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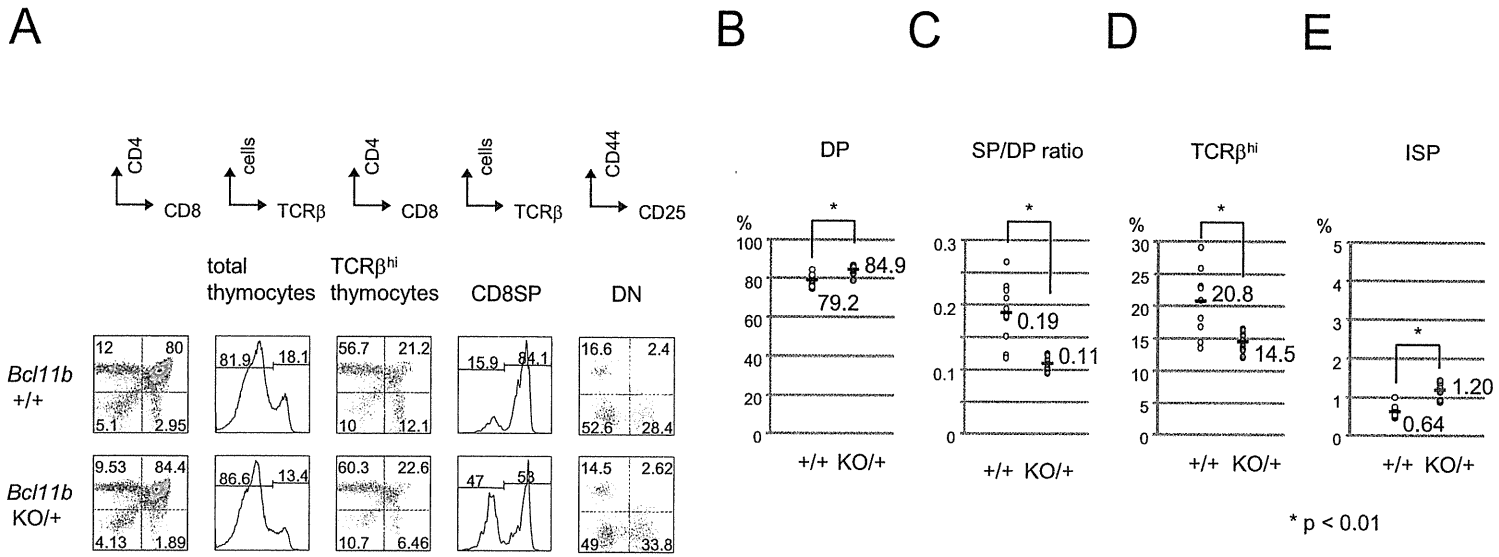
Figure 1
Fig.1



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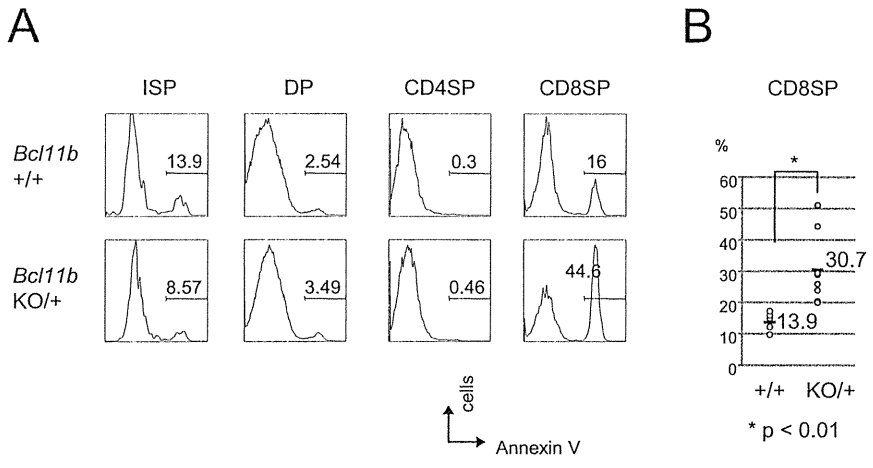

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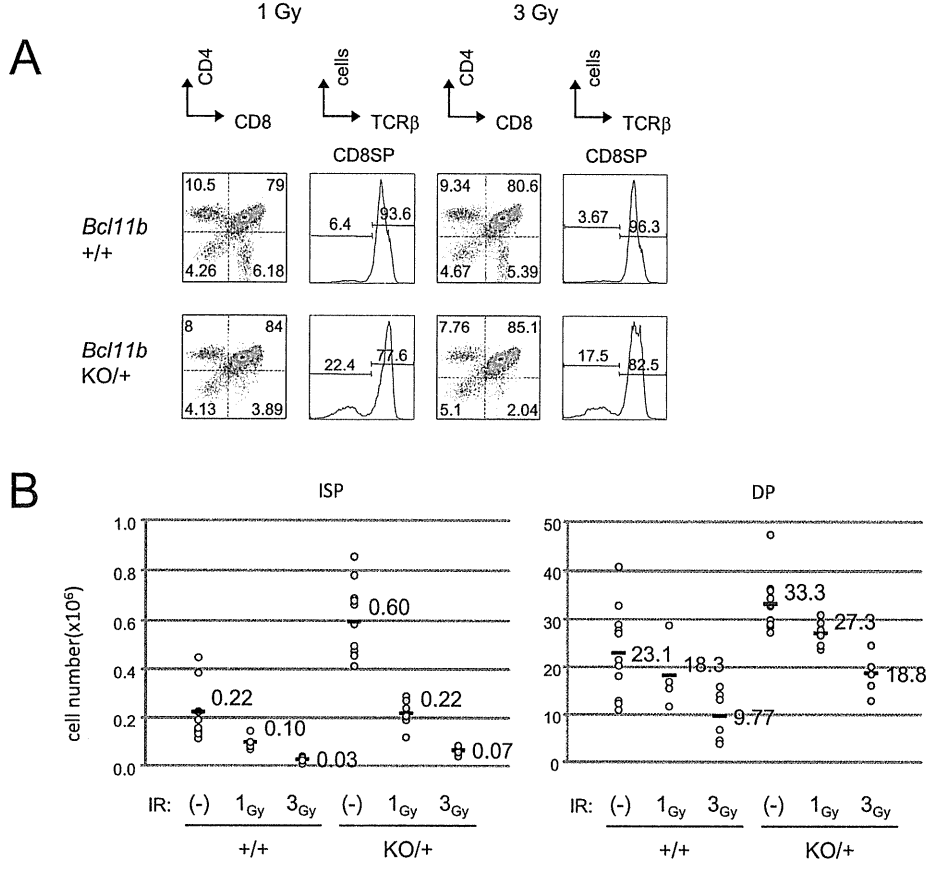

Figure 4

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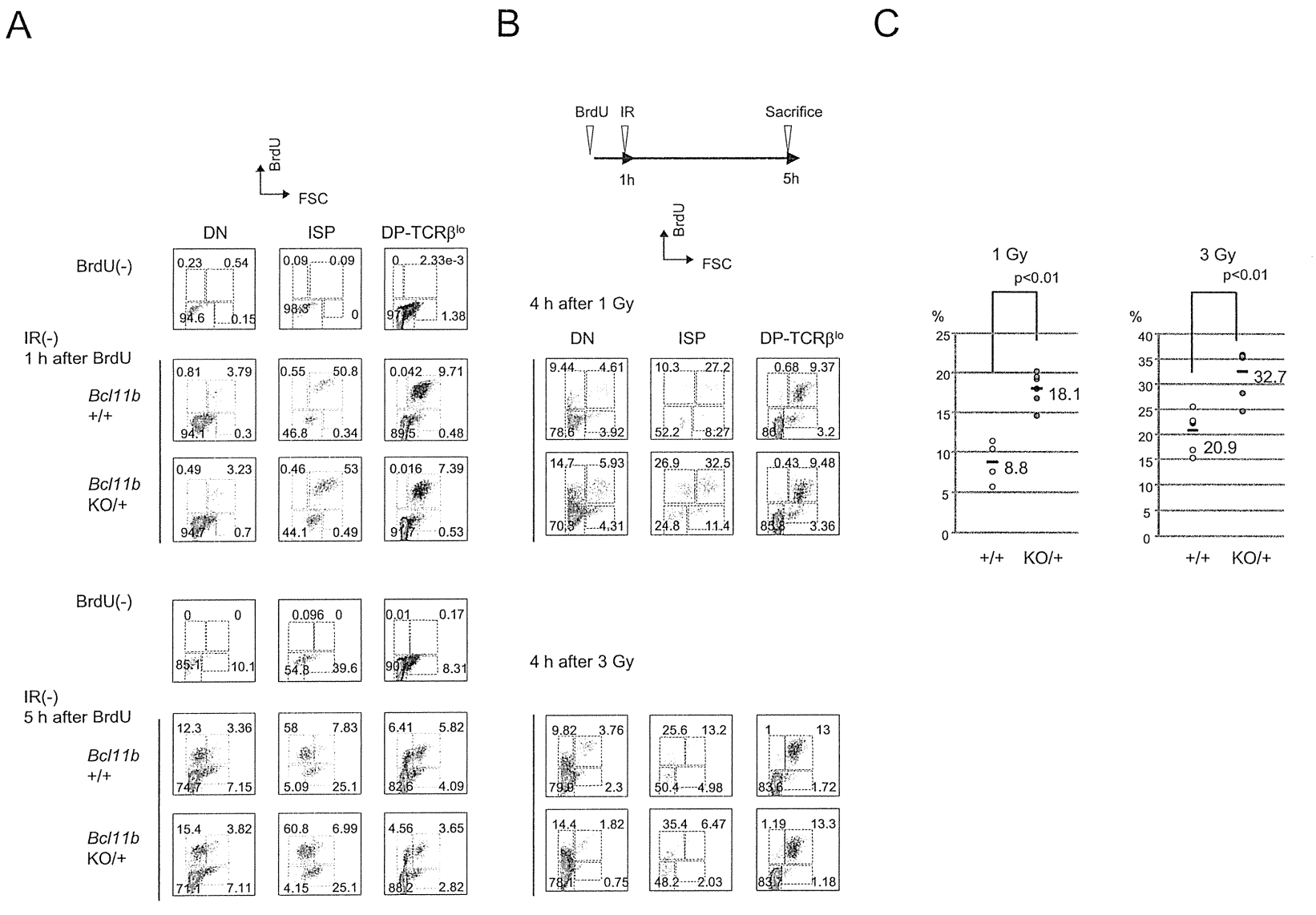

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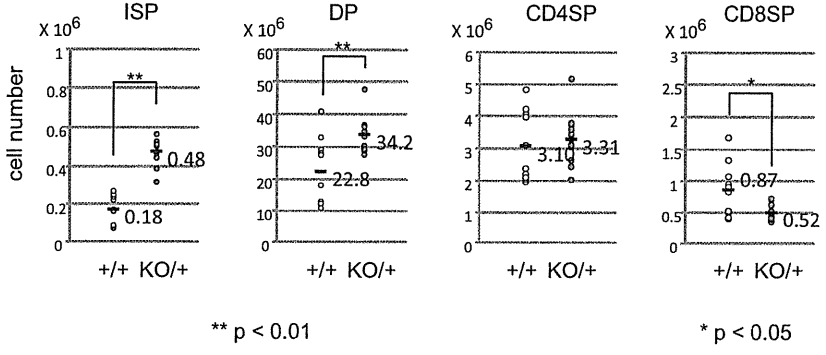

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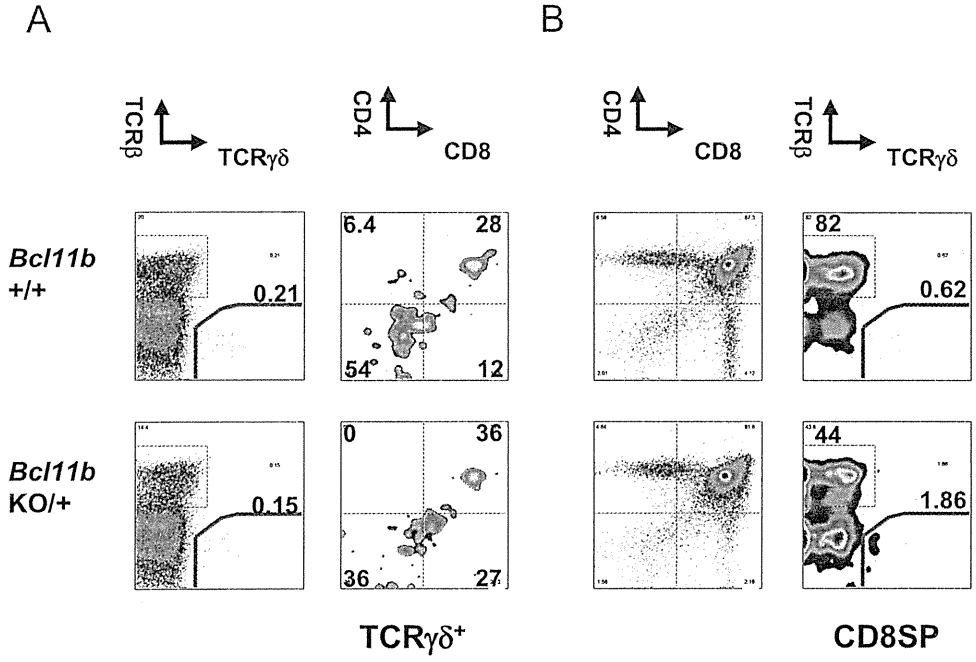

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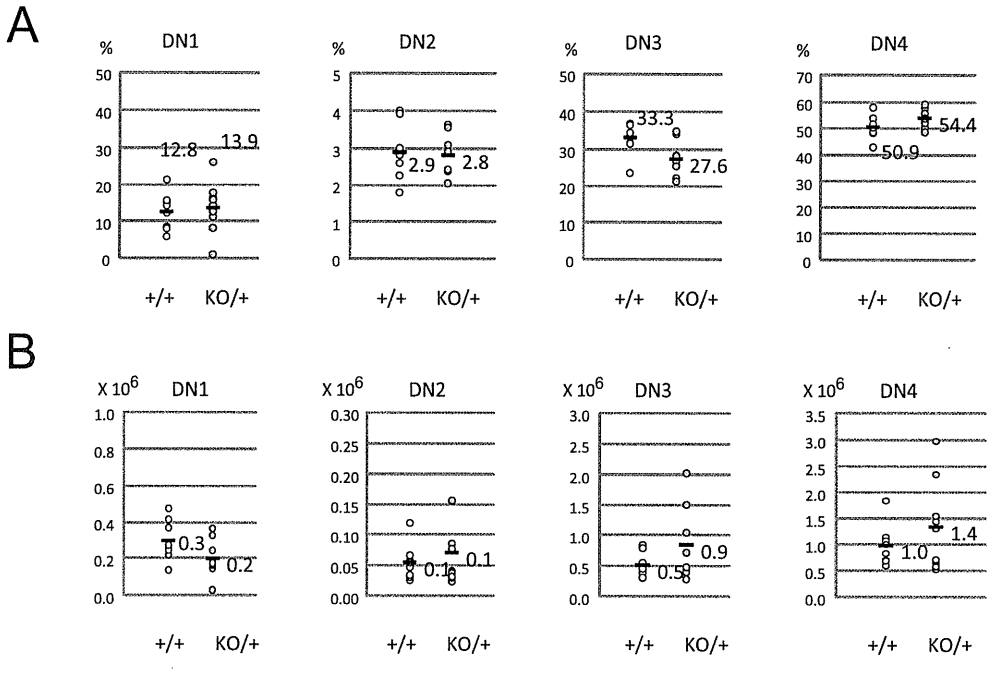

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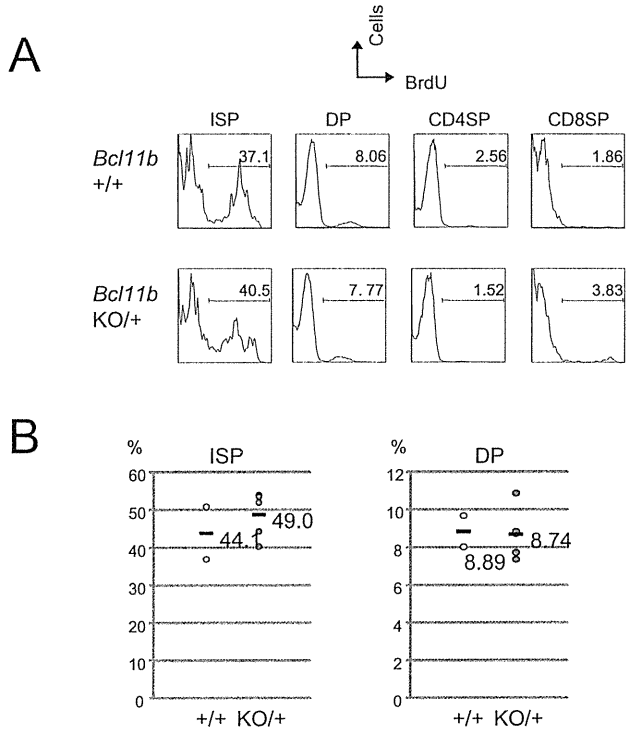

Figure Legends

Fig. 1. Effect of *Bcl11b*$^{KO/+}$ genotype on differentiation of thymocytes. (A) Flow cytometric analysis of thymocytes from *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice using CD4, CD8, TCRβ, CD25 and CD44 markers. The markers used are indicated above in each panel. As for TCRβ expression on thymocytes, total thymocytes (left) and cells in the CD4^{-}CD8^{+} quadrant (right) were analyzed. (B, D, E) The percentage of cells and the absolute number in thymocyte subsets: B, DP cells; D, TCRβ^{high} thymocytes; E, ISP cells. C, The ratio of SP cells/DP cells. The absolute number in average is shown below each number of the percentage. Comparison was performed between *Bcl11b*$^{+/+}$ (n=9) and *Bcl11b*$^{KO/+}$ (n=11) mice. P value for difference in the percentage between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice is less than 5 % in each of B, C, D and E.

Fig. 2. Comparison of apoptosis in thymocytes from *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice. (A) Flow cytometric analysis of Annexin V-positive cells in the thymocyte subsets indicated above. The vertical axis shows cell numbers and the horizontal axis shows Annexin V-expression levels. (B) The percentage of Annexin V-positive cells in thymocyte subsets. Comparison was performed between *Bcl11b*$^{+/+}$ (n=6) and *Bcl11b*$^{KO/+}$ (n=8) mice. P values for difference in ISP and CD8SP subsets are less than 1 %.

Fig. 3. Effect of γ-irradiation on the cell number in thymocytes. (A) Flow cytometric analysis of CD4, CD8 and TCRβ expression on thymocytes from 1 Gy and 3 Gy irradiated *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice. Analysis was performed 4 h after irradiation. (B) The cell number of ISP and DP cells in thymic lobe after irradiation or without irradiation. The sample number is 4 in 1 Gy *Bcl11b*$^{+/+}$ mice and 6 in 1 Gy *Bcl11b*$^{KO/+}$ mice, and 6 in 3 Gy

irradiated mice.

Fig. 4. Effect of γ-irradiation on cell cycle in thymocytes of *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice. (A) Flow cytometric analysis of BrdU incorporation levels (vertical axis) and FSC values (horizontal axis) in thymocyte subsets in unirradiated mice. BrdU^{+}FSC^{Large} fraction represents cells in S or G2/M phases of cell cycle whereas BrdU^{+}FSC^{Small} fraction represents G1 cells that have passed S phase. (B) Flow cytometric analysis of BrdU incorporation levels and FSC values in thymocyte subsets in 1 Gy and 3 Gy irradiated mice. (C) The percentages of BrdU^{+}FSC^{S} cells in the ISP thymocyte subset in *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice after 1 Gy and 3 Gy irradiation. P values for difference between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice after 1 Gy and 3 Gy irradiation are less than 0.5%. The sample number is 4 in 1 Gy *Bcl11b*$^{+/+}$ mice and 6 in 1 Gy *Bcl11b*$^{KO/+}$ mice, and 6 in 3 Gy irradiated mice.

Supplementary Fig. 1. The absolute number of ISP, DP, CD4SP and CD8SP thymocytes in *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice (A-D). Comparison performed between *Bcl11b*$^{+/+}$ (n=9) and *Bcl11b*$^{KO/+}$ (n=11) mice showed significant differences in ISP ($P<0.01$), DP ($P<0.01$) and CD8SP ($P<0.05$).

Supplementary Fig. 2. (A) Flow cytometric analysis of thymocytes from *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice. TCRβ and TCRγδ markers are used for total thymocytes (left) and CD4 and CD8 markers are used for the TCRβ-low and TCRγδ-high thymocyte fractions indicated (right). (B) Flow cytometric analysis of total thymocytes from *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice using CD4 and CD8 markers (left) and of the indicated CD8^{+} cell fractions using TCRβ and TCRγδ markers (right). The percentage of γδ T cells was very low in the CD8^{+} thymocytes both from *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice.

Supplementary Fig. 3. (A) The percentage of DN1, DN2, DN3 and DN4 cells in DN thymocytes from *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice. (B) The cell number of DN1, DN2, DN3 and DN4 in *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice. Comparison performed between *Bcl11b*$^{+/+}$ (n=9) and *Bcl11b*$^{KO/+}$ (n=11) mice did not show significant differences.

Supplementary Fig. 4. (A) Flow cytometric analysis of BrdU incorporation in thymocyte subsets from *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice. (B) The percentage of BrdU-positive cells in ISP and DP cells. Comparison performed between *Bcl11b*$^{+/+}$ (n=2) and *Bcl11b*$^{KO/+}$ (n=5) mice did not show significant differences.

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Contributions: R.G. and K.T. contributed equally to this work and performed the majority of studies; S.H. performed studies in Fig.2; Y.K. performed studies in Fig.3; Y.A. and Y.M. helped design studies in Figs. 3 and 4; R.K. helped to plan and direct experiments and wrote the manuscript.

Review

Role of the transcription factor Bcl11b in development and lymphomagenesis

By Ryo KOMINAMI*1,†

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Abstract: Bcl11b is a lineage-specific transcription factor expressed in various cell types and its expression is important for development of T cells, neurons and others. On the other hand, Bcl11b is a haploinsufficient tumor suppressor and loss of a *Bcl11b* allele provides susceptibility to mouse thymic lymphoma and human T-cell acute lymphoblastic leukemia. Although there are many transcription factors affecting both cell differentiation and cancer development, Bcl11b has several unique properties. This review describes phenotypes given by loss of Bcl11b and roles of Bcl11b in cell proliferation, differentiation and apoptosis, taking tissue development and lymphomagenesis into consideration.

Keywords: Bcl11b, T-cell development, haploinsufficient tumor suppressor, T-cell leukemia, thymic lymphoma

1. Introduction

Bcl11b (B-cell CLL/lymphoma 11b) belongs to Kruppel-like C_2H_2 type zinc finger transcription proteins, the largest family of transcription factors in eukaryotes.[1] The gene encoding Bcl11b was first identified as a tumor suppressor gene by our study.[2] In fact, mice lacking one *Bcl11b* allele are susceptible to thymic lymphomas. On the other hand, mice lacking both *Bcl11b* alleles, which die shortly after birth of unknown causes, exhibit many defects in different organs of newborn mice, including immune system, central nervous system (CNS), skin, teeth, and hair cells in cochlea.[3)–6)] Therefore, Bcl11b plays critical roles in the development of those organs and possibly others.[7] Recently, Liu *et al.* have reviewed roles for Bcl11b in T-cell development and maintenance of T-cell lineage commitment.[1] Thus, this review provides a focus on the tumor suppressor role of Bcl11b rather than T-cell development.

Bcl11b is located on mouse chromosome 12 and on human chromosome 14. This gene is originally called *Rit1* (radiation-induced tumor suppressor gene 1), because *Bcl11b* was isolated by scanning γ-ray induced mouse thymic lymphomas for losses of specific chromosomal DNA.[8] More than 10 years ago, the scanning was performed for the 361 thymic lymphomas that were induced in mice crossed between BALB/c and MSM strains. The two strains belong to different mouse subspecies, *Mus musculus domesticus* and *Mus musculus molossinus*, respectively, and hence they carry many distinct alleles and DNA markers between the two. Genome-wide allelic loss or loss of heterozygosity (LOH) analysis using polymorphic DNA markers mapped several candidate tumor suppressor gene regions.[8] Further analysis localized one of the regions on mouse chromosome 12 to a 2.9 cM interval between the D12Mit53 and D12Mit279 marker positions.[9] Construction of a physical map consisting of 15 BAC clones in the vicinity contained informative boundaries of allelic losses, which allowed us to finally localize a 35 kb interval with a high frequency of allelic loss (62%). Sequence analysis of this interval led to the finding of *Bcl11b* gene, and mutation analysis identified this gene responsible for thymic lymphoma development.[2]

Another candidate region was mapped on mouse chromosome 11, which harbored *Ikaros* gene. Mutation analysis of this gene in thymic lymphomas

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identified it as a tumor suppressor gene.[10] *Ikaros* is the well-known gene that plays critical roles in the development of lymphoid tissues and lymphomas.[11),12)]

2. Bcl11b and Bcl11a

Bcl11a is another member of the Bcl11 family in the mouse and human genomes.[13),14)] Although Bcl11a and Bcl11b share some sequence homology, they are located on different chromosomes and have different exon-intron structures. Bcl11a and Bcl11b are also called Ctip1 and Ctip2, respectively,[15)] because they were independently isolated for their interaction with the chicken ovalbumin upstream promoter transcription factor (COUP-TF) of orphan nuclear receptors. COUP-TF family members play important roles in development,[16)] and they usually mediate transcriptional repression by recruiting nuclear receptor co-repressor (NCoR) and/or silencing mediator for retinoid and thyroid hormone receptor (SMRT) to the template.[17)] As a transcription factor, Bcl11a and Bcl11b are also associated with the nucleosome remodeling and histone deacetylase (NuRD) complex to repress target promoters.[18),19)] However, functional association between Bcl11a/Ctip1 or Bcl11b/Ctip2 and COUP-TF remains open.

Phylogenetic analysis of Bcl11-like genes suggests that a homolog of Bcl11b first appears in cartilaginous fishes.[20)–22)] On the other hand, homologs of Bcl11a are already present in the genomes of amphioxus and sea lamprey. As previously pointed out,[21),22)] though not by Guo and Cooper *et al.*,[20)] the Bcl11 protein in sea lamprey, a jawless vertebrate, can be categorized into the Bcl11a cluster. This suggests that Bcl11b is segregated form the Bcl11a homolog at the vertebrate stage, and no Bcl11b homolog is present in invertebrates. This may be compatible with that Bcl11a is involved in the transcription of hemoglobin genes (see below) whereas Bcl11b regulates the development of T cells that are not present in sea lampreyor or in other invertebrates like sea urchin.

Bcl11a was originally called Evi9, named after a retroviral insertion site (Evi9) in myeloid leukemia tumors in BXH-2 recombinant inbred mice. Detailed analysis of the Evi9 site discovered this gene.[23)] Mice lacking *Bcl11a* exhibited neonatal lethality and impairments in B cell and lymphoid cell development.[14)] However, recent genetic studies of *BCL11A* in humans have shed new light on a complex regulatory process of fetal hemoglobin (HbF) expression. *BCL11A* is associated with persistent fetal hemoglobin in adult humans,[24),25)] which was provided by genome-wide association studies. This analysis identified *BCL11A* as a new HbF-associated gene on chromosome 2, by taking advantage of the natural variation in the level of HbF in various human populations. Subsequent studies established that BCL11A is a central mediator of γ-globin silencing and hemoglobin switching.[26)] An example of the finding in these studies is that down-regulation of BCL11A expression in adult human erythroid precursors led to robust induction of HbF,[27)] and mechanistically, BCL11A interacts with the Mi-2/NuRD chromatin remodeling complexes, as well as the erythroid transcription factors GATA1 and FOG1, in erythroid progenitors.[27)] Very recent studies revealed a network of transcription factors that the transcription factor KLF1 is a key activator of the BCL11A gene.[28),29)] Knockdown of KLF1 in human and mouse adult erythroid progenitors markedly reduced BCL11A levels and increased human gamma-globin/beta-globin expression ratios.

3. Bcl11b and trancription

Bcl11b/Ctip2 was initially identified as a transcriptional repressor that either directly bound to a GC-rich consensus sequence of target genes and/or interacts with NuRD complex.[18),19),30)] On the other hand, Bcl11b was shown to activate the transcription of NF-κB target genes,[31)] suggesting that Bcl11b acts both as a transcriptional repressor and activator in a context dependent manner. Figure 1 displays the Bcl11b structure including DNA binding and protein-interacting regions. The *Bcl11b* gene consists of 4 exons and encodes two different isoforms, α-isoform consisting of 884 and β-isoform consisting of 812 lacking exon 3 in the mouse.[2)] The long exon 4 comprises all six zinc-finger domains, and the 2nd and 3rd domains are responsible for DNA binding. Recently, structural homology modeling has been performed as to canonical DNA binding of Bcl11b zinc fingers, which is based on the high-resolution crystal structure of the zinc finger domains of the transcription factor Egr1 in complex with DNA.[32)] The result reveals that mutations identified within the 2nd and 3rd domains disrupt the structure comprising conserved amino acids that are modeled to be required for the stability of the zinc finger domain or its binding to DNA. Apart from the DNA binding region, Bcl11b possesses domains responsible for interaction with proteins and protein complexes. Their catalogue has grown recently, including histone deacetylases (HDAC1 and HDAC2), and the ubiq-

Fig. 1. Structure of Bcl11b protein. The bar represents the β isoform of Bcl11b including Zinc-finger and D/E-rich domains on the bar. All these domains are within the long exon 4, and the exons 1–3 are 214 amino acids in length. The human BCL11B is longer than the mouse one and consists of 894 amino acids in the β isoform. The 2nd and 3rd Zinc-finger domains are for DNA binding and regions shown by lines are for various proteins binding to Bcl11b.

uitous transcription factor Sp1, and the Rb-associating proteins (RbAp46 and RbAp48), the member of surtuin family proteins (Sirt1), the heterochromatin protein 1 (HP1), and the histone methyltransferase SUV39H1.[18),19),33)–36)]

Several target genes of Bcl11b were discovered in cultured cells. Genes encoding cyclin-dependent kinase inhibitiors, p21/Cip2/Waf1 and p57/Kip2, are examples, and they are transcriptinally suppresssed by Bcl11b.[19),37)] In addition, we have identified HDM2(MDM2) as a new target, a ubiquitin ligase that downregulates a key tumor suppressor p53.[38)] Interestingly, Bcl11b inhibits HDM2 expression in a p53-dependent manner and modulates responses to radiation-induced DNA damages. A study of thymocytes *in vivo* using Chip-seq (Chromatin immunoprecipitation followed by DNA sequencing) method revealed several new target sequences and genes such as Th-Pok (Zbtb7b) and Runx3,[39)] expression of which is required for immature DP thymocytes (see Section 4.1 and Fig. 2 for abbreviation of thymocyte subsets) to further differentiate into mature thymocytes (CD4SP or CD8SP cells, respectively). Those genes are upregulated in the DP thymocytes lacking Bcl11b expression, suggesting suppressive role for Bcl11b in their transcription. Induction of Th-Pok expression occurs downstream of T-cell receptor signaling,[40)] whereas Runx3 contributes to Th-Pok repression in CD8SP committed cells.[41)] Hence, these suggest essential roles for Bcl11b in early silencing of Th-Pok and Runx3 genes. It may be also possible that Bcl11b cooperatively works with ThPok or Runx in the activation or suppression of some target genes. However, it is elusive of the mechanism and whether or not these repressions by Bcl11b are done through the association with NuRD or others.

Recently, Chip-seq analysis for Bcl11b binding sequences was done in cells of striatal neurons. As a result, as many as 248 target genes were identified with the aid of gene expression profiling, which suggests the neurotrophic factor/neurotrophin signaling pathway as a primary target pathway for Bcl11b regulation.[42)]

4. Phenotypes of Bcl11b-deficient mice

Bcl11b is known to plays crucial roles in the development of several organs, including T cells, CNS, skin, and tooth. Bcl11b-deficient mice exhibit various developmental defects in these organs as follows.

4-1. T cells. As described above, the defect in T-cell development given by Bcl11b deficiency is the phenotype firstly discovered. At present, defects have been identified at several distinct stages of development of thymocytes and T cells.[3),22),43)–47)] Figure 2A illustrates T-cell development in thymus (see below for details) and indicates the stages of developmental arrest given by loss of Bcl11b. Bcl11b is a unique transcription factor that specifically functions for T-cell identity maintenance and another transcription factor of this type is Tcf1 (T-cell factor 1).[22),46)–48)] The T cell specification pathway involves many different signalings, one of which is the signaling by

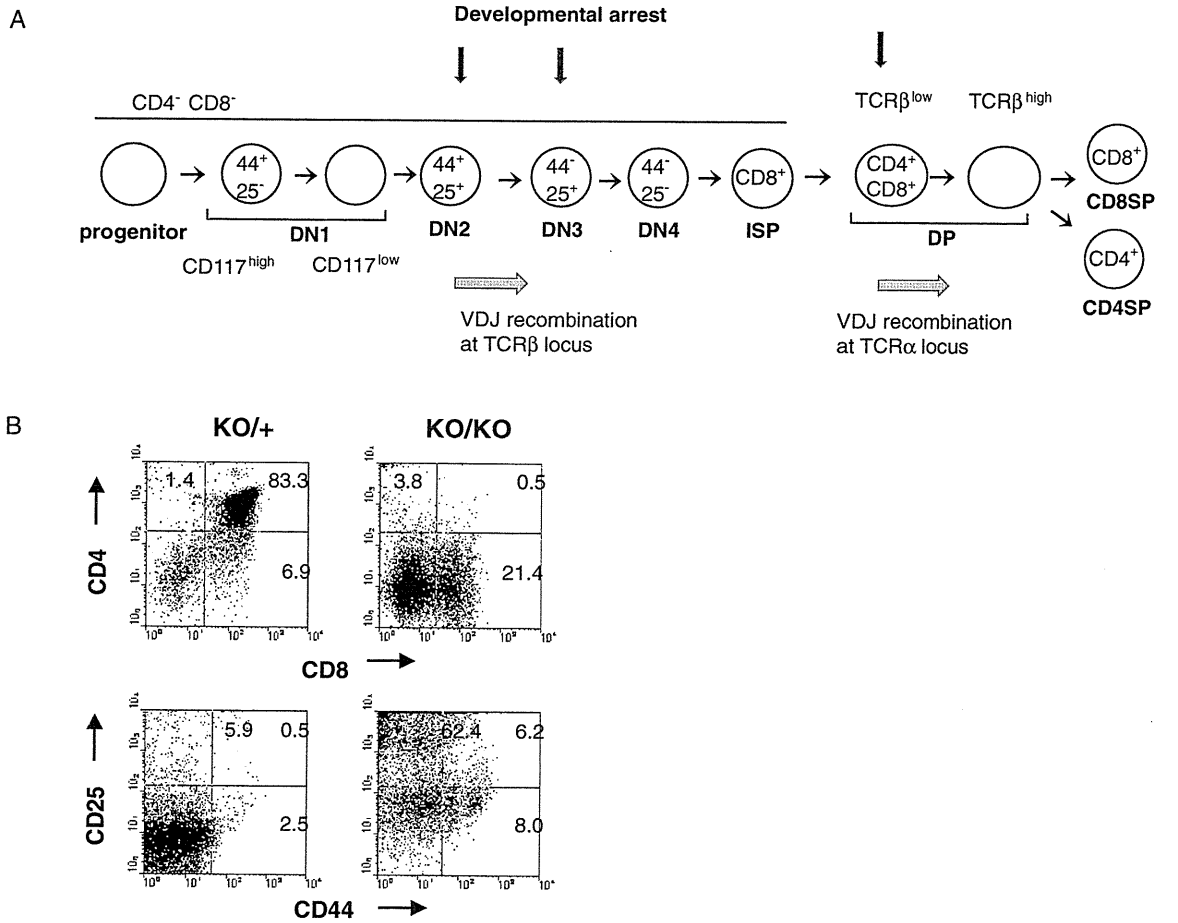

Fig. 2. (A) The diagram illustrates stages of T-cell development in thymus. Differentiation markers used here are CD117, CD44, CD25, CD4, CD8, and TCRβ on cell surface. See the text for details of development of $\alpha\beta$ T cells. In brief, it proceeds in order of maturity, $CD4^-CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive (DP), and $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive cells (CD4SP or CD8SP cells, respectively). Vertical arrows indicate stages of developmental arrest given by loss of Bcl11b. (B) Flowcytometry of thymocytes in $Bcl11b^{KO/+}$ and $Bcl11b^{KO/KO}$ mice using CD4, CD8, CD44, and CD25 markers. The vertical axis shows CD4 expression and the horizontal axis displays CD8 expression in total thymocytes (upper); the vertical axis shows CD25 expression and the horizontal axis displays CD44 expression in the CD4/CD8 double-negative quadrant (lower).

the Notch1 receptors. The receptors are expressed by early progenitors, and activated to function upon interaction with cognate ligands (Delta-like proteins) expressed by thymic epithelial cells.[49),50)] The review by Liu *et al.*[1)] and others[51),52)] describe tissue-specific signals that direct developmental fates of thymocyte progenitors in the thymus. To avoid redundancy, I touch briefly on thymocyte development and roles for Bcl11b in controlling thymocyte differentiation and expansion.

T cells arise from hematopoietic progenitor cells that migrate from the bone marrow to the thymus, where they proliferate as thymocytes (Fig. 2A). Development of $\alpha\beta$ T cells in the thymus proceeds through three major stages defined according to their expression pattern of CD4 and CD8 molecules on cell surface, i.e. in order of maturity, $CD4^-CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive (DP), and $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive cells (CD4SP or CD8SP cells, respectively). The CD4 and CD8 molecules are coreceptors of the T-cell receptor (TCR). Before DN thymocytes progress to the DP stage, they express CD8 but lack $\alpha\beta$ TCR on cell surface. Those cells are highly proliferative and called immature $CD8^+$ single positive (ISP) cells. Thymocytes at the DP stage express the $\alpha\beta$ T-cell receptor