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The multifaceted roles of Bcl11b in thymic and peripheral T cells - impact on immune diseases

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Abstract

The transcription factor Bcl11b is expressed in all T cell subsets and progenitors starting from DN2 stage of T cell development, and regulates critical processes implicated in development, function and survival of many of these cells. Among common roles of Bcl11b in T cell progenitors and mature T cell subsets are the repression of the innate genetic program and to some extent expression maintenance of TCR signaling components. However, Bcl11b also has unique roles in specific T cell populations, suggesting that its functions depend on cell type and activation state of the cell. Here we provide a comprehensive review of the roles of Bcl11b in progenitors, effector T cells, as well as in Tregulatory and iNKT cells, and the impact on immune diseases. While emphasizing common themes, including some that might be extended to skin and neurons, we also describe the control of specific functions in different T cell subsets.

1. Introduction

Bcl11b was initially discovered in neurons as Chicken Ovalbumin Upstream Promoter Transcription Factor (COUP-TF)-interacting protein 2 (CTIP2), along with its family member CTIP1 or Bcl11a(1), and it was later demonstrated to be critical for proper development and function of neurons(2–5). Bcl11b is a C_2H_2 zinc finger protein that binds GC-rich response elements(6) and has been described to associate with a variety of cofactors, including the corepressor complexes NuRD(7, 8) and Sirt1(9), as well as with the histone acetyltransferase (HAT) p300(10), functioning both as a transcriptional repressor and activator(1, 6–13).

Bcl11b expression is initiated in a Notch1- and TCF-1-dependent manner at the DN2 stage of T cell development(14–17) and is maintained in mature T lymphocytes, including iNKT and Tregulatory (Treg) cells(16, 18–23). It is expressed at lower levels in NK cells, but not in B cells, myeloid or dendritic cells(16, 18, 20). Bcl11b was also identified as a radiation-induced tumor suppressor gene 1 (Rit1) in $p53^+$ thymic lymphomas(24). Years of in depth study revealed that Bcl11b is necessary for several developmental checkpoints, including T cell commitment at DN2 stage(16, 25, 26), survival of DN3 and DP thymocytes(18, 27), β selection at DN3 stage, positive selection of CD4 and CD8 single positive (SP)

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thymocytes(18, 27, 28), development of Treg(22) and iNKT(23) cells, and most recently development of a subpopulation of γδ T cells(29). In mature T cells Bcl11b was demonstrated to control expansion and effector function of cytotoxic T cells (CTLs)(19), to restrict plasticity of Th17 cells by blocking expression of the Th2 program(21), as well as to control generation of iTreg cells from conventional $CD4^+$ T cells, and overall to control the suppression function of Treg cells(22).

Dissecting the roles of Bcl11b in different cellular contexts and the factors that regulate Bcl11b provides valuable information for the growing transcriptional networks that involve Bcl11b, as well as for its implications in immune diseases. Furthermore, as new populations of immune cells are identified, such as the innate lymphoid cells, novel Bcl11b targets and functions may be discovered.

2. Bcl11b is essential for multiple checkpoints during T cell development

2.1. Bcl11b in early stages of T cell development - repression of multipotency and alternate lineage potential, enforcement of commitment

The development of T cells is a multi-step process, requiring environmental cues including Notch signaling and cytokines, expression of key transcription factors necessary for T cell commitment, as well as downregulation of factors that support multipotency and alternative lineages. Ellen Rothenberg's group showed that Bcl11b expression is initiated at DN2a stage and further continues to increase, reaching a peak at DN2b stage, when self-renewal and multipotency are suppressed(14, 17, 25). Using the OP-DL1 system, they demonstrated *ex vivo* that absence of Bcl11b caused accumulation of DN1 and DN2a stage thymocytes, which expressed myeloid and NK lineage genes and failed to shut off stem cell and multipotency genes(25) (Figure 1). At the same time, Pentao Liu's group demonstrated that loss of Bcl11b *ex vivo* caused reprogramming of DN2, DN3 and DP thymocytes to the NK-like cells, designated induced T-to-natural killer (ITNK), which upregulated NK lineage genes such as Id2, NK1.1 and NKp46 and possessed increased anti-tumor activity(16) (see bellow). In a parallel study, Hiroshi Kawamoto's group, despite earlier reports of a developmental block at the DN3 stage in the global $Bcl11b^{-/-}$ mice(27) (see bellow), concluded that the developmental block is rather at DN2 stage(26). Despite the developmental block, the absolute numbers of $Bcl11b^{-/-}$ DN2 thymocytes was not increased, though they vigorously proliferated and differentiated to NK and myeloid cells *ex vivo*(26), similar to what was communicated in the other two studies(16, 25). Thus, these three studies all point to a critical role of Bcl11b at the DN2 stage in controlling expression of genes which support T cell commitment and suppress multipotency and alternative lineage genes (Figure 1). It remains to be established whether $Bcl11b^{-/-}$ DN2 cells generated *in vivo* remain blocked at DN2 or differentiate to NK and myeloid cells.

2.2. Bcl11b at DN3 stage and beta selection

As thymocytes enter the DN3 stage, TCR rearrangements occur at the TCRβ locus. Ryo Kominami's group analyzed neonatal thymi of global $Bcl11b^{-/-}$ mice, which die soon after birth due to neuronal defects, and showed that thymocyte development was blocked at the DN3 stage, concomitant with diminished cellularity and increased apoptosis(27) (Figure 1).

Additionally, TCRβ mRNA and protein levels were reduced due to impaired Vβ to Dβ rearrangements(27), restricting formation of the pre-TCR and halting T cell development. Provision of a transgenic TCR only partially rescued the defect(30), suggesting additional alterations. It remains to be established whether NK receptors as well as other innate receptors are expressed on *in vivo* on Bcl11b$^{-/-}$ DN3 thymocytes, similar to what was observed on *ex vivo* generated Bcl11b$^{-/-}$ DN3 thymocytes(16), which may interfere with appropriate signaling required for selection. Though γδT cells were reported to develop in normal numbers in the absence of Bcl11b(26, 27), more recent studies demonstrated that IL-17-producing γδT cells were absent in these mice(29).

2.3. Bcl11b in double positive thymocytes - role in positive selection, survival, sphingolipid metabolism and repression of genes involved in CD4 and CD8 lineage commitment

Due to the developmental block at DN2–DN3 stages, CD4-cre mediated deletion of Bcl11b, in which the Cre recombinase expression accumulates at DP stage, was utilized to elucidate the role of Bcl11b in DP thymocytes(18, 28). We showed that these mice had a significant reduction in CD4 and CD8 SP thymocytes and peripheral CD4^{+} and CD8^{+} T cells, concomitant with increase in some immune cell populations, including NK and myeloid cells. The increase in NK and myeloid cell numbers was associated with elevated splenic and bone marrow hematopoiesis through a bystander mechanism mediated by TNFα, however without indication of increased expansion(18, 31) (see bellow). The elevation in other immune populations, including B cells and γδ T cells could be attributed to increased hematopoiesis and/or homeostatic expansion(18, 31). The reduction in peripheral T cells was caused by defective positive selection, associated with impaired TCR signaling(18) (Figure 1). Though TCRα was normally rearranged, Bcl11b$^{-/-}$ DP thymocytes had increased spontaneous apoptosis, which occurred even in the absence of TCR signaling, on a TCRα$^{-/-}$ background(18). A slight decrease in the Bcl2 family member BCLXL and an increase in pro-apoptotic factors was observed, however, provision of the prosurvival factor Bcl2 only partially rescued survival, suggesting that additional factors are implicated (see bellow). Interestingly neither provision of transgenic TCRs or Bcl2 rescued defective positive selection of Bcl11b$^{-/-}$ DP thymocytes(18). Though reduced survival of DP thymocytes in the absence of Bcl11b is a common theme with DN3 thymocytes from the global Bcl11b$^{-/-}$ mice, Bcl11b$^{-/-}$ DN2 thymocytes did not suffer of this defect, possibly related to their *ex vivo* generation on OP-DL1.

A large number of genes were found dysregulated in preselected Bcl11b$^{-/-}$ DP thymocytes ((18, 28) and Albu and Avram, 2007, unpublished observations and GSE56714), among which genes implicated in modulation of TCR signaling(18). Additionally, genes with role in commitment to CD4 and CD8 lineages, such as Th-POK and Runx3(32–34) were found upregulated((28) (Figure 1). Although Th-POK mRNA was one of the most upregulated in our microarrays as well, the protein could not be detected in Bcl11b$^{-/-}$ DP thymocytes (Albu and Avram, 2007, unpublished observations, and GSE56714), making it unlikely that its premature expression is the cause of altered positive selection and spontaneous apoptosis. mRNA for Id2, important for the NK program(35), was also upregulated(28), similar to Bcl11b$^{-/-}$ DN2 thymocytes(16, 25), together with mRNAs for the NK receptors Nkp46,

Nk1.1, CD244, several killer cell lectin-like receptors, including Klrd1, Fc receptors, Ly6C and the inhibitory receptors PD-1 and CD160 ((18, 28) and Albu and Avram, 2007, unpublished data and GSE56714). Surprisingly, Bcl11b$^{-/-}$ DP thymocytes did not present surface Nk1.1, Nkp46 and CD244 proteins (Uddin and Avram, 2014, unpublished observation), but had elevated levels of surface CD160, PD-1 and Ly6C(18). We hypothesized that in addition to deregulated expression of some TCR signaling components, presence of Ly6C and CD160 and PD-1 inhibitory receptors on the surface of DP thymocytes may perturb TCR signaling and cause impaired positive selection. Interestingly, we also found alterations in several genes implicated in sphingolipid metabolism, which caused accumulation of sphingolipids, gangliosides and cholesterol, affecting glycolipid presentation by DP thymocytes to iNKT precursors(23) (see below). Such alterations, in addition to being associated with enlarged lysosomes in Bcl11b$^{-/-}$ DP thymocytes(23), can potentially affect membrane composition, and consequently impair TCR signaling and selection, as well as survival (Figure 1). Interestingly, Indra's group found later that Bcl11b$^{-/-}$ embryonic skin had larger amounts of sphingomyelin(36). This raises the possibility that common pathways may be regulated by Bcl11b in skin and T cells, and potentially neurons.

Thus, Bcl11b has some common roles in DP thymocytes, DN3 and DN2 thymocytes, such as repression of innate genes, and supports survival of both DP and DN3 thymocytes. However, additionally Bcl11b plays several distinctive functions in DP thymocytes: (1) maintenance of expression of several components of TCR signaling, (2) control of the expression of genes with critical roles in sphingolipid metabolism and cholesterol, and (3) silencing of genes implicated in CD4 and CD8 T cell commitment, such as Th-POK and Runx3 (Figure 1).

3. Bcl11b in iNKT cells

iNKT cells are a subset of T cells that express semi-invariant TCRs, composed of Vα14-Jα18 chains complexed with Vβ chains of limited diversity. They are formed from DP thymocytes, which are also the cells that present self glycolipids on CD1d molecules to select the iNKT precursors(37–39). Kastner et al. showed that mice deficient for Bcl11b starting with DP thymocytes lack iNKT cells in the thymus and periphery(28). In a series of mixed bone marrow chimeras, which functionally separated DP thymocytes that present glycolipid from iNKT precursors, our group demonstrated that Bcl11b regulates iNKT development by both intrinsic and extrinsic mechanisms, specifically playing a dual role, both in the iNKT precursors and in the DP thymocytes which present glycolipids(23) (Figure 1). The defect in glycolipid presentation was due to impaired sphingolipid metabolism, glycolipid trafficking and loading on CD1d, with deregulated expression of cathepsin D and L genes, Neimann-pick disease type C1 and 2 (NPC1 and NPC2), as well as genes encoding lysosomal enzymes, such as beta-galactosidase-1 and the acid sphingomyelinase(23). Sphingolipid species, including lactosylceramide, galactosylceraminde, glucosylceramide and sphingomyelin, as well as cholesterol accumulated in Bcl11b$^{-/-}$ DP thymocytes, which had enlarged lysosomes, reminiscent of lysosomal storage disease(23).

The mechanisms by which iNKT cell development is intrinsically regulated by Bcl11b await clarification and are currently under investigation.

4. Bcl11b in Tregulatory cells

Naturally occurring or thymic T regulatory (nTreg) cells also develop from DP thymocytes. Inducible Treg (iTreg) cells are formed from conventional $CD4^+$ T cells in the periphery in response to TGFβ and TCR stimulation(40, 41). Foxp3 is a critical transcription factor in the development of both nTreg and iTreg cells, and acts in concert with other transcription factors and cofactors(42–44). Mutations at the Foxp3 locus cause dysregulation of Treg development and function, leading to lymphoproliferative diseases, fulminant autoimmunity and death(45, 46). We found that $Bcl11b^{F/F}$CD4-Cre mice developed inflammatory bowel disease (IBD), rescued by the transfer of wild type Treg cells, supporting the idea that inadequacy of $Bcl11b^{-/-}$ Treg cells plays a critical role in IBD development in these mice(22). Furthermore, $Bcl11b^{F/F}$Foxp3-Cre mice had a similar phenotype, albeit delayed, likely associated with reduced levels of Cre in the Foxp3-Cre strain(47). $Bcl11b^{-/-}$ Treg cells had reduced suppressive activity, reduced Foxp3 and IL-10 levels (both at protein and mRNA levels) and upregulated proinflammatory cytokines, including TNFα, IFNγ and IL17, both at mRNA and protein levels, acquiring overall a proinflammatory effector $CD4^+$ T cell phenotype(22) (Figure 1). In addition, genes identified before as being de-repressed in the absence of Bcl11b in DN2 and DP thymocytes, such as NK receptors and Id2, were upregulated in $Bcl11b^{-/-}$ Treg cells as well, however similar to $Bcl11b^{-/-}$ DP thymocytes (Uddin and Avram, 2014, unpublished observations) and $Bcl11b^{-/-}$ $CD4^+$ T cells(31), surface NK1.1 protein remained low(22). It is possible that DP thymocytes, $CD4^+$ T cells and Treg cells have additional mechanisms to restrict presence of surface NK1.1 protein and other NK receptors, even when mRNAs are expressed. Chromatin immunoprecipitation (ChIP) assays revealed that Bcl11b associated with the promoter and conserved noncoding sequences-1 and -2 (CNS1 and CNS2) at the Foxp3 locus, thereby playing a critical role in the control of Foxp3 expression both in nTreg and iTreg cells, and explaining why $Bcl11b^{-/-}$ Treg cells have reduced Foxp3 levels(22). CNS1 was shown to be required for induction of Foxp3 expression during generation of iTreg cells from conventional $CD4^+$ T cells(48). Supporting our observation, induction of Foxp3 in response to TCR activation and TGFβ treatment was reduced in the absence of Bcl11b, further establishing a role of Bcl11b in generation of iTreg cells(22) (Figure 1). $Bcl11b^{-/-}$ Treg cells had reduced mRNA levels for several TGFβ signaling components, including Tgfbr2, Smurf1 and Tgif2, suggesting the possibility that Bcl11b controls TGFβ signaling to induce Foxp3 in iTreg cells. It has been demonstrated that TGFβ and TCR stimulation induces trimethylation of the H3K4 at the CNS-1 region, thereby opening the chromatin and allowing access of transcription factors such as Smad3(49). Furthermore TGFβ stimulus inhibits the activity of DNA methyl transferase 1 (Dnmt1), reducing DNA methylation status at CNS-1, which is reversed once the stimulation stops(50). It is possible that Bcl11b acts similarly to Smad3, occupying the CNS-1 region only when chromatin is opened and not having access when stimulation is removed. In conventional $CD4^+$ T cells, in which Bcl11b is also expressed, the DNA is highly methylated at CNS-1, likely blocking the access of factors that can induce Foxp3 expression(40), including Smad3 and potentially Bcl11b. Bcl11b's relationship with Foxp3

is even more complex, as Bcl11b was found to be part of the Foxp3 complex in Treg cells(51). Interestingly we found that Bcl11b also associated with CNS-2(22), known to be bound by Foxp3 in a manner dependent on Runx-Cbfb(48, 52, 53). It remains to be established how Bcl11b works in concert with these complexes. Additionally, we found that Bcl11b regulates IL-10 gene expression by association with the promoter and CNS+6.5(22). Therefore in addition to the common role of restricting the expression of innate genes, Bcl11b plays specific roles in Treg cells, controlling Foxp3 and IL-10 expression and overall the suppression function of Treg cells, represses the effector $CD4^+$ T cell program, and controls the generation of iTreg cells from conventional $CD4^+$ T cells(22).

5. Bcl11b restricts Th17 helper cell plasticity by repressing Th2 program

In addition to T cell development, Bcl11b is also an important component of T cell effector function and differentiation. We recently demonstrated that Bcl11b restricts Th2 lineage gene expression in Th17 cells during experimental autoimmune encephalomyelitis (EAE), by repressing Gata-3 expression through direct interaction with the proximal Gata-3 promoter(21) (Figure 1). Bivalent histone modifications, namely trimethylation of H3K4 and H3K27 at the promoters of lineage determining transcription factors, such as Gata-3 and Tbet, which poise for transcriptional activation or repression, respectively, suggest that alternative lineage programs are not completely blocked following Thelper cell differentiation(54–56). Though previously Th17 cells were demonstrated to be plastic toward Treg and Th1 cells(56), our study was the first to demonstrate that Bcl11b restricts plasticity toward Th2 lineage genetic program(21). Despite expression of IL-4 and Gata-3 (including at protein level) in $Bcl11b^{-/-}$ Th17 $CD4^+$ T cells of mice with experimental autoimmune encephalomyelitis (EAE), a Th17-mediated disease, the Th17 lineage transcription factor RORγt(57, 58) and the cytokines IL-17 and GM-CSF remained normal(21). Importantly, treatment promoting a Th2 response of EAE wild type mice, still did not affect the Th17 cytokines, demonstrating for the first time that the Th17 program is permissive to the Th2 program(21), which has major implications for Th17-mediated autoimmune diseases. The main consequence of the common expression of IL-4 and Th17 cytokines by $Bcl11b^{-/-}$ Th17 $CD4^+$ T cells and by Thelper cells of EAE wild type mice vaccinated in Th2 conditions, was on their migration, through an extrinsic mechanism(21) (see bellow). Interestingly, even though Bcl11b repressed Gata-3 gene expression in Th17 cells(21), Bcl11b was not required to downregulate Gata-3 in developing thymocytes, where Gata-3 seems to be upstream of Bcl11b(59), suggesting that in such context, Bcl11b either does not have access or is competed out from Gata-3 promoter. Possibly signaling events initiated by Th17-promoting cytokines, such as IL-6, TGFβ, IL-1β and IL-23(60), which are not likely to act in the thymus, play a critical role, favoring recruitment of Bcl11b to the Gata-3 promoter along with the NuRD complex to deacetylate the histones and silence Gata-3 gene expression.

Specifically how Bcl11b is regulated to restrict T helper cell plasticity is unknown and whether Bcl11b is important in promoting or restricting plasticity of other effector $CD4^+$ T cell populations remains to be determined.

6. Bcl11b in mature CD8+ T cells

Bcl11b is indispensable for effector $CD8^+$ T cells as well. Using conditional knock-out mice, which express the Cre recombinase post selection, through the Lck distal promoter, we studied the role of Bcl11b in mature T cells, both Thelper cells (see above) and $CD8^+$ T cells. At steady state, mice lacking Bcl11b in mature $CD8^+$ T cells exhibited a reduction in CD8 expression and Bcl11b was found to occupy the enhancers E8I, IV, and V at the CD8 locus(19). Regulation of CD8 expression only occurred in $CD8^+$ T cells, but not in DP thymocytes(18, 19). Though expressed in $CD4^+$ T cells, Bcl11b was not found to control the repression of CD8 gene in these cells(18, 19, 21). It is possible that other transcription factors, such as Th-POK, which occupy similar CD8 enhancer regions and mediate CD8 gene repression in $CD4^+$ T cells via recruitment of HDACs(61), may block Bcl11b's access to these regions. Interestingly, at steady state there was also a decrease in the naïve $CD8^+$ T cell pool in the absence of Bcl11b, and $Bcl11b^{-/-}$ $CD8^+$ T cells acquired a memory-like phenotype (Avram, 2008, unpublished observation), pointing to another potential role of Bcl11b in these cells, in controlling the naïve state. Similar to $Bcl11b^{-/-}$ DP thymocytes the small number of $Bcl11b^{-/-}$ naive $CD8^+$ T cells upregulated Id2, PD-1 and CD160, in addition to NK1.1 and other NK receptors' mRNAs, but not Th-POK (Avram, 2008, unpublished observation and GSE56925).

$Bcl11b^{-/-}$ effector cytotoxic T-lymphocytes (CTLs) had reduced expansion during immune response to *Listeria monocytogenes* and influenza, and diminished killing activity associated with low levels of granzyme B and perforin(19). Similar to DP thymocytes, $Bcl11b^{-/-}$ CTLs had reduced TCR activation, with reduced levels of CD69, Zap70 phosphorylation and calcium flux, and diminished mRNA levels for several components of the TCR signaling, including CD3, CD28 and Plcg1(19) and Avram, 2008, unpublished observation and GSE56713). $Bcl11b^{-/-}$ CTLs from mice infected with *L. monocytogenes* upregulated mRNAs for NK receptors, but not for Id2, PD-1 and CD160 (Avram, 2008, unpublished observation and GSE56713). The fact that CTLs, known to express Id2(13, 62) failed to further increase Id2 mRNA levels in the absence of Bcl11b, can be due to the fact that Id2 levels are already maximal, though we cannot exclude implication of other mechanisms, including exclusion from locus and/or competition with other transcription factors/ complexes. Again, all these data, suggest common themes, as well as differing roles, depending on the cell type, and moreover, indicating that even in the same T cell type ($CD8^+$ T cell, in this case), depending on the activation/effector state of the cell (naïve versus CTL), Bcl11b differentially controls expression of specific genes in a context dependent manner.

7. Modulation of Bcl11b function is dependent on post-translational modifications

Interestingly, once Bcl11b is expressed at the DN2 stage of T cell development, it remains expressed in all T cells. Kastner et al. found that Bcl11b represses the expression of Th-POK and Runx3 genes in DP thymocytes(28), however Bcl11b remains expressed in SP thymocytes and in mature T cells, which require either Th-POK or Runx3, suggesting that the repressive function of Bcl11b at these genes must be blocked as T cells mature, similar

to what happens to Id2 in CTLs. In addition, though during development Bcl11b does not seem to have an impact on Gata-3, in Th17 cells it represses its expression(21).

We demonstrated that Bcl11b associates with the chromatin remodeling/HDAC-containing complex NuRD and with the HAT p300, to mediate transcriptional repression or activation, respectively(7, 10). Zhang et al. recently demonstrated that the ability of Bcl11b to recruit the NuRD complex or p300 largely depends on its post-translational modifications(13). Specifically, they show that in steady state thymocytes Bcl11b exists in several states, sumoylated, phosphorylated, and neither sumoylated or phosphorylated, which all associate with the NuRD complex and repress Id2 gene expression and potentially other target genes, such as Thpok and Runx3. Stimulation of thymocytes with Phorbol 12,13-dibutyrate and a calcium ionophore (P/I), causes rapid and high phosphoryation of all three forms through MAPK pathway, followed by de-sumoylation by SENP proteins. However, phopsphatases are rapidly activated and dephosphorylate Bcl11b, which causes dismissal of SENP proteins and allows again sumoylation of Bcl11b, which now recruits the HAT p300 to de-repress Id2, however without replacement of NuRD(13). It remains to be established whether this mechanism occurs in positive selection of DP thymocytes and whether Bcl11b functions as a transcriptional activator for Id2 gene, in addition to being a transcriptional repressor, as its absence so far in early and DP thymocytes, as well as in naïve $CD8^+$ T cells, resulted in increased Id2 mRNA, and no change in CTLs, but never downregulation in any tested $Bcl11b^{-/-}$ T cell populations ((16, 28) and Albu and Avram, 2007, unpublished data and GSE56714, GSE56925 and GSE56713). It would be also important to determine whether phosphorylation, sumoylation and other posttranscriptional modifications modulate Bcl11b in other immune populations, and at other loci, such as Th-pok, Runx3 and Gata-3 etc. The pattern that we have observed in terms of regulation of a specific gene, is transcriptional repression or transcriptional activation in a given immune population and lack of regulation in another immune population, such as in the case of Id2, Th-pok, Runx3 and Gata-3. Additionally, we recently found that certain genes can be negatively regulated in one immune population and positively regulated in other immune populations, however the two immune populations do not derive from each other (Califano and Avram, unpublished observations; Uddin and Avram, unpublished observations).

We postulate that specific signaling events that trigger such differential functions of Bcl11b can be initiated by Notch, for example in progenitors, TCR at selection stages, as well as TCR and/or cytokines during immune response, and further TCR and TGFβ in conversion of conventional $CD4^+$ T cells to iTreg cells, etc.

8. Bcl11b extrinsically alters function of several immune populations and disease outcomes

The above-discussed data demonstrate the importance of Bc11b in regulating a plethora of cellular functions in a cell autonomous manner. In addition, our most recent data has found that absence of Bc11b in T cells can extrinsically alter the function and development of bystander cells, and importantly greatly alter disease outcomes. The first evidence came from the role of Bcl11b in iNKT cell development, as described above, where Bcl11b deficiency in DP thymocytes resulted in a loss in iNKT selection due to impaired

sphingolipid metabolism and glycolipid presentation to iNKT precursors(23). Two more recent studies have also described extrinsically regulated defects in mice deficient in Bcl11b in specific T cell populations(21, 31). As discussed above, Bcl11b removal during Th17-induced EAE with the Lck distal promoter-Cre system, which acts only in mature T cells, enhanced Th17 cell plasticity by derepression of Gata-3 expression and IL-4 cytokine production, without restricting expression of the Th17 lineage transcription factor RORγt, and production of IL-17 and GM-CSF(21). In this study we demonstrated that co-production of IL-4 and GM-CSF made dendritic cells, which do not express Bcl11b, upregulate retinaldehyde dehydrogenase 2 (RALDH2) expression, a key enzyme for production of retinoic acid (RA), and subsequently enhanced RA production(21). Our study further demonstrated that during EAE, Bcl11b$^{-/-}$ CD4^{+} T cells upregulated the gut homing receptors integrin α4β7 and CCR9 through an extrinsic mechanism, dependent on RA and IL-4, causing their diversion from the draining lymph nodes/CNS to the mesenteric lymph nodes/small intestine. Furthermore, the diversion of Bcl11b$^{-/-}$ effector CD4^{+} T cells caused a major reduction in the EAE severity, and importantly, their presence in the small intestine did not result in symptoms of inflammatory bowel disease (IBD), due to acquisition of regulatory function, however without expression of Foxp3 ((21) and Califano & Avram, 2014, unpublished). Moreover, re-routing of the cells was recapitulated in EAE wild type mice that were treated with $MOG_{35–55}$ peptide in Th2-inducing conditions, bypassing the need for Bcl11b deficiency, suggesting a possible therapeutic avenue for human multiple sclerosis and other autoimmune diseases, due to allowance of Th2 cell program in the context of Th17-mediated autoimmune diseases(21). These results demonstrate that induction of IL-4 is possible during Th17-mediated EAE, and it does not impact the Th17 cell program in itself, but the combination of the two types of cytokines impacts trafficking of immune cells, which has major impact of the disease outcome(21).

As shown above, Pentao Liu's group provided exciting evidence that the deletion of Bcl11b at early stages of thymic development or in DP thymocytes with the use of an inducible ERCre system reprogrammed the T cells to NK-like cells. In their experiments Bcl11b$^{-/-}$ DP thymocytes were generated *ex vivo* by treatment with tamoxifen for 48hrs, and then transferred in Rag2$^{-/-}$gc$^{-/-}$ KO mice, in which they formed mostly CD8^{+} T cells with enhanced anti-tumor activity(16). When we removed Bcl11b *in vivo* with the CD4-Cre system, the mice also exhibited reduced tumor burden in several tumor models, however in a manner independent of CD8^{+} T cells, but dependent on NK cells, CD4^{+} T cells and TNFα, abundantly produced by Bcl11b$^{-/-}$ CD4^{+} T cells(31). In our study Bcl11b$^{-/-}$ CD8^{+} T cells, though upregulated NK activating receptors, had minimal granzyme B levels and low degranulation. Importantly, NK cells were in increased numbers in Bcl11b$^{F/F}$/CD4-Cre mice and the only ones having high granzyme B levels and elevated degranulation(31). The increased numbers of NK cells resulted through elevated hematopoiesis in the bone marrow and spleen in a manner dependent on TNFα(31). Bcl11b is not expressed in hematopoietic stem cells, but expressed in NK cells, however the CD4-Cre system does not allow its removal from NK cells, supporting the implication of another extrinsically regulated effect following Bcl11b removal, through TNFα. Importantly, low doses of TNFα recapitulated stimulation of extracellular hematopoiesis and increased NK cell formation, as well as elevated anti-tumor immune response(31). The differences in our study versus Pentao Liu's

study may be due to the fact that the initiation of ITNK cell generation *ex vivo* is more efficient, as the restrictions imposed by thymic selection which likely would eliminate highly reactive cells, do not exist. Additionally, in $Bcl11b^{F/F}$/CD4-Cre mice, the small number of $Bcl11b^{-/-}$ $CD8^{+}$ T cells which are formed are likely to be strictly controlled by other immune populations and exposed to a different environment than the ITNK cells transferred in $Rag2^{-/-}gc^{-/-}$ mice, including the fact they do not have the opportunity to expand so vigorously as in $Rag2^{-/-}gc^{-/-}$ recipient mice.

Conclusions

One common theme when Bcl11b is removed in T cells is the expression of innate cell genes, supporting the idea that overall Bcl11b represses such program in T cells. Another theme in several progenitors and mature T cells is the expression maintenance of genes implicated in TCR signaling. Remarkably, an important function of Bcl11b in DP thymocytes and developing skin is the control of sphingolipid metabolism, which remains to be determined if it is a common theme in other T cell progenitors and subsets, as well as in neurons. In addition to the common themes, we postulate that Bcl11b also has diverse roles, controlling specific genes, depending on the T cell developmental stage and on the T cell subset, and moreover having differing roles depending on the T cell activation state. Such diverse and complex roles support the idea that Bcl11b's activity is dependent on stage and subset specific transcription factors and cofactors, and on cell type-specific signaling events. How Bcl11b is integrated in such complex regulatory networks remains to be further elucidated.

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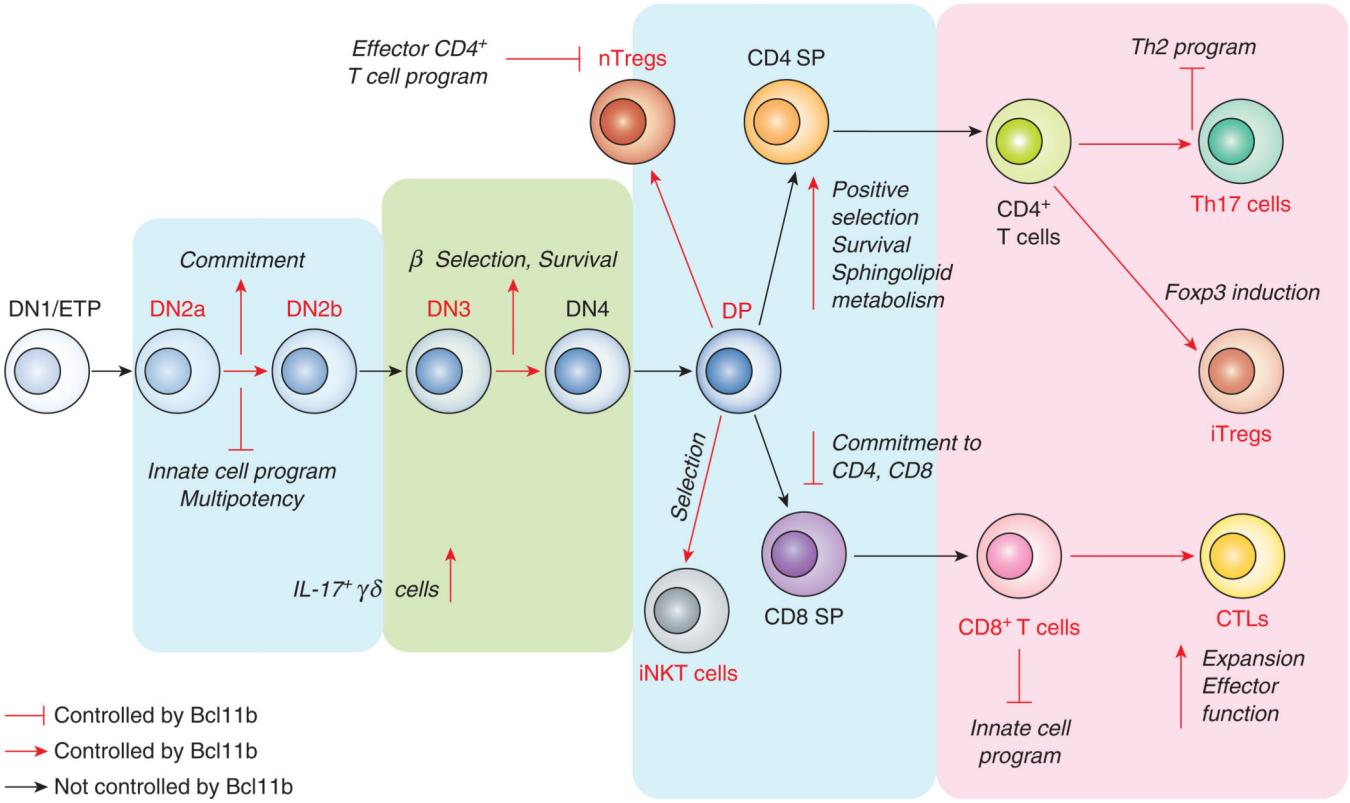

Figure 1. Roles of Bcl11b in thymic and peripheral T cells

Thymic developmental stages are indicated as double negative (DN1–DN4), double positive (DP) and single positive (SP). Specific T cell populations are indicated as invariant natural killer T (iNKT) cells, natural Treg (nTreg), inducible (iTreg) cells, $CD4^+$ T, Th17, $CD8^+$ T cells and cytotoxic T lymphocytes (CTL). T cell populations and processes in which Bcl11b plays a role are shown in red. Functions controlled by Bcl11b in each developmental stage or T cell subsets are indicated.