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Reprogramming of T Cells to Natural Killer–Like Cells upon *Bcl11b* Deletion

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Abstract

T cells develop in the thymus and are critical for adaptive immunity. Natural killer (NK) lymphocytes constitute an essential component of the innate immune system in tumor surveillance, reproduction and defense against microbes and viruses. Here we show that the transcription factor Bcl11b was expressed in all T cell compartments and was indispensable for T lineage development. When *Bcl11b* was deleted, T cells from all developmental stages acquired NK cell properties and concomitantly lost or decreased T cell–associated gene expression. These induced T-to–natural killer (ITNK) cells, which were morphologically and genetically similar to conventional NK cells, killed tumor cells in vitro and effectively prevented tumor metastasis in vivo. Therefore, ITNKs may represent a new cell source for cell-based therapies.

T cell development involves progenitor homing, lineage specification, and commitment and requires a complex interplay among key transcription factors (1, 2). In the periphery, the cytokine interleukin 7 (IL-7) and the constant interaction of T cells with self peptide–major histocompatibility complex (MHC) play a critical role in T cell maintenance (3). Reverse transcription–polymerase chain reaction (RT-PCR) analysis indicates that many genes important for T cell commitment start to increase their expression in the transition through the double-negative (DN) stages from DN1 to DN2, and Bcl11b is the most up-regulated transcription factor (4). In bony fish, Bcl11b is shown to be required for T cell–precursor

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Supporting Online Material www.sciencemag.org/cgi/content/full/science.1188063/DC1 Materials and Methods, SOM Text, Figs. S1 to S8, Tables S1 to S4, References

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homing to the thymus (5). In the mouse, Bcl11b has critical roles in fetal thymocyte development and survival, for positive selection, and in survival of double-positive (DP) thymocytes (6, 7).

To determine *Bcl11b* expression in T cells at the single-cell level, we produced and analyzed a *Bcl11b-tdTomato* knock-in mouse (fig. S1, A and B) (8). In hematopoietic lineages, *Bc11b* was not expressed in B or myeloid cells, whereas almost all DN2 to DN4 and DP thymocytes, $CD4^+$ and $CD8^+$ T cells, γδ T cells, and natural killer T cells (NKTs) expressed *Bcl11b* (figs. S2, A to C, and S3, A to C). In DN1 thymocytes, very little to no expression of *Bcl11b* was detected in $CD117^{++}$ cells [known as early T cell lineage progenitors (2)] (figs. S2A and S3A). During NK development, transient low *Bcl11b* expression was observed in immature NK cells but not in NK precursors or mature NK cells (figs. S2D and S3D). In contrast, the majority of thymic NK cells, identified by CD127 (9), expressed *Bcl11b* (figs. S2D and S3E). Moreover, using quantitative real-time PCR (QRT-PCR) analysis, we showed that, in both $CD4^+$ and $CD8^+$ splenic T cells, *Bcl11b* transcription in naïve ($CD44^-CD62L^+$) T cells was roughly two times that in activated T cells ($CD44^+CD62L^-$) (figs. S2E and S3F), and activated T cells exhibited a bimodal pattern of expression (fig. S2F).

To further determine Bcl11b functions in T cells, we generated the conditional knockout mice (*Bcl11b* $^{flox/flox}$) (fig. S4A), which were crossed to the *Rosa26Cre-ERT2* mice (10). Consequently, in *CreERT2; Bcl11b*$^{flox/flox}$ mice (the PLBD line, referred to hereafter as *flox/flox*), *Bcl11b* could be deleted by treating cultured cells or mice with 4-hydroxytamoxifen (OHT). Using OHT-treated whole thymocytes from these and the control (*CreERT2; Bcl11b*$^{flox/+}$, referred to as *flox/+*) mice, we sorted and subsequently cultured DN1 and DN2 cells in T cell media on OP9-DL1 stromal cells (fig. S4B) (11), which support T cell development but suppress NK cell development from the progenitors (12). All stromal cells were killed in the OHT-treated *flox/flox* DN1 thymocyte culture.

Flow cytometry showed that 24% of cells in this culture expressed NKp46, which is primarily expressed on NK cells (Fig. 1A) (13). These $NKp46^+$ cells did not express T cell genes for CD3 or the β T cell receptor (TCRβ) (fig. S4C) and had lost both alleles of the *Bcl11b* exon 4 (fig. S4D), which indicated that they did not acquire or had lost T cell features, even though they were cocultured with OP9-DL1 stromal cells for 14 days. However, the control OHT-treated *flox/+* and untreated *flox/flox* DN1 cells proliferated rapidly, and many acquired CD3 expression but not $NKp46^+$ (Fig. 1A and fig. S4E). These data demonstrated that *Bcl11b* deficiency caused production of the $NKp46^+$ cells from DN1 thymocytes and that Bcl11b was required early in T cell development. Similar to cultured DN1 thymocytes, OHT-treated *flox/flox* DN2 thymocytes also produced $NKp46^+CD3^-$ cells, which killed the stromal cells, whereas control DN2 thymocytes did not (Fig. 1A and fig. S4E). Growth of NK-like cells from *Bcl11b*-deficient DN1 or DN2 thymocytes appeared to be independent of Notch signaling, because $NKp46^+$ cells were readily produced from DN1 or DN2 thymocytes cultured on OP9 stromal cells without IL-2 or IL-15 (fig. S4F).

We subsequently deleted *Bcl11b* in DN3 thymocytes. Again, stromal cell–killing $NKp46^+CD3^-$ cells appeared (Fig. 1, B and C, and fig. S4G). The reprogramming also worked in myeloid or B cell culture media (fig. S4, H and I), which demonstrated that reprogramming to $NKp46^+$ cells was intrinsic to the *Bcl11b*-deficient thymocytes. To confirm the T cell origin of $NKp46^+CD3^-$ cells, we examined their *TCRβ* locus. These cells exhibited TCRβ V(D)J recombination [recombination of the variable (V), diversity (D), and joining (J) gene segments] even though TCRβ was not expressed (Fig. 1D). We therefore

named these killer cells that were reprogrammed from T cells induced T–to–natural killer or ITNK cells.

We next compared, using microarray analysis, the expression profiles of DN3 thymocytes; normal splenic NK cells that were expanded in vitro after enrichment (lymphokine-activated killer, or LAK cells, composed of >90% NK cells); and ITNKs reprogrammed from DN3 cells (Fig. 1E). Consistent with the killing ability of ITNK cells, their expression profile was more similar to that of LAK cells than to their parental DN3 thymocytes. QRT-PCR validation showed that expression of many T-lineage genes—such as *Notch1, Est1, Hes1, Gata3, Dtx1*, and *Tcf1*—was decreased, whereas expression of genes usually associated with NK cells—such as *Id2* (14), *IL2rβ* (*CD122*), *Zfp105* (15), and *E4bp4* (16)—was up-regulated (Fig. 1F and table S1). Zbtb32 (Rog, Repressor of GATA), which is not normally expressed in DN3 cells but plays important roles in regulating T cell activation and suppresses Gata3 activity (17), was highly expressed in ITNKs. Expression of *Cdkn1c* (*p57KIP2*), a putative direct downstream target gene of Bcl11b (18), was also drastically increased in ITNKs (Fig. 1F). These results collectively demonstrated that Bcl11b was essential for maintaining the T cell expression profile and for suppressing NK cell gene expression.

ITNKs could also be produced from mature T cells. We OHT-treated sorted DP thymocytes, $CD4^+$ and $CD8^+$ T cells, and γδ T cells from *flox/flox* mice. Many ITNKs ($NKp46^+$) were found growing in DP thymocytes and $CD8^+$ T cell cultures (fig. S4, K and L), which effectively killed stromal cells. These ITNKs, in contrast to those reprogrammed from DN1 to DN3 thymocytes, retained TCRβ on the cell surface. We were unable to obtain consistent production of $NKp46^+$cells from splenic or thymic $CD4^+$ T cells, or from γδ T cells, because these cells appeared prone to cell death in vitro once *Bcl11b* was deleted.

To estimate the reprogramming efficiency, we sorted single DN3 thymocytes from OHT-treated *flox/flox* thymocytes into individual wells of 96-well plates preseeded with OP9-DL1 stromal cells in T cell medium (fig. S5A). Out of the 79 wells that had cells growing, 36 wells had many fast-proliferating T cells (Fig. 2A), which deleted only one *flox Bcl11b* allele (Fig. 2B, lanes T1 and T2). These cells (*flox/−*) nevertheless served as excellent controls for Cre toxicity because they had activated Cre recombinase. In the other 43 wells, thymocytes were reprogrammed to $NKp46^+$ stromal cell–killing ITNKs (Fig. 2A). IL-2 was clearly able to greatly promote proliferation of ITNKs, because, from one DN3 thymocyte, up to 0.5 million ITNKs were obtained with IL-2, but only ~50,000 cells without IL-2. All ITNK cells had lost both *Bcl11b* alleles (Fig. 2B, lanes I1 and I2), and ITNKs of individual wells had unique rearranged TCRβ loci, which confirmed their independent origins (Fig. 2C). Therefore, once *Bcl11b* was deleted, the reprogramming efficiency of DN3 thymocytes to ITNKs could reach 100%. ITNKs from DN3 thymocytes not only expressed NK cell surface receptors and had similar cytotoxic functions, but were morphologically similar to LAK cells. ITNKs were larger than thymocytes and had granules and showed evidence of high protein-synthesis activity with abundant endoplasmic reticulum (Fig. 2, D and E). Besides NKp46, ITNKs expressed NKG2A/C/E, TRAIL, perforin, and interferon-γ, but not some other key NK cell function genes, such as members of the Ly49 family (fig. S5, B and C). Similar observations were made with in vitro reprogrammed ITNK cells from DP thymocytes (table S2 and fig. S5D). ITNKs were unlikely to be related to thymic NK cells because they did not express CD127 (fig. S5E). Moreover, unlike conventional mature NK cells, most ITNKs did not express CD11b, rather, they expressed CD27 and retained their killing ability even after being cultured in vitro for 1 month (fig. S5F).

We next measured the killing ability of the DN3-reprogrammed ITNKs by performing standard ^{51}Cr-release assays with three cell lines: B16F10 melanoma, which are MHC class

I (MHC I) low or negative (19); RMA lymphoma, which express MHC; and RMA-S lymphoma (TAP-1–deficient variant), which have reduced MHC I presentation (20, 21). LAK cells generally only killed MHC I–negative cells (Fig. 2F). Similar to LAKs, ITNKs also selectively killed MHC I–negative B16F10 and RMA-S cells, although they had slightly lower killing potency (Fig. 2F).

To exclude the possibility that ITNKs were in vitro artifacts, we deleted *Bcl11b* in vivo (fig. S6A). Two to 3 weeks after OHT treatment, ITNKs were detected in both the spleen ($NKp46^+CD3^+$) and the thymus ($NKp46^+$) from *flox/flox* mice but not from the *flox/+* controls (Fig. 3A). *Bcl11b* was found deleted in these in vivo reprogrammed ITNKs (fig. S6B). Both $CD4^+$ and $CD8^+$ ITNKs ($NKp46^+$) were found (fig. S6C). Some wild-type γδ T cells expressed NKp46; however, *Bcl11b* deletion caused a threefold increase in the $NKp46^+$ γδ T cells (Fig. 3B), which suggested that all T cell populations have reprogramming potential. The in vivo reprogrammed ITNKs could readily be expanded in NK culture conditions (fig. S6D), but they were not NKT cells (fig. S6, E and F). Besides expressing NK cell–associated genes, the in vivo reprogrammed ITNKs also lost or decreased some key T cell genes such as *Il7r, Tbx21* (*T-bet*), and *Cd8a* (fig. S6G). Consequently, TCR signaling in ITNKs appeared to be compromised (fig. S6H).

The in vivo analysis of ITNKs in *flox/flox* mice was complicated by the presence of many host T cells and NK cells (Fig. 3A). To address this problem and also to investigate whether in vivo reprogramming upon *Bcl11b* loss is cell autonomous, we transplanted OHT-treated DP thymocytes from *flox/flox* mice ($CD45.2^+$) into $Rag2^{-/-}Il2rg^{-/-}$ mice ($CD45.1^+$) that lack B, T, and NK cells (fig. S7A) (22). We chose DP thymocytes because they usually account for more than 75% of total thymocytes and could be efficiently reprogrammed in vitro to ITNKs (fig. S4K). Two weeks after transplantation, ~5% of splenocytes were found to be from the donor cells ($CD45.2^+$) (Fig. 3C), and ~47% of them expressed NKp46 and thus were ITNKs. ITNKs lost both copies of *Bcl11b*, and the majority of them expressed CD8 (fig. S7, B and C). The other 53% of cells ($NKp46^-$) were T cells and still retained the *Bcl11b* floxed allele (fig. S7C). ITNKs were also found in the bone marrow and peripheral blood (fig. S7D). We estimated that there were ~200,000 ITNK cells in the spleen. No $NKp46^+$ cells were found in control mice transplanted with untreated DP thymocytes (Fig. 3C). ITNK cells were maintained in the recipients for at least 3 months without change in cell number, perhaps representing a dynamic balance in their numbers. The recipient mice did not show any noticeable abnormality, which indicated that ITNK cells did not indiscriminately kill normal cells, nor were they malignantly transformed.

Compared with those reprogrammed in vitro, the in vivo reprogrammed ITNKs expressed NK surface receptors of the Ly49 family, including Ly49C/I and Ly49G2 (fig. S7E) (table S2), and could be extensively expanded ex vivo with IL-2 or IL-15 for at least 3 weeks while still retaining their killing ability (Fig. 3D).

The ex vivo expanded ITNKs were then assessed for their tumor cell–killing capability. These cells exhibited elevated cytotoxic potential and were also generally more potent than both in vitro ITNKs and LAKs against each of the target cells (Fig. 3E and Fig. 2F). Moreover, they killed RMA cells with almost the same efficiency as they killed RMA-S cells (Fig. 3E), despite expression of some inhibitory Ly49 receptors, which recognize MHC I.

Transplantable murine melanoma B16 cell lines are well-established models for studying experimental cancer therapies and NK cell tumor surveillance function (23). Injection of B16 cells into $Rag2^{-/-}Il2rg^{-/-}$ mice leads to rapid formation of metastatic foci in the lungs (24). To investigate the tumor-killing ability of the ITNK cells in vivo, we injected 2 million

OHT-treated or untreated DP thymocytes from *flox/flox* mice into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients to allow reprogramming of thymocytes to ITNKs in vivo (fig. S7F). Two weeks later, each recipient was injected with B16F10 melanoma cells. Four weeks after the initial thymocyte transplantation, recipients were sacrificed and analyzed. Mice injected with phosphate-buffered saline or with untreated DP cells had ~200 metastatic foci in the lungs. In contrast, mice injected with OHT-treated DP thymocytes had ~20 tumor colonies in the lung (Fig. 3F and fig. S7G). Therefore, ITNKs were potent killers of tumor cells in vivo and prevented cancer progression.

Deletion of *Bcl11b* led to the rapid disappearance of Bcl11b protein (Fig. 4A). Microarray analysis showed that in OHT-treated *flox/flox* thymocytes, expression of T cell genes such as TCRβ and CD3 was already down-regulated within 24 hours (table S3). In another 24 hours, many genes associated with NK cells were expressed (table S3). Bcl11b is proposed to be regulated by Notch signaling in T cell development (25). The *Drosophila* ortholog of Bcl11 genes, CG6530, is shown to be a direct downstream target gene of Notch signaling (26). To confirm this, we identified putative CSL-binding sites (CGTGGGAA) (27) at the *Bcl11b* locus, which were conserved between mouse and human Bcl11b genes (Fig. 4B) (table S4). A chromatin immunoprecipitation (ChIP) assay performed with a CSL polyclonal antibody pulled down genomic DNA fragments from T cells. Three genomic regions were greatly enriched in the T cell samples when we used the CSL antibody compared with the control (Fig. 4C). The ChIP result thus confirmed that the canonical Notch signaling directly regulated Bcl11b in T cells (fig. S8).

NK cell–based therapies hold promise in cancer treatment. We are now able to reprogramme T cells to ITNKs, which can be extensively expanded but are not malignantly transformed. Rather, they effectively killed tumor cells in vitro and eliminated metastatic cells in mice but did not appear to attack normal cells. Therefore, ITNK cells may serve as a new cell source for cancer immunotherapy and other cell-based therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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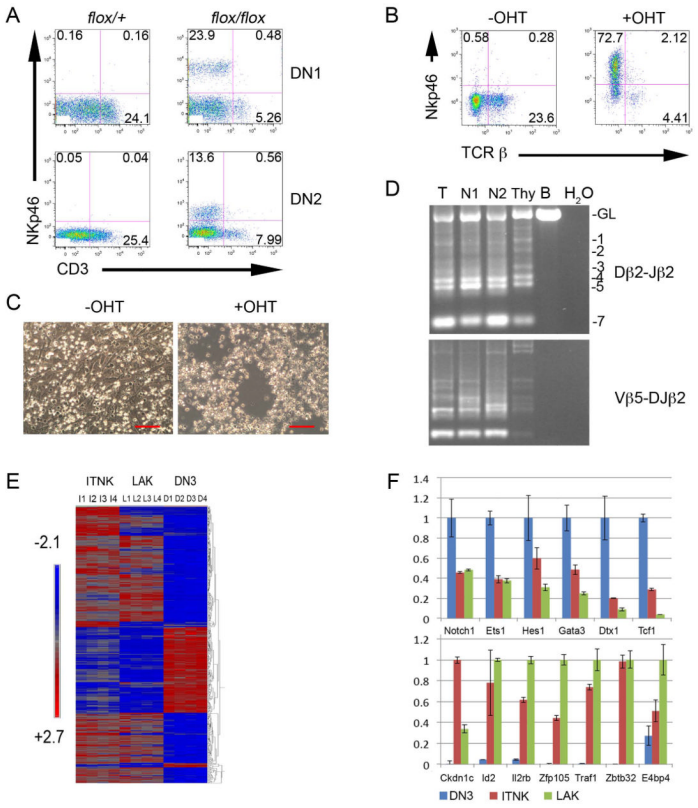

Fig. 1.

Bcl11b is essential for T cell development and for maintaining T cell identity. Thymocytes from *flox/flox* or *flox*/+ control mice were treated, or not, with OHT then sorted into DN1 or DN2 subsets, and cultured on OP9-DL1 stromal cells. (**A**) Flow cytometry profiles of cultured DN1 and DN2 thymocytes (+OHT) in the absence of IL-2. Numbers refer to percentage of cells in the gate. Data are representative of three experiments. (**B**) Flow cytometry profiles of cultured *flox/flox* DN3 thymocytes (±OHT) supplemented with IL-2. Data are representative of three experiments. (**C**) Killing of OP9-DLI stromal cells by OHT-treated *flox/flox* DN3 thymocytes. Scale bar, 40 μm. (**D**) DNA from purified NKp46^{+} cells was prepared and subjected to PCR to detect DJ (top) and V(D)J (bottom) recombination at the TCRβ locus. T, T cells growing from untreated DN3 thymocytes; N1 and N2, sorted NKp46^{+} cells growing from OHT-treated *flox/flox* DN3 thymocytes; Thy, wild-type whole thymocytes; B, B cells; and GL, germline band. H_2O: no DNA template in PCR. Numbers indicate DJ recombination products. (**E** and **F**) Microarray analysis of gene expression in NKp46^{+}CD3^{-} ITNK cells from DN3 thymocytes (columns I1 to I4), IL-2–expanded NK cells (LAK; L1 to L4) and sorted DN3 *flox/flox* thymocytes (DN3; D1 to D4) were subjected to expression. (E) Two-way hierarchical cluster map of the array data. Column numbers (I1 to I4 for instance) refer to four independent RNA samples for each cell type, and rows represent individual transcripts. Scale indicates the $\log_2$ value of normalized signal level. (F) QRT-PCR validation of gene expression of selected genes among ITNKs, LAKs, and DN3 cells. Bars are means ± SD of three samples.

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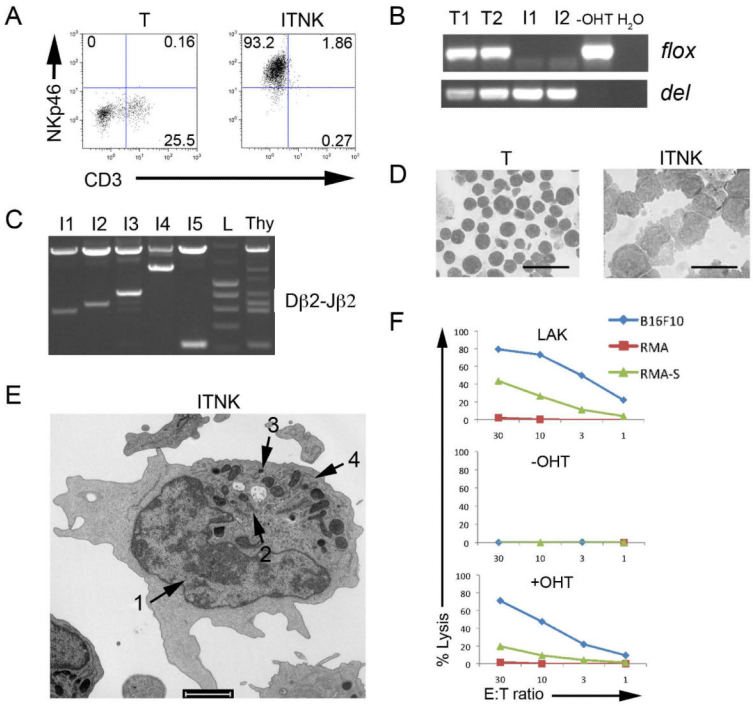

Fig. 2.

Efficient reprogramming of T cells to ITNKs. (**A**) Representative flow cytometry profiles of ITNKs reprogrammed from single *flox/flox* DN3 cells. Numbers refer to percentage in total cells. T: T cells that did not have complete *Bcl11b* deletion. Data are representative of three experiments. (**B**) PCR genotyping of *Bcl11b* deletion in two representative T cell (T1 and T2) and ITNK (I1 and I2) wells. *flox*, floxed allele; del, deletion allele. −OHT: no OHT treatment; H_2O: no template control. (**C**) DJ recombination at the TCRβ locus of five ITNK wells (I1 to I5) showing unique DJ recombination. L, DNA ladder; Thy, wild-type thymocytes. (**D**) Giemsa stain of parental DN3 thymocytes (T) and ITNK cells. Scale bar, 20 μm. (**E**) Transmission electron micrograph of an ITNK cell. 1, Nucleus; 2, Golgi body; 3, granule; 4, endoplasmic reticulum. Scale bar, 2 μm. (**F**) Cytotoxicity of ITNKs (labeled as "+OHT") and LAKs measured in standard ^{51}Cr-release assays with B16F10, RMA, and RMA-S tumor cell targets at the indicated effector-to-target (E:T) ratios. −OHT: *flox/flox* T cells. Data are means of triplicate wells.

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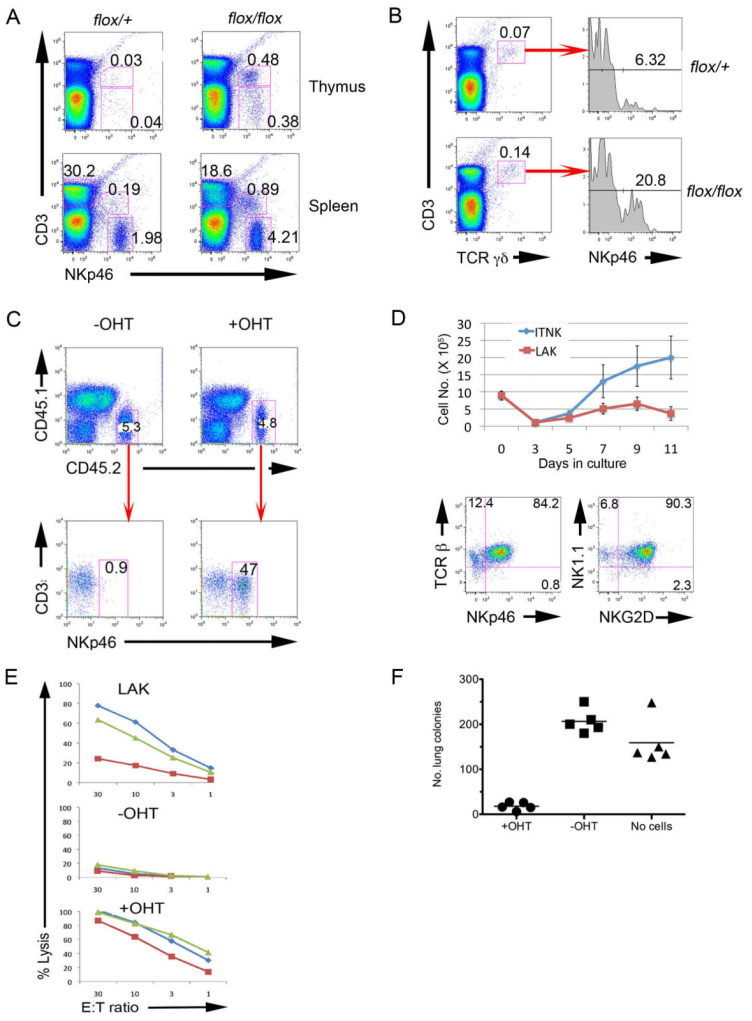

Fig. 3.

ITNKs reprogrammed in vivo are potent tumor cell killers. (**A**) Flow cytometric analysis of thymocytes and splenocytes from OHT-treated *flox/flox* and *flox*/+ mice. Numbers refer to the percentage in the lymphocyte gate. Data are representative of four mice. (**B**) Analysis of ITNKs from thymic γδ T cells in OHT-treated *flox/flox* mice. Data are representative of two mice. (**C**) ITNKs production in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients injected with *flox/flox* DP thymocytes. Two weeks after injection, donor (CD45.2^{+}) and host (CD45.1^{+}) splenocytes were analyzed. Numbers refer to the percentage of lymphocyte gate. Plots are representative of 15 mice from three independent experiments. (**D**) Ex vivo expansion of ITNKs in IL-2 from splenocytes of the recipient mice. Viable cells were counted (top) at the indicated time points and analyzed (bottom). Numbers refer to percentages. Most cells in the culture were ITNKs because they expressed NKp46, TCRβ, NK1.1, and NKG2D. Bars are means ± SD of four samples. Data are representative of three experiments. (**E**) The ex vivo expanded ITNKs (labeled as "+OHT") were used in ^{51}Cr-release killing assays with B16F10, RMA, and RMA-S tumor cell targets at the indicated effector-to-target (E:T) ratios. −OHT: *flox/flox* T cells. Data are means of triplicate wells. Results are representative of three experiments. (**F**) ITNKs prevented tumor metastasis. *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients were first transplanted with treated (+OHT) or untreated (−OHT) *flox/flox* DP thymocytes or phosphate-buffered saline. Recipients were subsequently injected intravenously with 50,000

B16F10 melanoma cells. Lung tumor colonies were enumerated 2 weeks after tumor challenge. Data are from individual mice, and bars represent the means.

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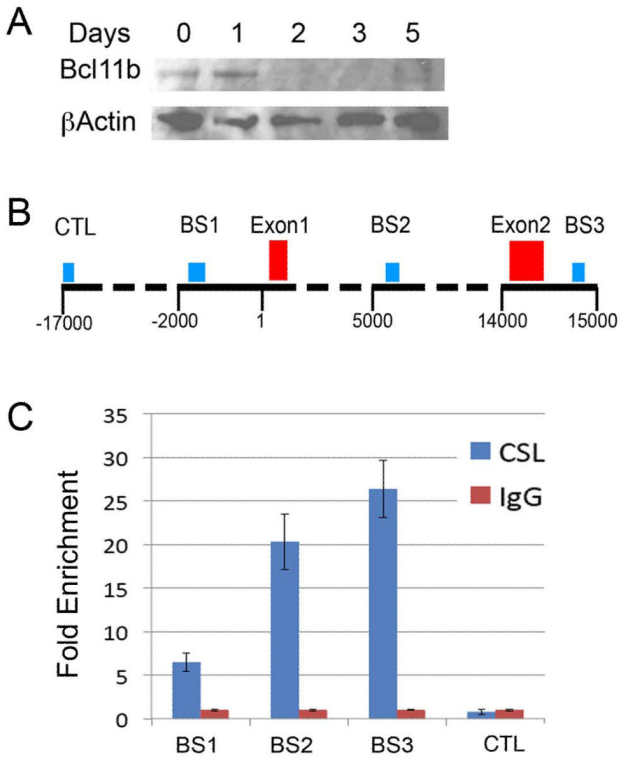

Fig. 4.

Bcl11b is a direct downstream target gene of Notch signaling. (**A**) *Bcl11b* protein in T cells after OHT treatment detected by Western blot. (**B**) Schematic of the *Bcl11b* locus showing putative CSL binding sites (BS) and that of an irrelevant control binding site (CTL). (**C**) Genomic DNA was prepared from immunoprecipitation of thymocytes, by using CSL or control immunoglobulin G (IgG) antibodies, and was amplified by using primers flanking the putative CSL or the control binding sites at the *Bcl11b* locus. Three *Bcl11b*-binding regions: Region 1, about 1.8 kb from start of the transcription; region 2, 5.4 kb downstream of exon 1; region 3, about 600 base pairs downstream of exon 2. CSL, CSL antibody; IgG, control IgG. Fold-enrichment was calculated relative to the IgG control (set to 1). Bars are means ± SD of triplicate samples.