. Gene expression results in panel A show

that repression of T-cell genes and activation of *Csf1r* are not, in fact, complete in the CD11c+ Mac1- cells. (B) Cells with CD11c+ Mac1- phenotype can be intermediates toward full diversion. Schematic shows the experimental plan. After sorting distinct subsets of transduced Scid.adh.2C2 cells based on their patterns of CD11c/Mac1 expression at 2 days (middle panel), further culture for 2 days more with or without GSI reveals that CD11c+ Mac1- cells are primed to progress to a fully diverted CD11c+ Mac1+ phenotype.

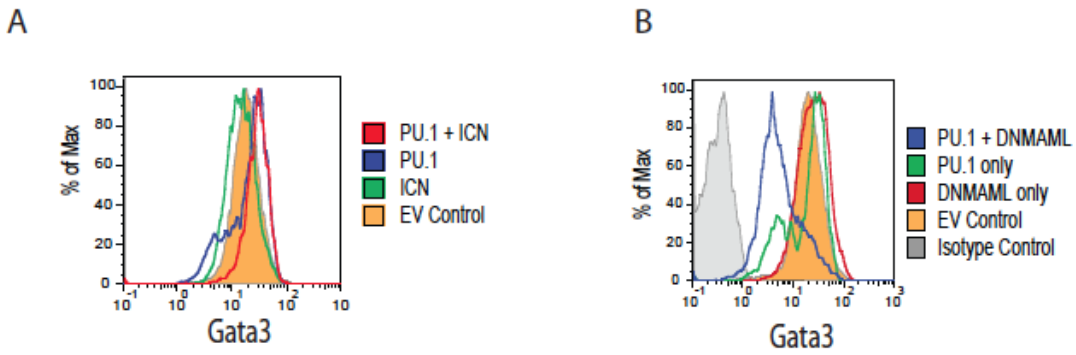
Fig. S4



Supplementary Figure 4. Scid.adh.2c2 cells co-expressing PU.1 with *Tcf7*, *Cebpa*, or *HEBalt* do not alter their expression of Mac1 in comparison to cells expressing PU.1 alone, while co-expression of PU.1 with *Gfi1* increases the percentage of Mac1+ cells

compared to PU.1 alone. Scid.adh2c2 cells were infected with (A) *Tcf7*, (B) *Cebpa*, (C) *Gfi1*, or (D) HEBalt (*Tcf12,* alternative promoter isoform) for 24 hours. The cells were then infected with PU.1 or empty vector and cultured for an additional 48 hours. The cells were analyzed for their expression of Mac1 and CD25 using flow cytometry.

Fig. S5



Supplementary Figure 5. GATA3 protein levels are decreased by PU.1 in the absence of Notch signaling, but are unchanged by PU.1 in the presence of high levels of Notch signaling. (A) Scid.adh.2c2 cells were infected with PU.1 and ICN and cultured for 48 hours. GATA3 intracellular staining shows that PU.1 alone can down regulate GATA3 protein in some cells, while PU.1 co-expressed with ICN and ICN alone do not down regulate GATA3 protein levels. (B) Scid.adh2c2 cells infected with PU.1 and dnMAML for 48 hours show increased down regulation of GATA3 proteins levels compared to cells expressing only PU.1. dnMAML alone does not down regulate GATA3 protein levels.

Table S1

A.

Gene	EV Delta DN2		EV Mig DN2		PU1 Delta DN2		PU1 Mig DN2	
	Average	Std. Dev.	Average	Std. Dev.	Average	Std. Dev.	Average	Std. Dev.
Aiolos	0.0026	0.0005	0.0056	0.0025	0.0025	0.0015	0.0039	0.0014
Bambi	0.0006	0.0000	0.0008	0.0002	0.0014	0.0004	0.0019	0.0005
Bcl11a	0.0017	0.0001	0.0008	0.0000	0.0049	0.0007	0.0068	0.0018
Bcl11b	0.0267	0.0105	0.0378	0.0044	0.0194	0.0116	0.0241	0.0114
C/EBPa	0.0004	0.0005	0.0001	0.0000	0.0001	0.0001	0.0005	0.0004
CD18	0.0099	0.0012	0.0159	0.0048	0.0202	0.0082	0.0242	0.0032
Csf2Ra	0.0052	0.0035	0.0024	0.0013	0.0056	0.0015	0.0054	0.0011
Deltex	0.0094	0.0011	0.0001	0.0000	0.0039	0.0032	0.0002	0.0003
E2A	0.0334	0.0119	0.0306	0.0062	0.0212	0.0132	0.0249	0.0094
Fes1	0.0046	0.0009	0.0062	0.0018	0.0250	0.0037	0.0267	0.0067
Flt3	0.0005	0.0004	0.0029	0.0014	0.0009	0.0002	0.0022	0.0009
Fog1	0.0118	0.0066	0.0141	0.0027	0.0036	0.0008	0.0023	0.0005
Gata3	0.0052	0.0015	0.0072	0.0024	0.0066	0.0027	0.0028	0.0005
Gfi1	0.0081	0.0014	0.0087	0.0003	0.0031	0.0016	0.0013	0.0002
Gfi1b	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HEBalt	0.0079	0.0057	0.0027	0.0017	0.0015	0.0013	0.0007	0.0006
HEBcan	0.0273	0.0093	0.0301	0.0110	0.0163	0.0057	0.0202	0.0100
Hes1	0.0174	0.0042	0.0085	0.0011	0.0062	0.0004	0.0014	0.0004
Hes5	0.0013	0.0011	0.0008	0.0005	0.0006	0.0004	0.0011	0.0008
Hhex	0.0042	0.0017	0.0034	0.0009	0.0086	0.0030	0.0091	0.0038
Id1	0.0086	0.0043	0.0055	0.0039	0.0054	0.0004	0.0051	0.0017
Id2	0.0096	0.0025	0.0140	0.0051	0.0080	0.0041	0.0156	0.0057
Id3	0.0326	0.0092	0.0499	0.0078	0.0195	0.0086	0.0524	0.0295
Ikaros	0.0067	0.0016	0.0097	0.0017	0.0032	0.0013	0.0038	0.0015
Il7Ra	0.0213	0.0059	0.0142	0.0007	0.0164	0.0057	0.0104	0.0018
Lef1	0.0479	0.0089	0.0275	0.0085	0.0271	0.0083	0.0149	0.0024
Limd1	0.0091	0.0016	0.0115	0.0023	0.0143	0.0059	0.0160	0.0026
Lmo2	0.0010	0.0002	0.0012	0.0001	0.0015	0.0005	0.0041	0.0006
Lyl1	0.0110	0.0061	0.0043	0.0010	0.0101	0.0058	0.0188	0.0078
Mac1	0.0009	0.0010	0.0008	0.0004	0.0013	0.0007	0.0020	0.0001
Meis1	0.0030	0.0020	0.0038	0.0010	0.0090	0.0029	0.0134	0.0051
Mint	0.0025	0.0011	0.0036	0.0007	0.0023	0.0011	0.0024	0.0009
Myb	0.0597	0.0237	0.0439	0.0052	0.0317	0.0184	0.0101	0.0016
Notch1	0.0779	0.0305	0.0340	0.0024	0.0594	0.0252	0.0252	0.0065
Nrarp	0.0064	0.0035	0.0020	0.0005	0.0015	0.0004	0.0008	0.0002
Pou5f1	0.0006	0.0005	0.0013	0.0011	0.0082	0.0048	0.0157	0.0063
Psen	0.0158	0.0010	0.0159	0.0030	0.0143	0.0006	0.0175	0.0009
Ptcra	0.0330	0.0113	0.0017	0.0005	0.0292	0.0080	0.0059	0.0012
Rag1	0.0863	0.0326	0.0708	0.0423	0.0314	0.0211	0.0485	0.0264
Runx1	0.0677	0.0323	0.0493	0.0061	0.0862	0.0195	0.0872	0.0258
Runx3	0.0478	0.0049	0.0532	0.0163	0.0364	0.0164	0.0296	0.0139
Smad3	0.0045	0.0012	0.0050	0.0022	0.0022	0.0008	0.0037	0.0008
SpiB	0.0254	0.0050	0.0226	0.0131	0.0114	0.0061	0.0086	0.0033
Tcf7	0.0851	0.0217	0.1427	0.0646	0.0266	0.0140	0.0207	0.0115
Tgfbr1	0.0399	0.0034	0.0304	0.0050	0.0320	0.0082	0.0349	0.0136
Zfp710	0.0053	0.0016	0.0085	0.0040	0.0174	0.0026	0.0222	0.0022

B.

Gene	EV Delta DN3		EV Mig DN3/4		PU1 Delta DN3		PU1 Mig DN3/4	
	Average	Std. Dev.	Average	Std. Dev.	Average	Std. Dev.	Average	Std. Dev.
Aiolos	0.0054	0.0024	0.0127	0.0025	0.0027	0.0012	0.0096	0.0028
Bambi	0.0007	0.0001	0.0008	0.0006	0.0012	0.0003	0.0016	0.0005
Bcl11a	0.0004	0.0003	0.0002	0.0002	0.0014	0.0008	0.0010	0.0006
Bcl11b	0.0552	0.0135	0.0701	0.0229	0.0393	0.0204	0.0442	0.0139
C/EBPa	0.0001	0.0000	0.0002	0.0001	0.0001	0.0001	0.0002	0.0002
CD18	0.0136	0.0088	0.0156	0.0039	0.0108	0.0037	0.0238	0.0053
Csf2Ra	0.0033	0.0017	0.0029	0.0009	0.0018	0.0009	0.0025	0.0010
Deltex	0.0272	0.0009	0.0006	0.0004	0.0153	0.0063	0.0003	0.0000
E2A	0.0488	0.0040	0.0342	0.0125	0.0349	0.0082	0.0263	0.0102
Fes1	0.0013	0.0006	0.0017	0.0007	0.0097	0.0027	0.0074	0.0023
Flt3	0.0001	0.0001	0.0005	0.0005	0.0002	0.0001	0.0003	0.0002
Fog1	0.0155	0.0030	0.0167	0.0087	0.0068	0.0034	0.0030	0.0008
Gata3	0.0052	0.0007	0.0053	0.0019	0.0109	0.0010	0.0024	0.0002
Gfi1	0.0137	0.0033	0.0137	0.0026	0.0052	0.0009	0.0024	0.0005
Gfi1b	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HEBalt	0.0104	0.0061	0.0028	0.0008	0.0053	0.0030	0.0004	0.0002
HEBcan	0.0493	0.0277	0.0713	0.0487	0.0318	0.0171	0.0371	0.0200
Hes1	0.0191	0.0062	0.0069	0.0026	0.0123	0.0034	0.0018	0.0009
Hes5	0.0015	0.0010	0.0011	0.0009	0.0008	0.0005	0.0010	0.0007
Hhex	0.0006	0.0001	0.0006	0.0004	0.0006	0.0003	0.0008	0.0004
Id1	0.0049	0.0007	0.0045	0.0028	0.0040	0.0009	0.0042	0.0017
Id2	0.0064	0.0003	0.0119	0.0073	0.0043	0.0015	0.0071	0.0022
Id3	0.0382	0.0087	0.0833	0.0332	0.0237	0.0087	0.0605	0.0285
Ikaros	0.0143	0.0056	0.0111	0.0052	0.0048	0.0020	0.0038	0.0013
Il7Ra	0.0271	0.0014	0.0157	0.0042	0.0255	0.0009	0.0119	0.0032
Lef1	0.2487	0.0477	0.1351	0.0243	0.1087	0.0532	0.0474	0.0169
Limd1	0.0133	0.0040	0.0115	0.0016	0.0112	0.0014	0.0128	0.0030
Lmo2	0.0012	0.0001	0.0015	0.0003	0.0012	0.0010	0.0018	0.0006
Lyl1	0.0028	0.0004	0.0027	0.0017	0.0059	0.0015	0.0096	0.0048
Mac1	0.0002	0.0002	0.0003	0.0002	0.0004	0.0002	0.0013	0.0003
Meis1	0.0030	0.0016	0.0030	0.0014	0.0064	0.0034	0.0085	0.0038
Mint	0.0031	0.0006	0.0031	0.0004	0.0028	0.0008	0.0032	0.0004
Myb	0.0849	0.0131	0.0584	0.0150	0.0538	0.0120	0.0156	0.0013
Notch1	0.1774	0.0141	0.0602	0.0057	0.1039	0.0261	0.0461	0.0144
Nrarp	0.0073	0.0011	0.0024	0.0007	0.0017	0.0001	0.0007	0.0002
Pou5f1	0.0041	0.0027	0.0060	0.0009	0.0041	0.0024	0.0266	0.0134
Psen	0.0173	0.0047	0.0167	0.0041	0.0145	0.0016	0.0158	0.0027
Ptcra	0.1064	0.0513	0.0313	0.0093	0.0905	0.0445	0.0317	0.0152
Rag1	0.2196	0.0646	0.2867	0.0168	0.0899	0.0205	0.1466	0.1180
Runx1	0.1223	0.0384	0.0731	0.0370	0.1171	0.0427	0.1113	0.0474
Runx3	0.0443	0.0067	0.0410	0.0115	0.0355	0.0102	0.0217	0.0097
Smad3	0.0119	0.0013	0.0098	0.0018	0.0047	0.0007	0.0054	0.0022
SpiB	0.0601	0.0400	0.0321	0.0118	0.0216	0.0067	0.0176	0.0040
Tcf7	0.1513	0.0909	0.1902	0.0490	0.0525	0.0078	0.0497	0.0239
Tgfbr1	0.0309	0.0132	0.0407	0.0209	0.0435	0.0052	0.0359	0.0108
Zfp710	0.0074	0.0049	0.0068	0.0036	0.0086	0.0003	0.0121	0.0021

C.

DN2 Cells - P-Values from Two-way ANOVA							
	DN2 Bcl11a	DN2 Hhex	DN2 Lmo2	DN2 Nrarp	DN2 Tcf7	DN2 Myb	DN2 Gata3
PU.1	0.0003	0.0002	0.0464	0.0035	0.0008	< 0.0001	0.0138
Notch Signaling	0.9167	0.8514	0.0152	0.0286	0.7622	0.0794	0.4349
Interaction	0.015	0.175	0.161	0.2986	0.1071	0.0018	0.0028

DN3 Cells - P-Values from Two-way ANOVA				
	DN3 Nrarp	DN3 Tcf7	DN3 Myb	DN3 Gata3
PU.1	0.0005	0.0175	0.0003	0.933
Notch Signaling	0.0007	0.647	0.0047	0.0011
Interaction	0.4525	0.4247	0.0055	0.0042

Table S1: Actual measured RNA expression levels in DN2 (A) and DN3 (B) thymocytes 16 hours after transduction with PU.1 or empty vector. Results shown are averages from

two or three independent experiments, and are given in units relative to *Actinb* expression levels in the samples. Delta = culture on OP9-DL1. Mig = culture on OP9-control ("OP9-Mig"). (C) Genes showing significant effects of PU.1 and/or interaction with Notch signaling, based on two-way ANOVA analysis.

Table S2: Compilation of actual gene expression values from all Scid.adh.2c2 perturbation experiments in Figs. 3–7. Individual worksheets (A-E) provide average values for the measured genes, in units relative to *Actinb*, from 2–4 independent experiments per analysis. (A) Gene expression effects during PU.1-mediated diversion to Mac1+, in presence or absence of GSI. Patterns of response are summarized in tabular form in main figure 3D. (B) Gene expression effects of empty vector or PU.1 together with forced Notch activation (ICN1) or forced Notch inhibition (dnMAML). A summary of distinct patterns of response is shown to the right of the columns of measured gene expression values, and referred to by "response group" number in the text. (C) Gene expression effects of PU.1 together with Id2. A summary of distinct patterns of response is tabulated on the right as in worksheet B. (D) Gene expression effects of PU.1 together with Myb. A summary of distinct patterns of response is tabulated on the right as in worksheet B. (E) Gene expression effects of PU.1 together with Gata3 shRNA. A summary of distinct patterns of response is tabulated on the right as in worksheet B.

Table S3: Primers for qRT-PCR

Chapter 4: Discussion

Numerous studies support the potential of pro-T cells to give rise to myeloid lineage cells when placed under permissive conditions (Laiosa et al., 2006b; Bhandoola et al., 2007; Bell and Bhandoola, 2008; Wada et al., 2008). This thesis argues that the presence of the multi-lineage factor, PU.1, in early T-cells helps maintain their lineage plasticity (Anderson et al., 2002b; Dionne et al., 2005). We also support the view that Notch signals from the thymic microenvironment channel the activity of PU.1 so that the T-cell lineage is chosen over diversion to a myeloid fate (Franco et al., 2006; Laiosa et al., 2006b). Until the data presented here, the transcriptional regulatory partners involved in this decision were unknown. This thesis described the roles of the transcription factors Myb and Gata3 in collaborating with Notch signaling to encourage T-cell fidelity in an environment with high PU.1. We also detailed a mechanism for shutting down Notch signaling that is driven by PU.1 and Id2 inhibition of E-proteins.

A popular method of studying the developmental potentials of progenitor cells involves sorting a certain progenitor population and then injecting the cells back intravenously in mice (Schlenner and Rodewald, 2010). The developmental potential of these cells is then assigned based on the mature/committed cell types generated by the donor cells. While these experiments are important and valuable, they could miss observing some additional developmental potential because the cells were not able to reach some critical microenvironment. For the studies presented here we used the bone marrow co-culture system OP9-Dl1 to provide a T-cell supportive environments and used the OP9-control system as a way to compare the effects of Notch signaling developing pro-T cells. It is known that signals received from cytokines are also important for the development of immune cells (Miranda et al., 2005; Schwartz et al., 2006; Gentle et al., 2012) and so it is

common practice to add IL7 and Flt3L to cultures when trying to develop T-cells in-vitro. In Chapter 2 we presented preliminary studies showing that the addition of the myeloid cytokines, MCSF and G-MCSF, to the OP9-Dl1 and OP9-control (with IL7 and Flt3L) did not change the percentages of Thy1+Mac1+ cells generated from PU.1 transduced fetal thymocytes. Mac1 up-regulation does coincide with the up-regulation of the Mcsf receptor gene, *Csf1r*, but perhaps 2 days is not enough to switch the cells dependence on IL7 and Flt3L to myeloid cytokine signaling provided by Mcsfr. Therefore, for subsequent experiments we felt it was unnecessary to add myeloid cytokines to create an environment permissive for the initiation of diversion from a T-lineage pathway to a myeloid lineage pathway.

We used the early T-cell line, Scid.adh.2c2, for much of the data presented in this thesis. The permissiveness of the Scid.adh.2c2 cell line to initiate myeloid diversion in response to PU.1 was first described in Dionne et al. (Dionne et al., 2005). There they describe another related cell line, Scid.adh.6d4 that could not up-regulate Mac1 in response to PU.1 even when no major differences in the gene expression profiles of Scid.adh.2c2 and Scid.adh.6d4 were found. This data serves as a reminder that sometimes several cell lines have to be tested before one is identified to be appropriate for the experimental questions addressed. Since the Scid.adh.2c2 cell line is a thymic lymphoma (Carleton et al., 1999b) there are some abnormalities to keep in mind when interpreting data. One such abnormality is the low but non-trivial levels of Mac1 mRNA normally expressed in non-transduced Scid.adh.2c2 cells (data not shown). Mac1 protein is not detected on the cell surface of non-transduced cells and not until 48 hours after PU.1 transduction. We do not

know what is driving this Mac1 up-regulation in cells that are devoid of PU.1 and other myeloid gene regulators.

We believe that the Scid.adh.2c2 cell line is a good system to study the changes in the regulatory gene network with high PU.1 and high or low levels of Notch signaling. The major focus in Chapter 3 was the gene regulatory changes. The highlights included the repression of *Myb*, *Gata3*, E-proteins, and Notch target genes in samples transduced with PU.1 with low Notch signaling and in both fetal thymocyte and Scid.adh.2c2 data (Chapter 3) (Dionne et al., 2005; Franco et al., 2006). In this discussion I provide some interpretations of the results as well as propose future experimental directions.

Discussion of the interactions between PU.1 and other components of the lymphomyeloid developmental switch

PU.1 and Notch signaling

It was previously described that Notch signaling could alter PU.1 activity in early T-cells and dampen its ability to inhibit T-cell genes and up-regulate myeloid genes (Franco et al., 2006). Changes in the experimental design allowed us to better separate the effects of PU.1 transduction from the effects of Notch signal deprivation during the infection process (4 hour infection protocol vs. 12 hour infection protocol). Here data describing the ability of PU.1 to inhibit Notch signaling target genes in Scid.adh.2c2 cells and fetal thymocytes was presented (see Chapter 3). The Notch signal inhibition driven by PU.1 was shown to be due in part to E-protein inhibition caused by the enhanced levels of the E-protein inhibitor, Id2, combined with the inhibition of E-proteins themselves. Recently a mechanism was presented for the repression of T potential in B-cell/natural

killer cell bipotent progenitors via Notch1 receptor inhibition driven by Id2/3 inhibition of E2A (Pereira de Sousa et al., 2012). E2A has also been shown to regulate Notch1 expression (Ikawa et al., 2006; Yashiro-Ohtani et al., 2009). In our studies the enhanced diversion seen by ID2 and PU.1 co-expression was overcome by the addition of ICN. The up-regulation of *Id2* and severe repression of E proteins is not detected until the cells have been transduced with PU.1 for 48 hours (later than some gene changes that seen with only 16 hours after PU.1 transduction). This suggests that Notch signaling inhibition via E-protein inhibition may not be the gatekeeper of diversion, but rather a lockdown mechanism used to keep Notch signaling down once the cells have made the decision to divert. We attempted to repeat these types of experiments in fetal thymocytes where the control of Notch signals is made simpler by the use of the OP9-stromal cell line system, but we could not successfully detect enough co-transduced cells to accurately interpret the results. E-protein studies have been notoriously difficult because of devastating survival effects when they are over-expressed. Knockdown studies are complicated by the interference of redundant E-proteins found in the cells. Nonetheless we would like to try a co-transfection experiment with PU.1 and an E47 construct whose level of expression can be controlled chemically via a tamoxiphen inducible promoter (Schwartz et al., 2006). We predicate a reduction in the percentage of Mac1+ cells with PU.1 and E47 compared to PU.1 alone. We also hypothesize to see a reduction in the inhibitory effect of PU.1 on Notch target genes, but not necessarily on T-cell regulatory genes like *Tcf7*, *Myb*, or *Gata3*. Finally, there is some evidence that PU.1 could also be inhibiting Notch signaling through other mechanisms like regulation or repression of the components of the Notch signaling machinery (Ch3: Fig. 3C and 6D- *Psen2*). Additionally, PU.1 binding sites have been

identified up-stream of a Notch1 regulatory region although direct regulation of Notch1 by PU.1 has not been shown (Zhang et al., 2012).

We also detected the differential ability of Notch signaling to block Mac1 up-regulation but not CD11c up-regulation. PU.1 can drive the up-regulation of the dendritic cell marker, CD11c, but Notch signaling does not inhibit its up-regulation in fetal thymocytes or Scid.adh.2c2 cells (Chapter 2: Fig. 11; Chapter 3: Fig. 4). This suggests that Notch signaling can protect against diversion to a macrophage program but not a dendritic cell program. More extensive characterization of the CD11c+ cells that were generated is required for classification of these dendritic type cells. These results are physiologically relevant considering data showing that Notch signaling regulates the development of a new type of Thy1-expressing dendritic cell in the thymus (Ishifune et al., 2011).

PU.1, Myb and GATA3

Myb and PU.1 co-expression decreases the percentage of cells that divert compared to PU.1 alone (Chapter 3: Fig. 6A, B). *Myb* down regulation is part of the diversion process, although the protection of *Myb* does not elicit the same strong protection response mediated by Notch signaling. Gene expression analysis of these cells showed that introduction of Myb to PU.1 over-expressing cells generally lessened the extent to which some genes were inhibited (e.g., *Rag1*, *Aiolos*, *Psen2*, *Nrarp*). A striking exception to this was the T-cell transcription factor Gata3. *Gata3* levels were up-regulated in Myb expressing cells with or without PU.1 (Chapter 3: Fig. 6D). We later showed that Myb also protected Gata3 protein levels from PU.1-driven inhibition (Chapter 3: Fig. 6G). The protection of Gata3 levels was shown to be important in Gata3 knockdown experiments because this increased the percentage of Mac1+ Scid.adh.2c2 cells after PU.1 transduction.

Myb has been shown to repress key myeloid genes like *C/EBPβ, JunD, Runx1* and PU.1 in Myb ChIPseq studies using a myeloid cell line (Zhao et al., 2011). However, it is not clear how Myb is protecting *Gata3* from PU.1 in Scid.adh.2c2 cells where high levels of Notch signaling do not seem to be inhibiting the expression of these factors (such as Runx1). Samples with Myb alone had increased *Gata3* levels and Myb could be driving *Gata3* expression directly or indirectly and in this way be supporting T-cell lineage fidelity. The ability of Myb to regulate *Gata3* expression has not been previously shown in early T-cell, but has been described in more mature cells. In Th2 T-cells, a complex composed of Myb, Gata3, and Menin (men1), is used to regulate Gata3 expression (Nakata et al., 2010). *Myb*, *Gata3*, and *men1* are all expressed in Scid.adh.2c2 cells and could therefore also be playing a role in *Gata3* regulation (*JZ and EVR unpublished data*). Although Myb and PU.1 have been shown to cooperate in the activation of a neutrophil gene, their direct interaction has not been shown (Oelgeschläger et al., 1996) and therefore there is no published evidence that suggests Myb could be inhibiting PU.1 activity in early T-cells via direct interaction.

We have shown that *Gata3* is important in the myeloid vs. T-cell lineage decision during early T-cell development, but the mechanism/s through which this occurs remain to be discovered. Gata3 binding sites near *Myb, Tcf7, E2a, Gfi1, Runx3,* and *Zfpm1* regulatory regions in both Scid.adh.2c2 and primary cells have been detected (M.M.DR and EVR, unpublished data) (Zhang et al., 2012). Knockdown of Gata3 using and shRNA did not lead to the inhibition of any of the previously mentioned proposed Gata3 target genes, except for *Runx3* (Ch. 3: Fig. 7E). Gata3 knockdown in combination with PU.1

expression mostly amplified the gene expression changes driven by PU.1. One idea for the driving mechanism of this was that Gata3 was further inhibiting Notch signaling by its up-regulation of *Id2*. This idea was supported by the fact that *Lyl1* expression is lowered in PU.1 samples with Gata3 knockdown compared to PU.1 alone. This suggested lowered levels of Notch signaling since we showed that PU.1 could not turn on *Lyl1* as well when Notch signaling is low (Ch.3: Fig. 4B). However, this hypothesis seems less likely when one notes that *Csf1r* and *Bcl11a*, which also depend on the status of Notch signaling for their expression, are not affected by the knockdown of Gata3 (Ch. 3: Fig. 7E). We would also expect to see a reduction of Notch target genes when Id2 is up-regulated in the Gata3 knockdown samples, but this is not detected. It could be beneficial to perform a Gata3 ChIPseq experiments in Scid.adh.2c2 cells transduced with PU.1 or an empty vector to detect if PU.1 has the ability to influence Gata3 DNA binding. This could help us identify candidate genes that could explain how PU.1 is interfering with the ability of Gata3 to support T-cell development.

Changes in gene expression can also be driven by modifications of chromatin structure that can either: allow access of regulatory factors to DNA regions thus allow gene activation or deny access of regulatory factors to DNA regions and in that way mediate repression of those genes (Budd, 2012). PU.1 and Gata3 have both been shown to reorganize chromatin structures which influence developmental decisions (Lee et al., 2001; Stopka et al., 2005). PU.1 marks the enhancers of macrophage regulatory regions and is able to partially recreate an H3K4me1 macrophage pattern in fibroblasts (Ghisletti et al., 2010). Performing histone modification ChIPseq studies on cells expressing PU.1 with

high or low levels of Notch signaling could show that there is a difference in chromatin modification that could help explain the ability of Notch signaling to modify PU.1 activity.

In Ch.2 we presented some preliminary data the MAP kinase signaling could also be protecting Scid.adh.2c2 cells from diversion. There is evidence that investigating this complex question may prove fruitful. For example, MAP kinases have been shown to positively regulate *Gata3* in Th2 T-cells and interestingly, Myb is required for this regulation to occur (Gimferrer et al., 2011a). It could be the case that something driven by a MAP kinase pathway, separately or in conjunction with Notch signaling, is protecting *Gata3* from PU.1-driven inhibition. A simple experiment to test this hypothesis would be to transduced Scid.adh.2c2 cells with PU.1 in the presence or absence of MAP kinase inhibitors and perform Gata3 intracellular staining to detect changes in Gata3 protein levels. If the MAP kinase pathway is involved in T-cell lineage fidelity against PU.1, then we would expect to see an increased reduction of Gata3 protein levels in samples with PU.1 and the MAP kinase inhibitor compared to cells with PU.1 alone.

Finally, although Gata3 over-expression in our hands did not inhibit Mac1 up-regulation in PU.1 co-transduction experiments, Gata3 over-expression in Thy1- cells (DN1 cells) dramatically down-regulated the expression of an important myeloid cytokine receptor, Mcsfr (Anderson et al., 2002a). The level of Gata3 expressed in these experiments may be too high and we know that this can be toxic to the cells as well as have the ability to divert pro-T cells to a mast cell fate (Taghon et al., 2007). The use of a Gata3 retroviral vector whose expression is tightly controlled chemically may provide different results. However, it is true that global Gata3 binding studies show that even when Gata3 protein levels remain fairly consistent, the binding pattern of Gata3 is highly stage specific.

This could mean that a collaborator of Gata3 that is differentially expressed during T-cell development could be playing a huge role in the results obtained from Gata3 over-expression studies.

PU.1 and unknown partners

Previous studies using Scid.adh.2c2 showed that a mutation in the transactivation domain of PU.1 lowers the generation of Mac1+ cells by nearly half compared to wild-type PU.1 (Dionne et al., 2005). The PU.1s dependence on co-factors for its ability to act in cell specific ways has been well documented (Behre et al., 1999; Ji et al., 2008). For example, Runx1 cooperates with PU.1 during macrophage development by inhibiting the ability of PU.1 to interact with co-repressors, such as Eto2 (Hu et al., 2011). There is the possibility that a co-repressor required by PU.1 is inhibited by Notch signaling. If this were to occur the result would be the inability or decreased ability of PU.1 to repress T-cell regulatory genes. PU.1 co-immunoprecipitation experiments with subsequent mass spectrometry could reveal differential binding partners between cells that were cultured in high vs. low levels of Notch signaling.

Evidence presented here shows that PU.1 is mediating the repression of Notch target genes and important T-cell regulatory genes (Ch. 3). Ongoing studies by Dr. Ameya Champhekar (Postdoctoral scholar at Caltech) suggest that PU.1 is not mediating these effects directly, but rather indirectly via an unidentified repressor. In these studies, fetal thymocytes are infected with a construct expressing PU.1 fused with the Drosophila Engrailed repression domain such that PU.1eng can bind its target genes, but only mediate their repression. Comparing the gene expression changes that occur with PU.1eng and normal PU.1 can help clarify if PU.1 is repressing these genes directly or via a target gene.

For example, if PU.1 were repressing gene X directly, then PU.1eng and normal PU.1 would both drive the inhibition of gene X. If gene X was repressed by a transcription factor turned on by PU.1 (PU.1 is indirectly repressing gene X), then we would expect normal PU.1 to inhibit gene X, while PU.1eng may cause increased gene X expression. This is because PU.1 is not able to turn on the direct repressor of gene X. In this way Dr. Champhekar has shown that most of the inhibitory gene expression effects on Notch target gene and T-cell regulatory genes are indirect (*AC and EVR unpublished data*). *Gata3*, *Myb*, and *Tcf7* are included in those thought to be indirectly repressed by PU.1. Notch signaling could in fact be blocking the ability of PU.1 to activate this mysterious repressor and in that way modify the activity of PU.1 during early T-cell development so that these important T-cell genes are not inhibited.

Suggestions for additional studies

Aspects of the studies presented here would benefit from further study and several of these have been discussed in the previous sections. Here I would like to propose additional experiments that could enhance the understanding of lymphoid vs. myeloid decisions during early T-cell development.

Many of the experiments presented in the results section of this thesis used the Scid.adh.2c2 system as a model for early T-cells. Although the factors that were the major focus of the proposed network, Myb and Gata3, acted in a similar fashion in fetal thymocytes and Scid.adh.2c2 cells, it would enhance the results of this study if we repeated the double transduction experiments in the primary cells. Several of these double transduction experiments with PU.1 and another transcription factor were attempted (*data not shown*), but failed to generate adequate numbers of doubly transduced cells and

therefore the interpretation of the results was complicated and unreliable. Often the problem faced with these experiments was not low infection rates, but poor survival of the fetal thymocytes over-expressing two factors simultaneously. It may be beneficial to clone the transcription factors into an inducible retroviral system where the levels of over-expression can be controlled by the addition of a chemical activator. For example, the tamoxifen inducible estrogen receptor system has been used successfully to control the over-expression levels of transcription factors like PU.1 (Laslo et al., 2006).

Previous morphological studies of fetal thymocytes 4 days after PU.1 transduction showed that Mac1+ cells resembled macrophages while PU.1 transduced Mac1- cells continued to resemble T-cells (Dionne et al., 2005). This thesis contained studies looking at gene expression and phenotypic changes of fetal thymocytes transduced with PU.1 for no more than 3 days. At this point the cells were not tested for the generation of functional macrophages because of the early time points. Functional studies of fetal thymocytes that up-regulate Mac1 could provide additional evidence that we are detecting pro-T cell divergence to a myeloid fate and not just a studying Mac1 (*Itgam*) up-regulation. These functional studies could use fluorescent bacterial particles to test the ability of PU.1+Mac1+Thy1+ cells to perform phagocytosis.

The importance of microRNAs in development has been shown in numerous cell types and at all stages of maturity (Eisenberg et al., 2009; Bonev and Papalopulu, 2012; Dumortier and Van Obberghen, 2012). MicroRNA function in early T-cell development is unclear and not widely studied, but mir182 and mir150 are expressed in and important for mature T-cells (Stittrich et al., 2010; Ghisi et al., 2011). PU.1 is heavily involved in microRNA expression during myeloid development (Rosa et al., 2007; Ghani et al., 2011).

In multipotent progenitor cells PU.1 can drive the expression of the mir-23a cluster, which inhibits B-cell development in favor of myeloid development (Kong et al., 2010). Antagonistic effects of microRNAs on PU.1 in mature macrophages have also been published (Ponomarev et al., 2011). There is the possibility that microRNAs also play a role in the lineage fate choices during early T-cell development. There are several methods used for the detection of microRNA expression including microarrays and Q-PCR (Wark et al., 2008) that could be used to detect differences in microRNA expression in samples with high PU.1 and low or high levels of Notch signaling.

In Chapter 3 we described data showing that co-transduction of PU.1 and Gfi1 increased the percentage of Mac1+ cells in fetal thymocytes and Scid.adh.2c2 cells. This result was surprising given that studies featuring Gfi1 and PU.1 are usually about how they antagonize each other's functions. Gfi1 has been shown to antagonize PU.1 via protein-protein interaction (Dahl et al., 2007) and can displace PU.1 from PU.1 regulatory targets (Spooner et al., 2009). Also, Gfi1 in collaboration with C/EBPα supports neutrophil development at the expense of PU.1-driven macrophage development (Laslo et al., 2006; Laslo et al., 2008). In order to try and understand what is happening in our PU.1 and Gfi1 co-transduction studies we could first start with comparing the gene expression changes in cells transduced with PU.1 or Gfi1 alone vs. cells expressing both PU.1 and Gfi1. We would be interested in identifying changes in Notch target genes and T-cell regulatory genes, especially *Myb* and *Gata3*. In Chapter 3 we described a sharp decrease in Gata3 protein levels in samples that up-regulated Mac1 in response to PU.1 transduction. Gfi1 has been shown to decrease the ubiquitination of Gata3 protein in Th2 T-cells (Shinnakasu

et al., 2008) and it would be interesting to do a comparison of Gata3 protein levels and Gata3 protein modifications in PU.1 and Gfi1 transduced cells.

Finally, it has been shown that the regulation of PU.1 binding to gene regulatory regions in B cells and myeloid cells depends on the expression of lineage exclusive factors such as C/EBPβ, E2A, and EBF (Heinz et al., 2010). At physiological levels, PU.1 seems to bind similar regulatory regions in the presence of high and low levels of Notch signaling (Heinz et al., 2010; Zhang et al., 2012). However, the regulatory targets in PU.1-transduced cells could be different than when PU.1 is at physiological levels. A direct way of exploring these topics would be to do chromatin immunoprecipitation using a PU.1 antibody (with subsequent q-RTPCR of genome wide sequencing) in samples transduced with PU.1 and cultured with high or low levels of Notch signaling. The levels of Notch signaling would be better controlled by retroviral expression of ICN or dnMAML (see Ch.3) vs. Notch signaling inhibition by GSI. The identification of regulatory regions with differential PU.1 binding could identify additional candidates that could be involved in the lymphomyeloid lineage decision regulatory network.

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Appendix A:

Exploration of interactions between Bcl11b, PU.1, and Lyl1 in an early T-cell line

Abstract

T-cell development is driven by the combinatorial expression of transcription factors that are often important for the development of other blood cell types. *PU.1* and *Lyl1* are expressed in the earliest intrathymic T-lineage precursors and although they are required for T-cell development, they have also been implicated in maintaining alternative cell lineage options in these early cells. The transcription factor Bcl11b is expressed later in development and has been shown to prevent cells from adopting a natural killer cell fate. In these preliminary studies we explored the effects of the combinatorial expression of these factors in relationship to lineage decisions and to gene expression changes in a DN3-like cell line. We report that neither Lyl1 nor Bcl11b affected the ability of PU.1 to drive myeloid diversion. We also found that while effects of Lyl1 were marginal when expressed alone, when co-expressed with PU.1 or Bcl11b, Lyl1 was more potent in mediating gene expression changes. Gene expression analysis of PU.1 and Bcl11b co-expression showed that both of these factors were able to inhibit some of the key gene expression effects achieved when they are expressed alone.

Introduction

Early T-cell development is driven by the combinatorial expression of several transcription factors and most of these factors are also important for the development of other blood cell types (Rothenberg, 2012). Early T-cell progenitors are identified by their lack of the mature T-cell markers CD4 and CD8 and thus are termed double negative (DN). The DN stages can be simply characterized by their cell surface expression of Kit, CD44 and CD25 (DN1 through DN4), although these populations can be have further subdivided using other cell surface markers (Yui et al., 2010). For the studies presented here we used

the early T-cell line, Scid.adh.2c2, (which resembles a DN3 staged cell) to ask questions about the interactions between PU.1, Bcl11, and Lyl1 in relation to gene expression and T-lineage fidelity.

The zinc finger transcription factor Bcl11b is required for early T cell development and initially not expressed in the earliest DN1 stage, but sharply up-regulated at the DN1 to DN2 transition where it remains highly expressed in all the subsequent stages of development (Tydell et al., 2007; David-Fung et al., 2009; Li et al., 2010a). Although Bcl11b is also expressed in several cell types like skin cells (Liang et al., 2012), neuronal cells (Arlotta et al., 2005), tooth enamel-forming cells (Golonzhka et al., 2009), brain cells (Simon et al., 2012), and leukemic cells (Go et al., 2012), among all blood lineages, it is uniquely expressed in T cells. While a low-level expression of Bcl11b is detected in some natural killer (NK) cells (Liu et al., 2010), the expression of Bcl11b does not seem to be necessary for the development of the NK lineage. Most Bcl11b studies related to hematopoiesis have been knock-out/knock-down studies and Liu et al. provides a good review of these results (Liu et al., 2010). Briefly, Bcl11b deficient cells show increased proliferation and have a developmental block at the DN2 stage. Bcl11b deficient cells fail to turn off many progenitor/stem cell genes and generate an increased percentage of NK cells. Bcl11b is mostly characterized as a repressor, but has also been documented as an activator of transcription (Cismasiu et al., 2006). ChIPseq studies in neuronal cells have identified Bcl11b target genes (Tang et al., 2011), but Bcl11b target genes in T-cells remain elusive. The Bcl11b over-expression studies presented here agree with some of the gene expression changes seen in previously reported knockdown studies; such as the effects of Bcl11b on the progenitor/stem cell genes *Bcl11a* and *Lyl1*. In addition, we describe

differential gene expression changes caused by the exogenous expression of 4 different isoforms of Bcl11b. These results support data suggesting that the Bcl11b exon 2 is needed to mediate repression of its target genes.

In this appendix we also test the hypothesis that Bcl11b is involved in blocking the potential for pro-T cells to divert to a myeloid lineage during early development. In the presence of Notch signaling an Ets family transcription factor, PU.1, supports T-cell development (Spain et al., 1999), but in the absence of Notch signaling, PU.1 can divert early T cells to myeloid fate (Ch.3) (Franco et al., 2006; Laiosa et al., 2006b). Gene expression analysis of a DN3-like thymoma cell line, Scid.adh.2c2, used to study the interactions of PU.1 and Notch signaling, showed that Bcl11b is down-regulated in cells that up-regulate the myeloid marker Mac1 (Ch. 3: Fig. 3C). Bcl11b has been shown to be important for maintaining T-cell lineage fidelity and so we tested its ability to block Mac1 expression in PU.1-transduced cells. Here we show that Bcl11b cannot block PU.1 mediated up-regulation of Mac1 in Scid.adh.2c2 cells, but surprisingly has the ability to inhibit Notch target genes. Interestingly, we also detected the ability of Bcl11b to block the PU.1-driven up-regulation of the stem/progenitor cell genes *Lyl1* and *Bcl11a*.

Finally we describe gene expression changes caused by the stem/progenitor cell gene, *Lyl1*, in Scid.adh.2c2 cells co-expressing PU.1 or Bcl11b. Lyl1 is a basic-helix-loop-helix transcription factor expressed in myeloid cells, B cells, and important for the maintenance of adult hematopoietic stem cells (Souroullas et al., 2009). Zohren et al also showed that Lyl1 plays an important role for the maintenance/survival of uncommitted precursors in the thymus. Lyl1 has been reported to form heterodimers with E proteins and these can either act as activators or inhibitors of transcription (Massari and Murre, 2000;

Kee, 2009). During early T cell development, *Lyl1* is expressed during the earliest stages before commitment, but is abruptly shut off at the DN3 stage (Yui et al., 2010; Zohren et al., 2012). As we show in Ch.3, *Lyl1* can be up-regulated by PU.1 transduction. We therefore wanted to explore the possibility that Lyl1 expression could prime cells for PU.1-driven diversion. We found that Lyl1 did not enhance Mac1 up-regulation when co-expressed with PU.1. We also found that effects of Lyl1 on gene expression in Scid.adh.2c2 cells were modest and mostly inhibitory effects on Notch target genes. When expressed in combination with PU.1 or Bcl11b these effects were largely enhanced beyond the levels of PU.1 or Bcl11b alone.

Results

Gene expression changes in Scid.adh.2c2 cells transduced with different Bcl11b isoforms

The Bcl11b gene is made up of 4 exons, with exon 4 contains all 6 zinc finger domains of the Bcl11b protein as well as the putative DNA binding site (Kominami, 2012), while exons 1, 2 and 3 contain domains most likely used for interactions with other proteins. Bcl11b was first described as having 3 isoforms: the full-length (FL) isoform with all 4 exons, another isoform with exons 1-2-4, and a 1-4 isoform (Sakata et al., 2004). Full length Bcl11b (1-2-3-4) appears to represent a minority of Bcl11b protein, but is co-expressed in T-cells with the 1-2-3 form. The 1-4 form is also generated in-vivo and may be associated with leukemia (Go et al., 2012). The 1-2-4 Bcl11b isoform was found to be the most highly expressed isoform in progenitor T cells (L.L*, E.V.R unpublished data*), however differences in the ability of these isoforms to mediate gene expression changes have yet to be explored.

In order to describe the effects of Bcl11b transduction and to test the role that different exons may play in driving gene expression, we used retroviral constructs with different combinations of Bcl11b exons for over-expression experiments. All of the Bcl11b isoforms used contained exon 4 and include 1-2-3-4 full length, 1-2-4, 1-3-4, and 1-4 isoforms. These different isoforms were singly transduced into Scid.adh.2c2 cells for 2 days. The cells were then sorted for their expression of the retroviral vector and their RNA was analyzed using qRT-PCR. A heatmap generated from 2 or 3 independent experiments showed that effects on Scid.adh.2c2 gene expression with Bcl11b transduction did depend on the particular isoform that was used (Fig. 1).

Zeb2 and *Gfi1b* were up-regulated by all Bcl11b isoforms, although this occurred at differing intensities with the 1-4 isoform giving the weakest response. *Egr3* was inhibited in all isoforms with the 1-4 isoform giving the strongest response. For most of the gene expression responses the isoforms paired well according to their inclusion of exon 2; meaning that the 1-2-3-4 and 1-2-4 isoforms were more similar to each other than the 1-3-4 and 1-4 isoforms. Exon 2 seemed to be required for the inhibition of *Runx3*, *Hes5*, and *Il7r* as well as for the up-regulation of *Pou6f1* and *Aiolos*. While there were some changes in expression with the 1-3-4 and 1-4 isoforms only, the most dramatic was their down-regulation of *Id2* and the up-regulation of the Tgfb pseudo receptor, *Bambi*, by the 1-3-4 isoform. Even though many of the gene expression changes in the 1-2-3-4 and 1-2-4 samples were similar, the different isoforms were also able to differentially affect gene expression. In general, the 1-2-3-4 Bcl11b isoform up-regulated genes while the 1-2-4 isoform inhibited them. For example, *Notch3, Bambi, Ptcra, Lef1, E2a*, and *Myb* were all up-regulated with the full length isoform compared to the decreased up-regulation or no up-

regulation with the 1-2-4 isoform. The genes down-regulated by the 1-2-4 isoform include many Notch target genes like *Dtx1, Nrarp, Notch1*, and *Tcf7*. These results suggest that exon 2 is important for most of the gene expression changes, especially those where repression is seen. There is published data suggesting that the first 45 amino acids of Bcl11b (encoded by exon2) are important for mediating its repressive effects because they were found to bind a classic repression complex, NuRD (Cismasiu et al., 2005). The lack of Notch target gene down-regulation in the 1-2-3-4 isoforms suggests that Bcl11b exon 3 maybe block the ability of exon2 to recruit NuRD.

Gene expression effects of Bcl11b and PU.1 co-expression in Scid.adh.2c2 cells

T-cell commitment occurs during the DN2 stage of T-cell development (Yui et al., 2010). PU.1 is repressed during this stage and this is required for T-cell development to continue properly because if miss-expressed it could divert the cells away from the T-lineage (Anderson et al., 2002b; Laiosa et al., 2006b). Bcl11b has the opposite expression pattern of PU.1 as it is sharply up-regulated at the DN2 stage, but Bcl11b has also been shown to influence cell fate choices (Tydell et al., 2007; Li et al., 2010a). The importance for the temporal separation of these two factors during early T-cell development have not been explored. We were interested in looking at the gene expression analysis of cells co-transduced with PU.1 and Bcl11b to document any interference with the gene expression effects of the factors when they are expressed alone. To achieve this we co-transduced PU.1 and Bcl11b in Scid.adh.2c2 cells and cultured them for 2 days before sorting cells positive for both retroviral vectors as well as cells transduced with only one of the retroviral vectors. The sorted samples were prepared for RNA analysis using qRT-PCR and bar graphs are used to display the singly vs. doubly transduced cells while (Fig. 2A) a heatmap

was generated using the results obtained from the doubly infected cells (Fig. 2B). We found that the gene expression profile of single infected populations (Bcl11b only or PU.1 only) was, as expected, similar to the expression profile of cells doubly infected with a retroviral construct expressing a transcription factor and another expressing an empty vector. Note that because Scid.adh.2C2 cells do not express PU.1, and it was necessary for PU.1 expression to be induced using a retroviral vector, we could not use this system to assay for any repression activity of Bcl11b on PU.1 itself. Also, Bcl11b was approximately 10x over-expression in the transduction experiments compared to normal levels of Bcl11b expressed in Scid.adh.2c2 cells.

Like in the previous experiments, Bcl11b alone was able to shut down several genes (*Dtx1, Notch1, Runx3, Hes5*, and *Egr3*) with or without the co-expression of exogenous PU.1. As many of these genes are Notch targets (see Ch.3), this implies that high-level Bcl11b, like PU.1, can interfere with the Notch pathway. However, Bcl11b also up-regulated select genes that are normally induced in DN3 stage, including *Id3* and *Notch3*. Up-regulation of genes by Bcl11b was mostly enhanced by PU.1 co-expression (*Zeb2*, *Scl*, *Id3*). Interestingly, Bcl11b was able to block PU.1 from driving both up-regulation and down-regulation of genes (Table 1). PU.1 was unable to inhibit *Id3* and *Smad3* or to enhance *Lyl1* and *Bcl11a* expression in the presence of high Bcl11b. This suggests that Bcl11b is playing an important role for multiple aspects of DN development. Bcl11b can antagonize the ability of PU.1 to turn on a progenitor or stem cell program by blocking the PU.1-driven up-regulation of *Lyl1* and *Bcl11a* (Fig. 2A, B). Bcl11b is also inhibiting the ability of PU.1 to down-regulate genes needed during the DN3 stage that are

important for the β-selection checkpoint (such as *Id3* and *SpiB*). Since Bcl11b has the ability to block the ability of PU.1 to mediate gene expression changes potentially important for myeloid vs. lymphoid lineage decisions we next wanted to explore the ability of Bcl11b to block Mac1 up-regulation in PU.1-tranduced Scid.adh.2c2 cells.

Bcl11b does not protect against PU.1 mediated diversion

In Bcl11b knockout mice it was shown that T-cell progenitors had Natural Killer cell potential, even in the presence of Notch signals (Ikawa et al., 2010; Li et al., 2010a; Li et al., 2010b). These cells could also be encouraged to adopt a myeloid-like identity if cultured with myeloid cytokines in the absence of Notch signals. Here we were interested in testing if Bcl11b and PU.1 co-expression in Scid.adh.2c2 cells protect them from PU.1-driven diversion. We co-transduced Scid.adh.2c2 cells with PU.1 and the FL (1-2-3-4) or 1-2-4 isoforms of Bcl11b and cultured them for 2 days before measuring Mac1 and CD25 expression using flow cytometry. Once again we detected lower levels of the Notch target gene, CD25, in the samples over-expressing Bcl11b-1234 and Bcl11b-124 (Fig. 1; bottom 2 left and right panels), a response that is often a symptom of Notch pathway inhibition. However, we found that co-expression of Bcl11b-1234 or Bcl11b-124 with PU.1 did not change the percentage of cells that up-regulated Mac1 compared to cells co-transduced with PU.1 and an empty vector (Fig. 3). Co-transduction experiments with PU.1 and Bcl11b in fetal thymocytes, cultured in the presence or absence of Notch signaling, were difficult to analyze because most of the doubly transduced cells did not survive (*data not shown*). When fetal thymocytes transduced with Bcl11b only were cultured in the absence

of Notch signaling the percentage of cells expressing Mac1 was similar to the percentage in the empty vector controls (*data not shown*).

The Bcll1b results discussed in the previous sections are somewhat less complicated to interpret because of their reproducibility, larger data set and our ability to compare them to Bcl11b knockdown studies. The results discussed in the following sections describe experiments done to analyze interactions between the progenitor/stem cell gene Lyl1 and the two factors already discussed, PU.1 and Bcl11b. These results were generated from a limited number of experiments and this makes their interpretation complicated, but they are included here as interesting preliminary data.

Lyl1 does not increase PU.1-driven Mac1 up-regulation in Scid.adh.2c2 cells

PU.1 and Lyl1 expression is carried over from multipotent progenitors that seed the thymus and are expressed when progenitor T cells still retain lineage plasticity. Lyl1 is one of the factors that are enhanced in response to PU.1 over-expression in both Scid.adh.2c2 cells and fetal thymocytes (Ch. 3: Fig. 2D, Fig. 3C). ChIPseq studies have revealed two sizeable PU.1 binding sites: one around the *Bcl11a* promoter-proximal and another just downstream of the 3'UTR (Zhang et al., 2012). In Chapter 3 we described how the antagonism of E-proteins by Id2 was able to increase the percentage of Mac1+ cells in PU.1-transduced Scid.adh.2c2 cells as well as drive the inhibition of Notch target genes (Chapter 3: Fig. 5A and B). Lyl1 can also bind to E-proteins and as such has the potential to antagonize their actions (Miyamoto et al., 1996; Zhong et al., 2007). Since we know that Lyl1 is expressed in cells that still retain alternate lineage potential (from the DN1 to DN2 stages of development), we were interested in exploring the possibility that co-expression of Lyl1 with PU.1 could further promote myeloid diversion of Scid.adh.2c2 cells.

Scid.adh.2c2 cells were co-transduced with Lyl1 and PU.1 and cultured for 2 days. We found that the percentage of Mac1 positive cells was not increased with Lyl1 and PU.1 co-expression compared to PU.1 expression alone (data not shown). High levels of Notch signaling block diversion in response to PU.1 (Franco et al., 2006). If Lyl1 up-regulation were important for diversion we would expect it to be blocked by high levels of Notch, but we failed to detect a Notch-mediated block in Lyl1 up-regulation. Supporting evidence that Lyl1 may not be involved in the diversion response comes from the observation that Lyl1 expression is increased in the presence of high PU.1 and high Notch signaling (Ch. 3: Fig. 4B). Although Lyl1 did not seem to be involved in enhancing diversion in these Scid.adh.2c2 cells, we were interested in probing interactions between PU.1, Bcl11b, and Lyl1that could be occurring during early T-cell development.

Gene expression effects of PU.1 and Lyl1 co-expression in Scid.adh.2c2 cells

We know that Lyl1 does not enhance diversion in Scid.adh.2c2 cells when co-expressed with PU.1, but it may be collaborating with PU.1 to mediate other gene expression changes within the early T-cell program. Scid.adh.2c2 cells serve as a model system for cells that are more restricted to the T-cell lineage and have already turned off their own endogenous *Lyl1* as well as PU.1, and so we transduced PU.1 and Lyl1 along with empty vector or with each other in Scid.adh.2c2 cells to try and detect possible interactions. After transduction we cultured the cells for 2 days and then sorted them according to their co-expression of PU.1 and Lyl1. Gene expression analysis using qRT-PCR was performed on these samples and a heatmap was generated using these results (Fig. 4).

Lyl1 over-expression caused mostly moderate changes in the gene expression profiles of Scid.adh.2c2 cells with the majority of these changes being inhibitory. We detected the down-regulation of several Notch target genes (*Il2ra* (CD25), *Dtx1*, *HEBalt*, and *Ptcra)*. This data suggests that Lyl1 over-expression may be saturating E-protein binding. Inhibition of E-protein activity by Id2 has also been shown to repress Notch target genes (Ch.3)(Pereira de Sousa et al., 2012). The Notch target gene down-regulation by Id2 over-expression was stronger than the results obtain by Lyl1 suggesting that the Notch target gene inhibition was weaker with Lyl1. Unlike Id2, however, Lyl1 mediated the repression of *Runx3*, *Id2*, and *Il7r* as well as the up-regulation of *Il2rb* (Fig.4).

PU.1 over-expression slightly up-regulated the E-protein inhibitor, *Id2*, and more robustly up-regulated *Bcl11a*. *Bcl11a* is another 'progenitor' gene expressed at the same time as PU.1 and *Lyl1*. In Ch.3 we showed that Id2 blocked the ability of PU.1 to up-regulate *Bcl1a*, however, PU.1 and Lyl1 co-expression did not have the same effect and PU.1 was able to up-regulate *Bcl11a* (Table 2). PU.1 and Lyl1 inhibited the expression of several genes when they were expressed apart or together. However, their inhibition was greater in the PU.1 alone samples and the samples expressing PU.1 and Lyl1 together compared to the samples with Lyl1 alone. Notch target genes (*Ptcra, Dtx1, Hes5*) and genes important for T cell development (*HEBalt, Tcf7, Rag1, Myb*) were included in this group (Table 2). Several of these genes were tested for their involvement in the diversion response to PU.1 over-expression and the results suggested that they were not acting along with or downstream of PU.1 to drive diversion (Ch. 3: Fig. S4B, D).

Gene expression effects of Bcl11b and Lyl1 co-expression in Scid.adh.2c2 cells

Lyl1 is expressed earlier than *Bcl11b* at a time when the cells naturally retain lineage

plasticity. Since experiments that knock-down Bcl11b in early thymocytes show that progenitor/stem cell genes like *Lyl1* failed to properly shut off (Li et al., 2010a), we wanted to investigate gene expression changes in Scid.adh.2c2 cells co-expressing Bcl11b and Lyl1. Bcl11b and Lyl1 were co-transduced in Scid.adh.2c2 cells for 2 days and the double transduced cells were sorted for qRT-PCR analysis. A heatmap was generated from this data (Fig. 5). Table 3 summaries the responses to co-expression and several of the gene changes were similar to the results in the previous section such that co-expression of Lyl1 increased the inhibition of genes. The expression of early T cell genes *Runx3, Il7ra, Hes5, Il2ra, Tcf7*, and *Dtx1* are inhibited in samples containing Lyl1 and Bcl11b alone, but are more strongly inhibited in samples co-expressing both factors. Note that several of these genes are Notch target genes (*Dtx1, Hes5, Il2ra*). The progenitor cell gene, *Gfi1b,* goes up with Bcl11b knockdown in 'DN2a' like cells (Li et al., 2010a), but in these samples Gfi1b also goes up with Bcl11b over-expression. However, the presence of Lyl1 slightly inhibits the ability of Bcl11b to up-regulate *Gfi1b*. The ability of Bcl11b to up-regulate *Notch3* (and to a lesser extent *Gfi1*) is also inhibited by Lyl1 co-expression. Alternatively, the ability of Lyl1 to mediate the up-regulation of *Il2rb* is inhibited by Bcl11b. Possible interpretations of this data are discussed below.

Discussion

The experiments described in this appendix are preliminary studies that can serve as a foundation for further/future research. It may be difficult to draw conclusions from several of the data sets included here because of the limited number of independent experiments, but in this section we will discuss this data as it would relate to more complete data sets.

Lyl1 over-expression moderately inhibited Notch targets genes possibly via driving E-protein inhibition. In Ch. 3 we showed that PU.1-driven *Bcl11a* up-regulation is enhanced by high levels of Notch signaling and that Id2 was able to block this up-regulation; possibly by lowering the levels of Notch signaling in the cells. However, unlike the E-protein inhibition caused by Id2, Lyl1 was not able to block PU.1-driven *Bcl11a* up-regulation. Possible explanations for these results are that Lyl1 is a weaker inhibitor of E-proteins compared to Id2 or that the Lyl1 levels generated in the cell are not sufficient to inhibit the high levels of E-proteins in the Scid.adh.2c2 cells. Here we also showed the inability of Lyl1 to enhance Mac1 up-regulation in response to PU.1 transduction in Scid.adh.2c2 cells and this will probably be shown to be true for thymocytes as well. After these experiments were performed we found that Scid.adh.2c2 cells co-transduced with PU.1 and the intracellular Notch (ICN) expressed Lyl1 at a high level (Ch. 3: Fig. 4B). It is unlikely that a factor important for the diversion of the cells to be greatly enhanced when PU.1 and Notch signaling are both expressed at high levels. Additionally, several of the effects of Lyl1 in Scid.adh.2c2 cells involved reduction of important T-cell genes, such as *Tcf7* and *HEBalt*, which are not involved in blocking diversion (Ch. 3: Fig. S 4B,D), but perhaps part of the ability of PU.1 to shut down the T-cell program in the absence of Notch. However, it is interesting to note that both PU.1 and Lyl1 cause the down regulation of *Myb*. In Ch.3 we discussed the ability of Myb co-expression with PU.1 to block the inhibition of GATA3 by PU.1. This leads to a small, but reproducible reduction in the percentage of Mac1 positive cells (Ch. 3: Fig. 6B, C). *Gata3* has been found to be a Myb target gene in later T-cell development (Maurice et al., 2007). It seems however, that the slight down-regulation of *Myb* caused by PU.1 and Lyl1 co-

expression is not enough to dramatically affect *Gata3* expression levels in Scid.adh.2c2 cells. Gata3 most likely has other regulatory inputs; perhaps including Gata3 itself (Ouyang et al., 2000). GATA3 intracellular protein staining in these cells might provide a much clearer picture of any changes in GATA3 since this was also true in the experiments discussed in Ch. 3 (Ch. 3: Fig. 6E, F).

The Bcl11b and Lyl1 interaction studies presented here are complicated because of the limited data set. Also, Bcl11b and Lyl1 expression patterns are fairly removed from each other during development and therefore any effects seen are difficult to interpret. However, an interesting thing to point out is the inhibition of *Id2* by cells expression Lyl1 and Bcl11b. Id2 over-expression, like Bcl11b and Lyl1, can inhibit Notch target genes. The strong down-regulation of Notch target genes in the Bcl11b and Lyl1 samples may therefore be mediated via a non-E-protein inhibition pathway since *Id2* levels are low in these cells. The inhibitory effects on the Notch target gene *Dtx1* by Bcl11b and Lyl2 expressing cells are also interesting. During normal DN2b thymocyte development, *Dtx1* expression is expressed at lower levels and then shoots up during the DN3 stage of development. In the DN2b stage *Bcl1b* and *Lyl1* endogenous expressions overlap and could be mediating the repression of *Dtx1*. Only when Lyl1 expression is extinguished in the DN3 stages do *Dtx1* levels go up.

Most of the gene expression changes caused by Lyl1 transduction in Scid.adh.2c2 cells were very subtle. An explanation for this may be that Lyl1 binds DNA as part of complexes that often include *Scl, Gata2, Gfi1b, Meis1*, and *Fli1* (Wilson et al., 2010). Although there are binding sites for Lyl1 near *Nrarp, Fog1, Cdk6, Notch 1*, and *Id2,* the down-regulation of these genes by Lyl1 over-expression alone is minimal. It is true that

simply binding to DNA does not prove regulation of a gene, but there is the possibility that Lyl1 is not functioning at full capacity because it is missing collaborators. The Wilson et al. study was done in a multipotent progenitor cell line, but in Scid.adh.2c2 cells these collaborating transcription factors are often never expressed (*MMDR and EVR, data not shown*).

The second major focus of this appendix was centered on over-expression studies with the transcription factor Bcl11b. Before discussing that data we would like to point out that there was approximately a 10x over-expression of Bcl11b compared to normal levels of Bcl11b expressed in Scid.adh.2c2 cells. This could have caused aberrant changes in gene expression and perhaps a controllable vector, like a tamoxifen inducible system, that can provide lower levels of over-expression would generate different gene expression results.

All of the major studies published about Bcl11b have been knockdown or knockout studies (Li et al., 2010a; Li et al., 2010b) and the data presented here agrees with several of their observations. *Runx3* and *Nrarp* expression levels go up in Bcl11b knockdown studies (Kastner et al., 2010; Li et al., 2010a) and in our over-expression studies Bcl11b inhibits these genes. Our studies also agreed with the list of genes that were inhibited by loss of Bcl11b (*Ptcra, Id3, and Spib*) since they were enhanced with Bcl11b transduction. These previous over-expression studies are valuable and the PU.1 and Bcl11b co-expression studies shown here may provide more insight into the importance of Bcl11b during early T-cell development.

Two interesting patterns emerged from the Bcl11b study with PU.1. The first was a set of genes that Bcl11b inhibits (*Runx3*) or turns on (*Spib*) that are blocked by co-

expression with PU.1. *Runx3* is, similarly to PU.1, expressed at higher levels during earlier stages of development, but is down-regulated as T-cell development progresses. *Spib* has an opposite expression pattern where it is low initially, but goes up after PU.1 is off. These events required for passing an important checkpoint during T-cell development are enhanced by Bcl11b, but blocked by PU.1. The second pattern relevant to early T-cell development was the ability of PU.1 to up-regulate progenitor/stem cell genes *Bcl11a* and *Lyl1* and the ability of Bcl11b to block this up-regulation (Fig. 2, Heatmap and bar graph of qRT-PCR results). Bcl11b knock-down studies have shown that *Bcl11a* and *Lyl1* are abnormally up-regulated (Li et al., 2010a) and these results support the idea that Bcl11b helps shut down the program that allows access to non-T-cell lineage developmental options. We have shown however that Bcl11b is not enough to block the myeloid option on its own. Bcl11b and PU.1 co-expression experiments done in fetal thymocytes and fetal liver precursors also indicated that Bcl11b could not block Mac1 up-regulation in the absence of Notch signaling (*data not shown*).

Bcl11b has been described mostly as a transcriptional repressor (Liu et al., 2010). The first 45 amino acids of Bcl11b form a complex with a classic inhibition complex, NuRD, and this has been shown to mediate the repression of a targeted promoter (Cismasiu et al., 2005). This agrees with the data presented here showing that the Bcl11b constructs without exon 2, which contains this 45 amino acid region, do not cause repression of genes to the same extent as the 1-2-3-4 and 1-2-4 isoforms. Co-immunoprecipitation studies in Scid.adh.2c2 cells with the different Bcl11b isoforms and subsequent mass spectrometry to identify proteins differentially bound to Bcl11b could also help in teasing out domain functions. Bcl11b over-expression experiments in fetal thymocytes proved to be fairly

toxic (1-2-3-4 seemed to be better than 1-2-4), and so cell culture studies have been difficult to carry out (*L.L, M.M.DR and E.V.R observations*). The severe survival effects seen with the 1-2-4 form could be related to its ability to strongly inhibit Notch target genes. Here we have presented valuable preliminary data generated in the Scid.adh.2c2 cell line that would have been difficult to generate in primary cells and could give us clues as the interactions of PU.1, Bcl11b, and Lyl1 during early T-cell development.

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Materials and Methods

Cloning/Sub-cloning

Lyl1 coding sequences were purchased from Genscript and subcloned into a LZRS retroviral vector with an NGFR marker. Bcl11b vectors were isolated using PCR and subcloned into a MIGR1 vector with a GFP marker.

See Chapter 3 for a full list of the materials and methods used in this section.

Figures

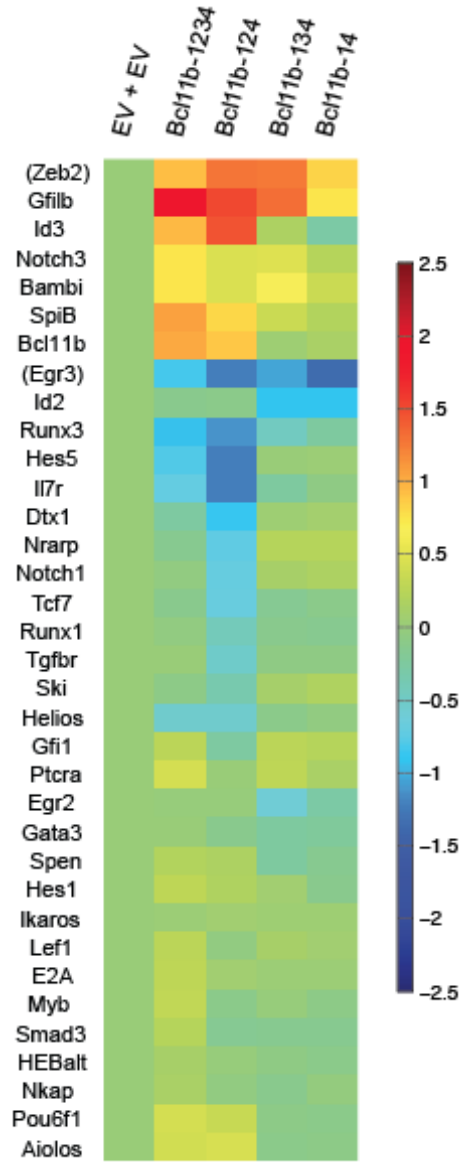

Figure 1. Gene expression comparisons between Scid.adh.2c2 cells expressing individual Bcl11b isoforms.

Scid.adh.2c2 cells were transduced with Bcl11b -1234, -124, -134, or -14 and cultured for 2 days. The transduced cells were sorted and a heatmap was generated from qRT-PCR data averaged from 2 or 3 independent experiments.

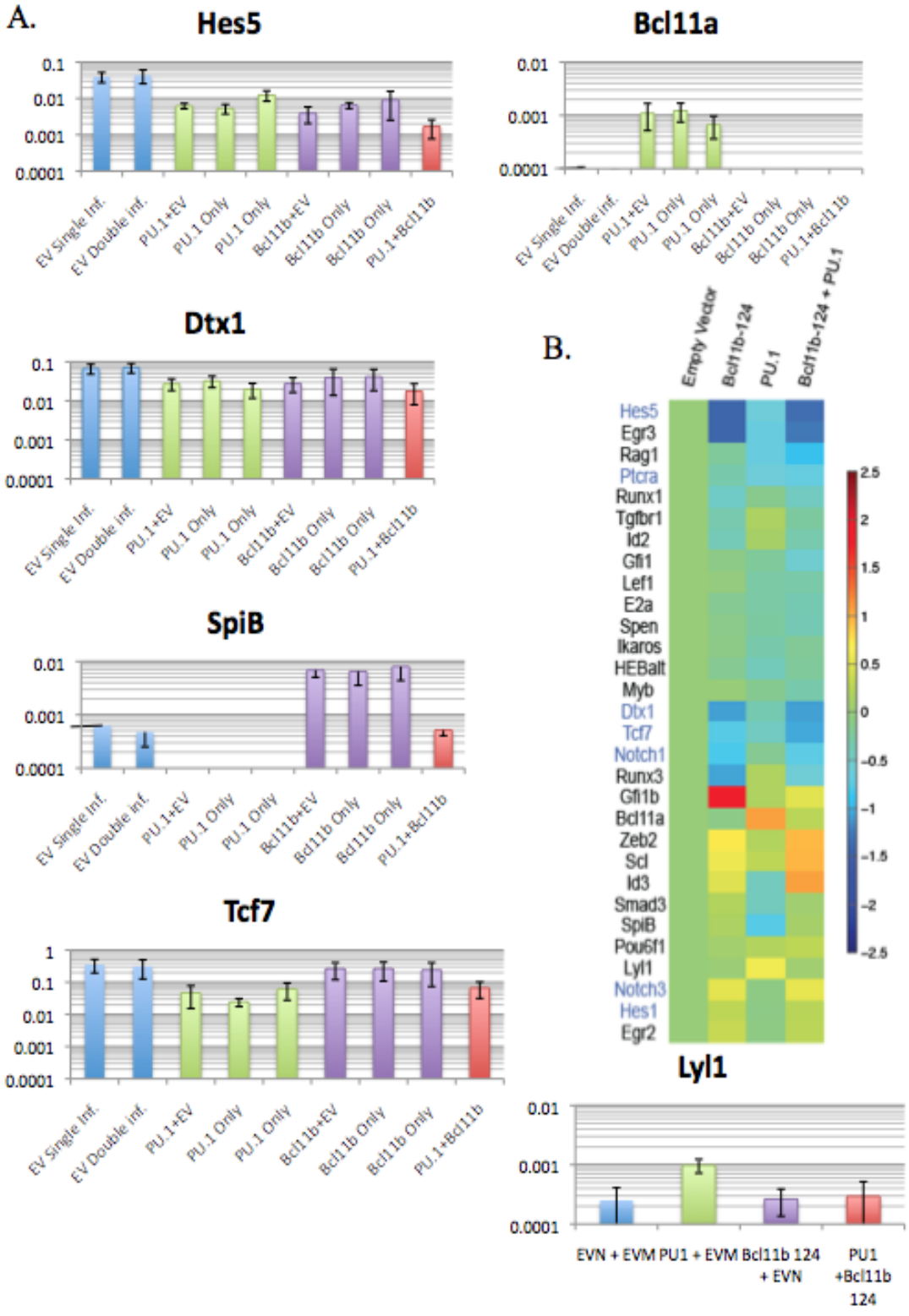
A.
Hes5
0.1
0.01
0.001
0.0001
EV Single Inf.
EV Double Inf.
PU.1+EV
PU.1 Only
PU.1 Only
Bcl11b+EV
Bcl11b Only
Bcl11b Only
PU.1+Bcl11b
Bcl11a
0.01
0.001
0.0001
EV Single Inf.
EV Double Inf.
PU.1+EV
PU.1 Only
PU.1 Only
Bcl11b+EV
Bcl11b Only
Bcl11b Only
PU.1+Bcl11b
Dtx1
0.1
0.01
0.001
0.0001
EV Single Inf.
EV Double Inf.
PU.1+EV
PU.1 Only
PU.1 Only
Bcl11b+EV
Bcl11b Only
Bcl11b Only
PU.1+Bcl11b
SpiB
0.01
0.001
0.0001
EV Single Inf.
EV Double Inf.
PU.1+EV
PU.1 Only
PU.1 Only
Bcl11b+EV
Bcl11b Only
Bcl11b Only
PU.1+Bcl11b
Tcf7
1
0.1
0.01
0.001
0.0001
EV Single Inf.
EV Double Inf.
PU.1+EV
PU.1 Only
PU.1 Only
Bcl11b+EV
Bcl11b Only
Bcl11b Only
PU.1+Bcl11b
B.
Empty Vector
Bcl11b-124
PU.1
Bcl11b-124 + PU.1
Hes5
Egr3
Rag1
Ptcra
Runx1
Tgfbr1
Id2
Gfi1
Lef1
E2a
Spen
Ikaros
HEBalt
Myb
Dtx1
Tcf7
Notch1
Runx3
Gfi1b
Bcl11a
Zeb2
Scl
Id3
Smad3
SpiB
Pou6f1
Lyl1
Notch3
Hes1
Egr2
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
Lyl1
0.01
0.001
0.0001
EVN + EVM
PU1 + EVM
Bcl11b 124 + EVN
PU1 +Bcl11b 124

Figure 2. Gene expression comparisons between Scid.adh.2c2 cells expressing Bcl11b-124, PU.1, or Bcl11b-124 and PU.1 separately or together.

Scid.adh.2c2 cells were co-transduced with a combination of empty vector, PU.1 and Bcl11b-124 and cultured for 2 days and then sorted. The gene expression profiles of these cells were generated using qRT-PCR results averaged from 3 independent experiments. (A) These samples were sorted into populations that were either doubly or singly transduced. (B) The gene expression profiles of the samples represented in this heatmap are of sorted cells doubly transduced with a combination of PU.1, Bcl11b, and empty vector.

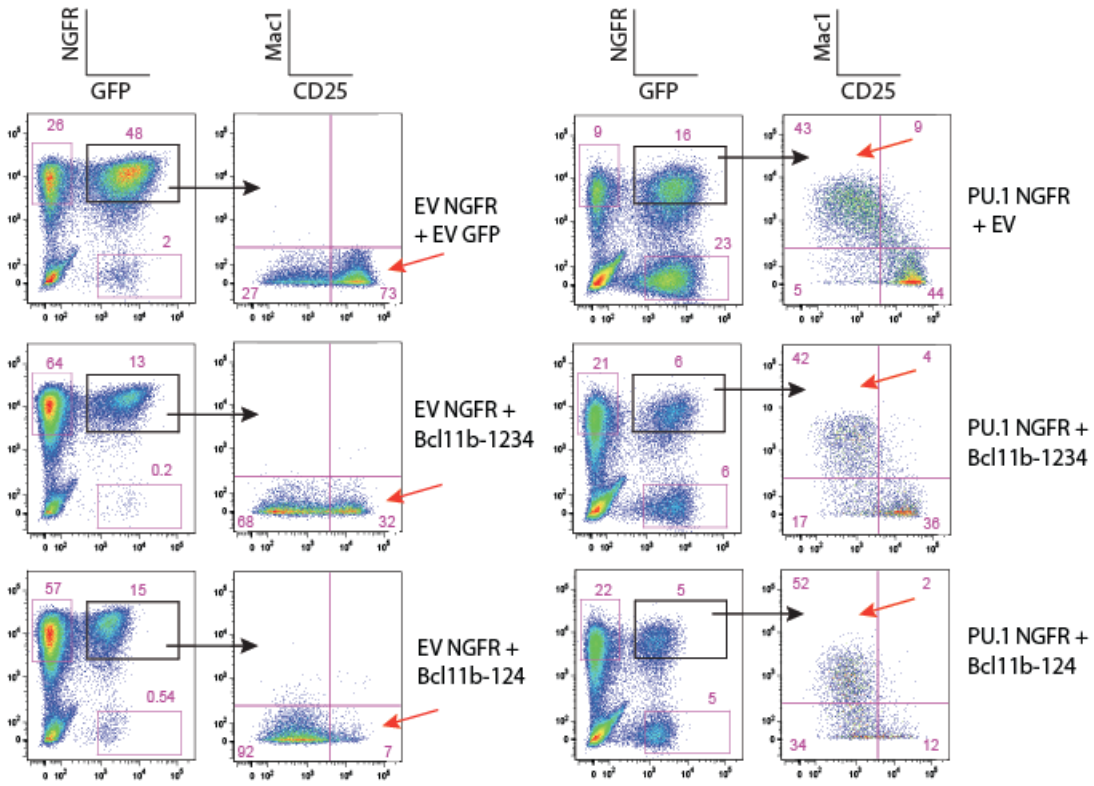

Figure 3. Scid.adh.2c2 cells co-expressing Bcl11b and PU.1 driven by MSCV IRES NGFR ("NGFR") are not inhibited from up-regulating Mac1 in response to PU.1. Scid.adh.2c2 cells expressing Bcl11b down regulate CD25 levels.

Scid.adh.2c2 cells were co-transduced with PU.1-NGFR and Bcl11b-1234-GFP or Bcl11b-124-GFP for 2 days. The NGFR+GFP+ cells were analyzed for their expression of Mac1 and CD25 using flow cytometry.

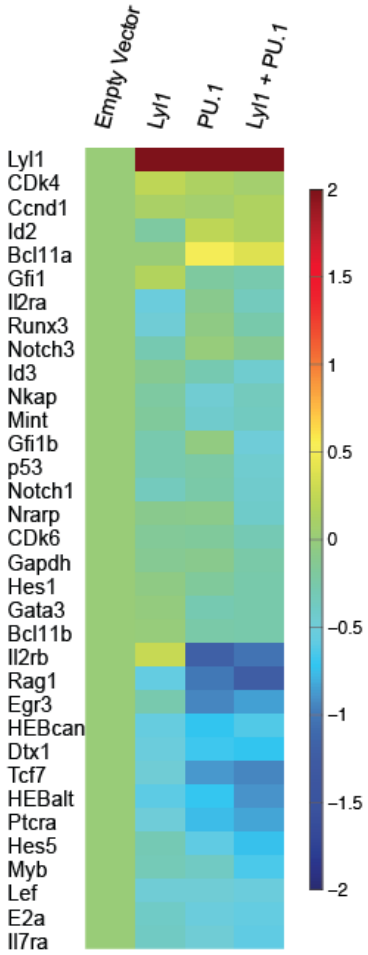

Figure 4. Gene expression comparisons between Scid.adh.2c2 cells expressing Lyl1, PU.1, or Lyl1 and PU.1 together.

Scid.adh.2c2 cells were co-transduced with a combination of empty vector, PU.1 and Lyl1 and cultured for 2 days. The cells were sorted according to their co-expression of PU.1 and Lyl1 (or other combination of retroviral vectors) and a heatmap was generated from qRT-PCR data averaged from 2 independent experiments.

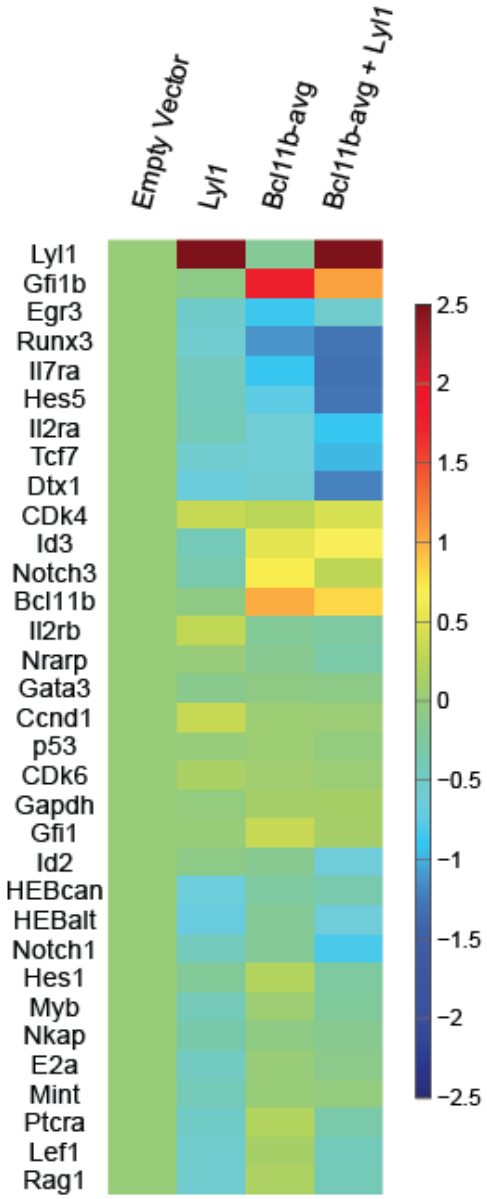

Figure 5. Gene expression comparisons between Scid.adh.2c2 cells expressing Lyl1 and Bcl11b.

Scid.adh.2c2 cells were co-transduced with a combination of empty vector, Lyl1 and Bcl11b and cultured for 2 days. The cells were sorted according to their co-expression of PU.1 and Bcl11b (or other combination of retroviral vectors) and a heatmap was generated from qRT-PCR data averaged from 2 independent experiments. The Bcl11b sample

averages are from two different isoforms (-124, and -1234). The generation of the heatmap requires a number greater than 0, so 0.0001 was added to the Lyl1 sample for Gfi1b so that it could be included in the analysis.

Tables:

Response:	Genes:
Down with Bcl11b and PU.1	*Hes5, Egr3, Dtx1, Tcf7, Notch1*
Down with PU.1, but rescued by Bcl11b	*Id3, Smad3, SpiB*
Up with with PU.1, but blocked by Bcl11b	*Tgfbr1, Id2, Lyl1, Bcl11a*
Down with Bcl11b	*Runx3*
Up with Bcl11b	*Notch3, Hes1, Egr2, Zeb2, Scl, Id3, Gfib*
Up with Bcl11b, but blocked by PU.1	*Gfib*

Table 1: Gene expression responses to PU.1 and Bcl11b co-expression

Response:	Genes:
Up with PU.1, Up less with PU.1 and Lyl1	*Bcl11a, Id2**
Up with Lyl1	Il2rb
Down with Lyl1	Id2*, Il2ra*, Runx3*, Rag1*
Down with PU.1 and Lyl1	*Myb*
Down with PU.1 alone and Down with PU.1 and Lyl1	*Il2rb, Rag1, Dtx1, Tcf7, Ptcra, Egr3, HEBcan, HEBalt, Hes5*

Table 2: Gene expression responses to PU.1 and Lyl1 co-expression

Response:	Genes:
Down with Bcl11b and down more with Bcl11b and Lyl1	*Runx3, Il7ra, Hes5, Il2ra, Tcf7, Dtx1,*
Down with Bcl11b and Lyl1	*Notch1, Id2*
Down with Lyl1	*, HEBcan*, Hebalt*, Ptcra*, Lef1*, Rag1*, Id3*, Notch3**
Up with Bcl11b, up less with Bcl11b and Lyl1	*Notch3, Gfi1b, Gfi1**
Up with Bcl11b and up more with Lyl1 and Bcl11b	*Id3*

Table 3: Gene expression responses to Bcl11b and Lyl1 co-expression