Innate T cells, including invariant natural killer T (iNKT) and mucosal-associated innate T (MAIT) cells, are a heterogeneous T lymphocyte population with effector properties preprogrammed during their thymic differentiation. How this program is initiated is currently unclear. Here, we show that the transcription factor BCL-6 was transiently expressed in iNKT cells upon exit from positive selection and was required for their proper development beyond stage 0. Notably, development of MAIT cells was also impaired in the absence of Bcl6. BCL-6-deficient iNKT cells had reduced expression of genes that were associated with the innate T cell lineage, including Zbtb16, which encodes PLZF, and PLZF-targeted genes. BCL-6 contributed to a chromatin accessibility landscape that was permissive for the expression of development-related genes and inhibitory for genes associated with naïve T cell programs. Our results revealed new functions for BCL-6 and illuminated how this transcription factor controls early iNKT cell development.
determine early iNKT cell development, we performed a global gene expression analysis in ST0 and ST1 iNKT cells. Pooled thymi from wild-type mice were magnetically enriched using CD1D tetramer, and were sorted according to their expression of CD24 and CD44 (Extended Data Fig. 1a). Our analysis showed that 764 genes were differentially expressed by at least twofold (DESeq, \( P < 0.05 \)) between ST0 and ST1 cells (Fig. 1a). Metascape analysis showed the pathways enriched among the differentially expressed genes (DEGs) (Fig. 1b). To gain further insight into the gene programs that were affected during progression from ST0 to ST1, we performed gene set enrichment analysis (GSEA)\(^7\), which revealed that ST1 cells were enriched for genes upregulated in memory CD8\(^{+}\) and CD4\(^{+}\) T cells compared to their naive counterparts (Fig. 1c). Thus, ST1 cells have a gene expression program distinct from their ST0 progenitors, which includes induction of genes associated with memory T cell transcriptional programs.

Our analysis revealed that genes associated with the earliest iNKT progenitors were highly expressed in ST0 cells, including \( Cd24a \), \( Cd8a \), \( Nr4a1 \) (encoding NUR77) and \( Cd69 \), and were all downregulated in ST1 cells (Fig. 1d). Several transcription factors that were associated with iNKT cell development (differentiation-associated genes) were upregulated during transition from ST0 to ST1.

**Fig. 1** The gene expression profile of ST0 and ST1 iNKT cells. a, Volcano plot showing genes that were differentially expressed between ST0 (sorted as tetramer\(^{-}\)TCR\(^{b+}\)CD24\(^{+}\)CD44\(^{-}\)) and ST1 (tetramer\(^{-}\)TCR\(^{b+}\)CD24\(^{-}\)CD44\(^{-}\)) iNKT cells. Lines indicate a twofold difference between ST0 and ST1 cells (DESeq, \( P < 0.05 \)). FC, fold change. b, Metascape analysis showing the pathways enriched among the genes that were differentially expressed. Metascape analysis was performed with a hypergeometric test coupled with Benjamini-Hochberg \( P \) value correction algorithm. c, GSEA analysis of RNA-seq data showing enrichment of T cell memory–related genes in ST1 cells as compared to ST0 cells. Gene sets were obtained from MSigDB for memory CD8\(^{+}\) (ref. \(^{49}\)) and memory CD4\(^{+}\) (ref. \(^{49}\)) (GSE11057) T cells. Normalized enrichment scores (NES) and false discovery rate (FDR) as implemented by GSEA, based on 1,000 permutations. d, e, Heat maps showing expression of select transcription factors (d, top) and known ST0-expressed genes (d, bottom) and cytokines, chemokines and their receptors (e) in RNA-seq data. \( n = 3 \) independent experiments with 4–6 pooled thymi each from 4–5-week-old wild-type mice. Differential gene expression analysis was performed with the DESeq algorithm. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \).
For example, Zbtb16 (encoding PLZF) and Id2 significantly increased in ST1 cells. In addition, PLZF target genes such as Runx3, Maf and Rora were expressed in ST1 but not in ST0 cells. Irf4, which is involved in the differentiation of conventional CD4+ T cell lineages, also increased in ST1 cells. Moreover, Zbtb7b (encoding Th-POK), a regulator of iNKT cell differentiation, was induced in ST1 cells. Expression of Tbx21 (encoding T-BET), which is highly expressed in mature NKT1 cells, started to be expressed in ST1 cells, consistent with the low expression of T-BET protein at this stage (Extended Data Fig. 1b). In addition to relevant transcription factors, ST1 cells exhibited higher expression of genes encoding multiple cytokines and cytokine receptors associated with mature NKT1 cells (Fig. 1c). In contrast, genes encoding receptors for immunoregulatory cytokines (for example Il10ra and Il4ra) were downregulated in ST1 cells. Taken together, these results revealed that a maturation program related to NKT1 cells emerged at the earliest immature stages of iNKT cell development in C57BL/6J mice.

Bach2, encoding a transcription factor associated with the maintenance of a naive T cell state that is downregulated in effector cells, was highly expressed in ST0, as compared to ST1 cells, in agreement with the activated T cell phenotype of iNKT cells (Fig. 1d). Genes encoding transcription factors related to innate lymphoid progenitors and early T cell precursors were preferentially expressed in ST0 cells, including Tox, Gfi-1, Runx1 and Sox4 (ref. 26). These genes were coexpressed in ST0 cells with other relevant transcription factor genes, including Tcf7, Gata3, Ets1 and Bcl11b, which continued to be expressed in ST1 cells (Fig. 1d). Genes encoding transcription factors related to TCR activation and co-stimulatory molecules (including Themis, Egr1, Egr2, Myb, Bila, Cd28, Cd69 and Rasgrp) were also highly transcribed in ST0 cells, probably as a consequence of iNKT cell positive selection and were downregulated as

**Fig. 2 | BCL-6 is expressed specifically in ST0 iNKT cells.** a, Graph showing average Bcl6 mRNA expression in the indicated fluorescence-activated cell sorting (FACS) populations. n = 3 independent experiments with 3–5 pooled thymi each from 4–5-week-old mice. Data represent mean ± s.e.m. b, Flow cytometry histograms showing expression of BCL-6 protein in the indicated populations. c, Flow cytometry plots showing expression of BCL-6 and CD24 in CD44−iNKT cells after magnetic bead-based enrichment. d, BCL-6 expression in pre-DP (CD69−TCRβ−CD4+CD8+), post-DP (CD69+TCRβ−CD4+CD8+) and ST0 iNKT cells. e, Graph showing gMFI of BCL-6 in the indicated thymic populations. Data represent mean ± s.e.m; n = 6 independent experiments. Statistical analysis was performed with a two-tailed unpaired Student’s t-test **P < 0.01, ***P < 0.001.
cells transitioned to ST1. Therefore, ST0 cells were characterized by expression of genes that were associated with adaptive and innate lymphocyte progenitors or naive T cells, consistent with their developmental potential.

**BCL-6 is expressed in ST0 iNKT cells.** We noticed that Bcl6 was highly expressed in ST0 cells, but its expression was lost in ST1 cells (Fig. 1d and Extended Data Fig. 1c) and this was confirmed by qPCR (Fig. 2a). Analysis of conventional thymocyte subsets showed that Bcl6 was highly expressed in preselected CD4+CD8+ double-positive (DP) cells, whereas it was dramatically reduced in post-selected DP and thymic CD4+ T cells (Fig. 2a).

BCL-6 protein mirrored messenger RNA expression; BCL-6 was highly expressed in DP cells but not in other conventional thymic...
subsets or in total thymic iNKT cells (Fig. 2b). CD69+ and EGR-2+ ST0 iNKT cells expressed high amounts of BCL-6, whereas BCL-6 was downregulated in ST1 cells (Fig. 2c and Extended Data Fig. 1d). ST0 cells expressed slightly higher amounts of BCL-6 than preselected DP cells (Fig. 2d,e). These observations indicated that ST0 iNKT cells, in contrast to conventionally post-selected DP cells and other iNKT cell subsets, exhibited high BCL-6 protein and Bcl6 mRNA.
BCL-6 was required for iNKT cell development. To investigate the function of BCL-6 in iNKT cells, we crossed Cd4^cre mice with Bcl6^f/f mice on the C57BL/6J background to generate mice that lack BCL-6, starting from the DP stage of thymic development. Efficient Bcl6 deletion in iNKT cells was confirmed in Cd4^cre Bcl6^f/+ (Extended Data Fig. 2a). Bcl6^+/Δ thymi exhibited a large reduction in the number and percentage of iNKT cells (Fig. 3a,b). iNKT cells were substantially reduced in peripheral tissues including the liver and spleen of Bcl6^+/Δ mice (Fig. 3a,b). The lack of iNKT cells in the thymus and periphery was a cell-intrinsic consequence of BCL-6 deficiency, as revealed by competitive bone marrow chimeras (Fig. 3c and Extended Data Fig. 2b,c). To investigate whether BCL-6 had a broad effect on development of innate-like T cell populations, we examined MAIT cells, which largely failed to...
Impaired progression from ST0 to ST1 in the absence of BCL-6. a, Volcano plots showing DEGs (twofold change, DESeq, *P < 0.05) between control and Bcl6Δ/Δ mice in ST0 and ST1 cells, based on RNA-seq data. b, Venn diagram showing genes that are both dependent on BCL-6 in ST0 and change their expression when transitioning from ST0 to ST1. Numbers in each compartment indicate the number of genes in that compartment. The percentage indicates the percentage of common genes relative to all ST0-specific genes (69 of 425). c, Scatter plots showing DEGs in the indicated comparisons. Dashed lines indicate twofold change thresholds. Numbers in each compartment indicate the number of genes in that compartment. The percentage indicates the percentage of common genes relative to all ST0-specific genes (33 of 339). NKT0-associated genes in ST1 cells as compared to ST0 cells. d, GSEA analysis showing enrichment of NKT1-associated genes in ST1 cells as compared to wild-type ST1 cells. NES and FDR are shown, as implemented by GSEA, and are based on 1,000 permutations. Statistical analysis of the overlaps in the Venn diagrams (b,d,f) was performed with a permutation test after 10,000 resamplings; *n = 3 independent experiments, with 4–6 pooled mice for each genotype. *** *P < 0.0001.

devlop in Bcl6Δ/Δ thymi (Fig. 3d). In contrast, conventional T cell development was normal in the absence of BCL-6 (Extended Data Fig. 3a,b), consistent with published results. Therefore, BCL-6 was essential for the development of innate-like T cells in the thymus. Accumulation of ST0 cells in BCL-6-deficient thymi. Because BCL-6 was expressed in both preselected DP cells and in ST0 iNKT cells, it was possible that BCL-6 influenced either selection of iNKT cells in the DP stage or iNKT cell development after
commitment to the innate T cell lineage. To distinguish between these possibilities, we first performed a thorough analysis of iNKT developmental stages in Bcl6Δ/Δ animals. To accurately calculate rare ST0 cells, we performed magnetic bead–based enrichment of thymic iNKT cells before flow cytometry (Fig. 4a and Extended Data Fig. 2d). Our analysis revealed that the number and percentage of ST0 iNKT cells increased in the absence of BCL-6 (Fig. 4b), indicating that iNKT cell positive selection was not altered. Corroborating these results, expression of several selecting molecules, including CD1D, CD150, LY108 and Sh2d1a, expression of Tcra Vα14-Jα18 transcripts and the number of DP cells were normal in Bcl6Δ/Δ mice (Extended Data Fig. 3b). In contrast, the numbers of ST1, ST2 and ST3 iNKT cells were severely reduced (Fig. 4c). However, the frequency of these populations among total iNKT cells was not altered in the absence of BCL-6, although ST1 cells were slightly enriched (Fig. 4c). Mixed bone chimeras also showed enrichment of Bcl6Δ/Δ ST0 and ST1 cells, with a concomitant loss of more mature cells, compared to their wild-type competitors (Fig. 4d,e and Extended Data Fig. 2e,f). Taken together, our results indicate that the developmental pathway of Bcl6Δ/Δ iNKT cells was blocked at ST0/ST1.

To examine whether BCL-6 influenced the fate of NKT1, NKT2 and NKT17 mature populations, we analyzed the thymi and the spleens of Bcl6Δ/Δ mice by flow cytometry for the expression of Tγ, related signature transcription factors. Our analysis revealed that the number of all mature iNKT subsets was reduced in Bcl6Δ/Δ mice (Fig. 5a,b and Extended Data Fig. 4). However, the frequency of NKT1 and NKT2 cells among the remaining iNKT cells was not altered, whereas the frequency of NKT17 cells was reduced in the absence of BCL-6 (Fig. 5a,b), consistent with the reduced expression of Rorc in ST1 Bcl6Δ/Δ iNKT cells (Extended Data Fig. 4c).

Our results show that the iNKT cell population in Bcl6Δ/Δ thymi was enriched for immature subsets, especially ST0 cells. To investigate the functional consequences of BCL-6 deficiency, we determined whether these cells were able to produce cytokines after phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation. The number of iNKT cells producing interferon (IFN)-γ, IL-4 and IL-17A was reduced in Bcl6Δ/Δ mice, consistent with their reduced total iNKT cell number (Fig. 5c,d). We also found a reduced frequency of cells producing IFN-γ and IL-17A, consistent with the reduced frequency of mature iNKT cells. However, the frequency of IL-4-producing cells was unaffected, in agreement with previous results showing that immature CD44+CD247+ iNKT cells were able to produce IL-4 (Fig. 5c,d)12,13. These findings were consistent with our phenotypic analysis and suggested an enrichment of immature cells among iNKT cells in the Bcl6Δ/Δ thymus.

**BCL-6-deficient iNKT cells showed normal proliferation and apoptosis.** Immature iNKT cells undergo a vigorous intrathymic proliferation phase that ultimately leads to an expansion of mature populations. Therefore, the loss of Bcl6Δ/Δ iNKT cells may be due to an impaired proliferative capacity. However, Ki67 staining and 5-ethynyl-2'-deoxyuridine (EdU) incorporation showed that Bcl6Δ/Δ iNKT cells did not have a proliferation defect (Extended Data Fig. 5a). In fact, a higher percentage of Bcl6Δ/Δ iNKT cells were EdU+/Ki67+ in ST1, compared to their control littersmates, consistent with increased expression of IL-7R and Lef1, two regulators of iNKT cell proliferation19 (Extended Data Fig. 5b). Bcl6Δ/Δ iNKT cells showed no evidence of increased apoptosis, as assessed ex vivo by staining with fluorescent pan-caspase inhibitor (Extended Data Fig. 5c). Therefore, the loss of iNKT cells in the absence of BCL-6 was not due to altered proliferation or apoptosis.

We noted that BCL-6–deficient adult mice still contained a few mature iNKT cells and hypothesized that increased proliferation may explain the appearance of these cells. Indeed, 10-day-old Bcl6Δ/Δ mice had a greater decrease in iNKT cell numbers in the thymus compared to adult Bcl6Δ/Δ mice (Extended Data Fig. 5d,e), indicating that iNKT cell numbers may increase with age due to their increased proliferation capacity.

**Progression from ST0 to ST1 required BCL-6.** We hypothesized that impaired development of BCL-6-deficient iNKT cells was due

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**Fig. 7 | BCL-6 is required for repression of a subset of PLZF target genes.**

**a.** Scatter plot showing the expression of PLZF target genes in the Bcl6Δ/Δ ST1 iNKT cells. Numbers in each quadrant indicate the number of genes in that quadrant. **b.** qPCR analysis showing Bach2 mRNA expression in sorted ST1 iNKT cells from control and Bcl6Δ/Δ mice. Data indicate mean fold change over the reference gene ± s.e.m., n = 3 independent experiments. **c, d.** FACS plots showing the expression of CD62L (c) and CD69 (d) in ST1 iNKT cells from the indicated mouse strains. Data represent the mean percentage ± s.e.m. of the indicated subset among ST1 cells, n = 5 (CD62L) and n = 3 (CD69) independent experiments. **e.** Histograms showing expression of ICOS in ST1 iNKT cells from the indicated mouse strains. Graphs show the mean gMFI of ICOS ± s.e.m. in control and Bcl6Δ/Δ ST1 iNKT cells, n = 5 independent experiments. Statistical analysis was performed with a two-tailed unpaired Student’s t-test (b–d) or one-sample two-tailed Student’s t-test (e). *P < 0.05, **P < 0.01.
to an early differentiation defect. To investigate this hypothesis, we examined the expression of the iNKT signature transcription factor PLZF at early stages of development. qPCR analysis from sorted ST0 and ST1 cells revealed that Zbtb16 mRNA was impaired in ST0 and ST1 Bcl6 Δ/Δ cells (Extended Data Fig. 6a). While approximately 15% of ST0 cells from control animals expressed PLZF, only 5% of Bcl6 Δ/Δ ST0 cells were PLZF-positive, as assessed by flow cytometry (Extended Data Fig. 6b). PLZF geometric mean fluorescence intensity (gMFI) was reduced by half in the absence of BCL-6 (Extended Data Fig. 6c). Consequently, only 60% of Bcl6 Δ/Δ ST1 cells were PLZF-positive, whereas 90% of ST1 cells in control littermates expressed PLZF (Extended Data Fig. 6d). However, PLZF was expressed appropriately in subsequent developmental stages (Extended Data Fig. 6d). These results indicated a blunted PLZF

Fig. 8 | Chromatin accessibility around developmentally regulated genes depends on BCL-6. a, Heat maps of ATAC-seq reads of DP, ST0, ST1 and mature NKT1 (mNKT) cells in wild-type mice. Statistical difference between profiles was estimated with a two-sided Kolmogorov–Smirnov test. b, Schematic diagram showing the number of ATAC-accessible regions in wild-type ST0 and ST1 iNKT cells. While the majority of regions were accessible both in ST0 and ST1 cells (number in the overlap), a number of regions were more accessible in ST0 cells (left circle) or in ST1 cells, based on a twofold differential accessibility (edgeR, P < 0.05). Percentages represent the percentage of peaks for the respective compartment relative to all peaks. c, Average genomic profiles of ATAC-seq reads of ST0 and ST1 cells in the indicated mouse strains. d, Scatter plot showing accessibility of differentially accessible (DA) loci between wild-type ST0 and ST1 in Bcl6 Δ/Δ ST1 cells. Numbers in each quadrant indicate the number of peaks in that quadrant. e, Dendrogram showing hierarchical clustering analysis of accessible loci in the indicated populations. f, Genome track view of the Zbtb16 locus in wild-type and Bcl6 Δ/Δ ST0 cells. g, Histograms showing the percentage of the developmentally regulated genes whose accessibility depends on BCL-6 in ST0 cells. Statistical analysis was performed with a permutation test after 10,000 resamplings; n = 2 independent experiments, with 4–6 pooled mice for each genotype. **P = 0.0023, ****P < 10^{-16}.
induction at the point of iNKT lineage specification after Bcl6 deletion, despite normal TCR signaling, as indicated by expression of CD5 and CD69, which are markers of TCR signal strength (Extended Data Fig. 6e).

To investigate how the gene expression program of early iNKT cell progenitors was influenced by deletion of Bcl6, we performed global transcriptomic analysis of ST0 and ST1 iNKT cells. Our analysis showed that 942 genes were differentially expressed between control and Bcl6Δ/Δ ST0 iNKT cells and 277 between control and Bcl6Δ/Δ ST1 cells (Fig. 6a). In both cell populations, 50% of DEGs were upregulated and 50% were downregulated in the absence of BCL-6. Of note, 21.5% of the DEGs between wild-type ST0 and ST1 showed altered expression in Bcl6Δ/Δ ST0 cells (Fig. 6b).

These genes belonged to pathways involved in regulation of leukocyte differentiation and effectors processes, cytokine production and cytokine–cytokine receptor interactions (Fig. 6c), which were also involved in iNKT cell differentiation. Interestingly, pathways involved in T cell activation were not regulated by BCL-6. Therefore, in ST0 cells, BCL-6 controlled a set of genes that contribute to iNKT cell differentiation.

Notably, 16.2% of the ST0-specific genes showed altered expression in BCL-6-deficient ST1 iNKT cells (Fig. 6d). The vast majority of these genes (62 of 69, 90%; Fig. 6e) increased expression in BCL-6-deficient ST1 cells, indicating that ST0-specific genes failed to be repressed in ST1 in the absence of BCL-6. In addition, 9.7% of the differentiation-associated genes were not properly induced in Bcl6Δ/Δ ST1 iNKT cells (Fig. 6f). Therefore, the differentiation program of iNKT cells is not fully deployed in the absence of BCL-6 and is also dissociated from downregulation of CD24.

To further explore this hypothesis on a global level, we performed GSEA analysis. In accordance with the known developmental trajectory of iNKT cells, wild-type ST1 cells were enriched for a gene signature associated with NKT1 cells, which constitute the vast majority of mature iNKT cells in C57BL/6 mice (Fig. 6g). Not surprisingly, wild-type ST0 cells were enriched for the NKT0 gene signature, defined as TetrCD24hiTCRβhiCD8αrCD44 NK1.1- cells (Extended Data Fig. 7a). Notably, Bcl6Δ/Δ ST1 cells showed a gene expression profile associated with NKT0 cells, but not NKT1 or NKT17 cells (Fig. 6h,i) and Extended Data Fig. 7b). However, Bcl6Δ/Δ ST1 cells were enriched for genes associated with the NKT2 signature (Extended Data Fig. 7c), consistent with previous reports showing that NKT2 cells resembled NKT0 progenitors. Taken together, these results demonstrate that progression of ST0 cells toward the ST1 developmental intermediate is dependent upon the function of BCL-6.

Because Bcl6 deletion affected expression of PLZF, we investigated whether PLZF target genes showed altered expression in Bcl6Δ/Δ iNKT cells. Our analysis of previously published microarrays confirmed that 134 genes were deregulated in the absence of PLZF in ST1 iNKT cells. Of those genes, 87 were also deregulated in Bcl6Δ/Δ ST1 cells by more than twofold, although only 22 genes were significantly changed (DESeq, P < 0.05, Fig. 7a). Notably, with the exception of five genes, all genes were concordantly deregulated in both BCL-6- and PLZF-deficient cells (Fig. 7a). Bach2, which is directly repressed by PLZF, was upregulated more than twofold in the absence of BCL-6, as confirmed by qPCR (Fig. 7b). L-selectin (CD62L), which is also repressed by PLZF and is a key homing receptor for naive T cells, was expressed at greater frequency in Bcl6Δ/Δ ST1 iNKT cells than in control littermate ST1 iNKT cells, as shown by flow cytometry (Fig. 7c). Similarly, CD69 was maintained on a greater percentage of Bcl6Δ/Δ ST1 iNKT cells (Fig. 7d).

Although our RNA-seq data did not reveal a decrease in Icos transcripts in the absence of BCL-6, flow cytometry analysis demonstrated that ICOS was reduced in Bcl6Δ/Δ ST1 iNKT cells (Fig. 7e). Our data revealed that PLZF expression was blunted at the point of iNKT cell specification and that multiple PLZF target genes failed to be repressed in the absence of BCL-6.

**BCL-6 regulated chromatin accessibility in ST0 iNKT cells.** To investigate whether the early stages of the iNKT maturation program were critical for determining the chromatin accessibility profile of mature iNKT cells, we performed assay for transposase-accessible chromatin (ATAC)-seq in ST0, ST1 and mature NKT1 (sorted as TetrCD24hiCD44NK1.1-) cells. To exclude loci that may be open transiently in ST0 cells due to positive selection and TCR signaling, we restricted our analysis in loci that were more accessible in mature NKT1 cells compared to DP thymocytes (iNKT-related regions). Our results showed that these regions were already accessible in ST0 cells (Fig. 8a and Extended Data Fig. 8a), indicating early opening of iNKT-related loci after iNKT cell lineage determination.

To gain further insight into how BCL-6 may regulate iNKT cell development, we examined chromatin accessibility profiles of ST0 and ST1 cells in the presence or absence of Bcl6 by ATAC-seq. Our analysis showed that 43,158 chromatin regions were open in wild-type ST0 and/or ST1 cells. The vast majority of these loci (37,227) were open in both ST0 and ST1 cells, including regions near key genes involved in iNKT cell development (Fig. 8b and Extended Data Fig. 8a). A total of 4,165 regions were more accessible in ST0 cells and 1,766 gained accessibility in ST1 cells (Fig. 8b).

ST0-accessible regions remained open in Bcl6Δ/Δ ST1 cells, whereas ST1-associated open chromatin regions were mostly closed in Bcl6Δ/Δ ST1 cells (Fig. 8c and Extended Data Fig. 8b). Specifically, 46% (1,915 of 4,165) of the ST0-accessible sites were still highly accessible in Bcl6Δ/Δ ST1 cells (Fig. 8d), including loci near genes associated with the naive T cell program, such as Bach2 and Sel (Extended Data Fig. 8c). In addition, 27.2% (481 of 1,766) of the ST1 accessible regions were less accessible in Bcl6Δ/Δ ST1 cells (Fig. 8d, Q3). Consistent with these results, hierarchical clustering analysis showed that the chromatin accessibility profile of Bcl6Δ/Δ ST1 cells clustered close to wild-type ST0 cells and away from wild-type ST1 cells (Fig. 8e). Collectively, these results indicated that BCL-6 shaped the chromatin accessibility profile of iNKT cells during transition from ST0 to ST1.

To investigate how BCL-6 may influence the chromatin landscape and gene expression of developing iNKT cells, we focused our chromatin analysis on the loci of genes with altered expression between wild-type ST0 and ST1 cells, which are thus relevant to iNKT cell development. Our analysis demonstrated that 16.2% (55 of 339) of differentiation-related genes (upregulated in wild-type ST1 compared to ST0) contained at least one region in which the accessibility was increased only in ST0 cells (Fig. 8f). Chromatin regions associated with several PLZF target genes or other important genes for iNKT cell biology were also affected, including Icos, Il18r1, Il18rap, Cxcr3, Maf, Il13 and Tbx21 (Extended Data Fig. 8d). In contrast, only 3.2% (11 of 339) of the differentiation-related genes contained at least one locus that was more accessible in wild-type ST0 cells than in wild-type ST1 cells, none of which were expressed in a BCL-6-dependent manner in ST0 cells (Fig. 8g). Therefore, BCL-6 was required, already at ST0, for increased chromatin accessibility near, and expression of, genes that were developmentally regulated in iNKT cells.

**Discussion**

The mechanisms that control the earliest stages of innate-like lymphocyte development are largely unknown. Here, we discovered that Bcl6 was highly expressed at the inception of iNKT cell development but downregulated by ST1, before the emergence of iNKT cell effector fates. We found a cell-intrinsic requirement
for BCL-6 for the development of iNKT and MAIT cells, whereas conventional T cell development was unperturbed. BCL-6 did not seem to influence positive selection of iNKT cells or the degree of Tcrα Vα14-Jα18 rearrangements in DP cells, despite its high expression at this stage and apoptosis and proliferation were not impacted by BCL-6 deletion. Our transcriptomic analysis showed that transition from ST0 to ST1 was associated with the emergence of gene programs associated with mature iNKT cells, such as genes for cytokines and cytokine receptors, homing molecules and T cell effector molecules and deployment of an innate-associated program. Indeed, a small fraction of ST0 cells upregulated PLZF, indicating that the innate T cell program may be specified even before CD24 is downregulated. Therefore, ST0 cells may be divided into two subsets, PLZF-negative ST0 (early ST0) and PLZF-positive ST0 cells (late ST0), which are becoming ST1 cells. In the absence of BCL-6, this program was not fully deployed and ST0 and ST1 iNKT cells had low expression of key innate T cell molecules, including PLZF and several PLZF target genes. In addition, naive T cell genes, such as Bach2, Lef1 and Sell, continued to be expressed in Bcl6ΔΔ ST1 cells. Therefore, BCL-6 promotes the PLZF-negative to PLZF-positive transition within ST0/ST1, placing BCL-6 as an essential early regulator of the innate T cell fate.

By combining RNA-seq and ATAC-seq in wild-type ST0 and ST1 cells, we found that innate-associated genes were induced in ST1 cells, while several loci surrounding these genes, including Zbbb16, Tbx21, Id2, Il4/Ili13, Ifng and Il2rb1, were already accessible in ST0 cells. In sharp contrast, these loci were not accessible in DP cells or naive CD4+ T cells, where they open only after T cell activation. These key loci had reduced accessibility in Bcl6ΔΔ ST0 iNKT cells, demonstrating that BCL-6 was required for the chromatin changes associated with induction of these innate genes. As differentiation proceeds toward ST1, several loci near genes associated with the naive T cell fate became less accessible. These regions remained accessible in Bcl6ΔΔ ST1 cells demonstrating that BCL-6 was necessary for their repression, either directly or indirectly. Because ST1 cells are heterogeneous (as indicated by the expression of T-BET and ROBY in a minor subset of cells), it remains unclear how each cell type may be affected by BCL-6 expression. Nonetheless, our results demonstrate that BCL-6 promotes a chromatin environment that is required to support the emergence of their ensuing innate program early in iNKT cell development.

Despite finding that PLZF fails to be properly induced in Bcl6ΔΔ ST0 cells, low expression of PLZF in a few ST0/ST1 may support their differentiation into effector subsets. In agreement with this, iNKT cells in mice with transiently reduced PLZF at early developmental stages showed comparable maturation to their littermate controls, despite their lower numbers. In addition, germline Bcl6−/− mice were shown to have near wild-type iNKT cell numbers in the spleen, in contrast to our results. However, those experiments were performed in fetal liver chimeric mice, where the competitor bone marrow lacked iNKT cells (derived from SAP-deficient mice), thus abolishing any iNKT cell competition. Our observations may explain the presence of BCL-6-deficient iNKT cells observed previously, as these cells may proliferate and accumulate with age; consistent with this hypothesis, we showed here that neonatal Bcl6ΔΔ mice had a greater defect in iNKT cell development than adult mice.

While BCL-6 is considered the signature transcription factor of follicular helper T (Tfh) cells, it has been broadly implicated in T cell differentiation. Previous studies showed that BCL-6 was transiently induced after activation of CD4+ and CD8+ T cells and it was required for the generation of memory T cells. BCL-6 supports the progenitor-like memory CD8+ T cell pool and these BCL-6-expressing CD8+ memory precursor cells share a molecular signature with hematopoietic stem cells and Tfh cells. We found that BCL-6 was expressed in early iNKT cell progenitors and was required for the development of T cells with an innate phenotype. However, it is not clear as to what extent activated/memory conventional T cells and innate T cells share a common gene network and how BCL-6 may regulate these programs. BCL-6 contains two transcriptional repressor domains and interacts with multiple co-repressor complexes; it is thus considered a bona fide transcriptional repressor. Notably, these domains are differentially required for the generation of Tfh and germinal center B cells, indicating cell-type-specific functions for BCL-6 (refs. 44,45). BCL-6 seems to promote Tfh fate by suppressing alternative lineage programs, including Tfh1, Tfh2 and Tfh17 (refs. 46–48). However, only a limited number of genes are bound and directly regulated by BCL-6 in Tfh cells. In addition, only 10% of the bound genes contain BCL-6 binding motifs, suggesting that BCL-6 may cooperate with other transcription factors to determine lineage fate. We postulate that BCL-6 may repress genes associated with the naive T cell fate or alternatively, BCL-6 may suppress expression of transcription factors that repress Zbbb16 and other innate-related molecules that repress this naive gene program. Identification of direct BCL-6 target genes in ST0 iNKT cells would help to clarify how BCL-6 regulates iNKT cell development. Taken together, our results reveal a new role for BCL-6 in enforcing the identity of early iNKT committed progenitors and shaping the genomic landscape for the subsequent unfolding of iNKT cell maturation programs and are consistent with BCL-6 being a broad regulator of T cell differentiation programs.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41590-020-0737-y.

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Methods

Mice. Wild-type, littermate controls and C57BL/6 mice were kept on a C57BL/6J background. C57BL/6J (referred to as B6) mice were described previously1. Both male and female mice were used in experiments. Competitive bone marrow chimeras were performed as previously described2. All mice were housed either at the University of Chicago Animal Resource Center or at the Biomedical Sciences Research Center (BSRC) Alexander Fleming Animal Facility. Experiments were performed according to the guidelines of The Institutional Animal Care and Use Committee of the University of Chicago and of the BSRC Alexander Fleming.

Flow cytometry, cell sorting and antibodies. Thymocyte suspensions from 10-day-old or 3–6-week-old mice were incubated with anti-FcγR before staining with fluorochrome-conjugated antibodies. Cells were acquired in a FACSCanto II or LSRII Fortessa or sorted in a FACSAria III and analyzed with FlowJo. Enrichment of thymocytes for INKT cells was performed by staining total thymocytes with APC- or PE-conjugated CD11b(+) tetramers (National Institutes of Health (NIH) Tetramer Facility at Emory University), followed by anti-APC or anti-PE microbeads and subjected to MACS-based magnetic cell separation. Similarly, enrichment of thymocytes for MAIT cells was performed by staining with APC-conjugated M1R-OP8RU tetramer (NIH tetramer facility at Emory University) for 1 h at 25°C. Propidium iodide was included in all samples to exclude dead cells from the analysis. Pan-caspase staining was performed with the carboxyfluorescein (FAM)-FLICA in vitro Caspase Detection kit (ImmunoChemistry Technologies), according to the manufacturer’s instructions.

To calculate ST0 cell numbers, a small fraction of the thymus was stained with Tetramer, TCRβ and CD24 before tetramer enrichment and analyzed by flow cytometry. From this staining, the total number of INKT cells was calculated, as Tetr (TCRβ)+ cells. Then, the number of ST0–ST3 cells was calculated according to the percentages in enriched samples. Antibodies specific for the following antigens were purchased from BD Biosciences, eBiosciences, BioLegend and Cell Signaling Technology: CD127 (A73-16.2), CD34 (GK1.5), CD69 (M5/114.15), CD86 (GL1), CD45.1 (A20), CD45.2 (104), CD44 (IM7), CD62L (MEL14), CD44 (IM7), CD24 (M1/69), IFN-γ (XMG1.2), IL-4 (11B11), αβ TCR (H57-597), βγ TCR (3B5-67), TCRγ (H2S1–9), TCRδ (AFKJS-9), T-BET (4B10), LEF1 (C12A5), PLZF (R17–809 or Mag.217F), K67 (SolA15) and BCL-6 (K11–91). Intracellular staining for PLZF, RORγt, LEF1, BCL-6, EGR2 and T-BET was performed using the Foxp3/Transcription Factor Staining Buffer Set (eBiosciences, 00-5523-00).

Cell culture, cytokine production and staining. Thymocytes were treated with 50 µM of PMA, 1 µg of ionomycin and brefeldin A for 4 h, before collecting and staining intracellularly for IFN-γ, IL-17A and CD69 (H1.2F3), NK1.1 (PK136), CD45.1 (A20), CD45.2 (104), CD62L (MEL14), CD44 (IM7), CD24 (M1/69), IFN-γ (XMG1.2), IL-4 (11B11), αβ TCR (H57-597), βγ TCR (3B5-67), TCRγ (H2S1–9), TCRδ (AFKJS-9), T-BET (4B10), LEF1 (C12A5), PLZF (R17–809 or Mag.217F), K67 (SolA15) and BCL-6 (K11–91). Intracellular staining for PLZF, RORγt, LEF1, BCL-6, EGR2 and T-BET was performed using the Foxp3/Transcription Factor Staining Buffer Set (eBiosciences, 00-5523-00).

In vivo EdU incorporation. In vivo EdU incorporation assays were performed with ClickIT EdU Alexa Fluor 488 according to the manufacturer’s instructions (Molecular Probes, C10420).

RNA analysis and real-time qPCR. RNA was extracted from sorted thymocytes using the RNeasy micro kit (QIAGEN, 74004), DNase-treated and reverse-transcribed using Superscript III (Invitrogen), qPCR was performed with gene-specific primers in STEPOne (Applied Biosystems), using the FAST START Universal SYBR Green Master (Roche, 4907491001), HPRT was used to normalize expression. Relative expression was calculated using the ΔCT method.

RNA next-generation sequencing and data analysis. Total RNA for RNA-seq was isolated from sorted ST0 (Tetramer−TCRβ−CD24−CD44+) and ST1 (Tetramer+TCRβ+CD24−CD44+) cells with RNeasy Micro Kit (QIAGEN) according to manufacturer’s recommendations. Libraries were constructed with NuGEN’s Ovation Ultralow Library System (NuGEN Technologies) and were subsequently subjected to 50–76 cycles of sequencing on a NextSeq500 (Illumina). RNA-seq analysis was performed with the Bioconductor package metaseqR, as previously described14. Differential gene expression analysis was performed with the DESeq2 algorithm. Genes were considered as a DESeq P < 0.05 and fold change >1.0 to <–1.0 in log, scale, corresponding to twofold up or down in natural scale respectively, were considered differentially expressed. Venn diagrams were generated by integrating lists of DEGs with the Bioconductor package VennDiagram. Statistical significance of the overlaps was assessed with permutation tests with an estimated P value after 10,000 resamplings. Heat maps were drawn with the Bioconductor package heatmap2. Scatter plots were generated with the Bioconductor package ggplot2.

ATAC-seq analysis. For each ATAC-seq experiment, 10,000 Tetramer+TCRβ+CD24−CD44+ cells and 10,000 Tetramer−TCRβ−CD24−CD44− cells from wild-type and B6 mice were sorted after magnetic bead–based tetramer enrichment. More INKT cells were sorted compared to MAIT cells due to lower cell yield. Sorted MAIT cells were further purified with PCR Cleanup kit (QIAGEN, 28204), before next-generation sequencing on an Illumina NextSeq500.

Quality control of the generated 50–hp single-end reads was performed with the FastQC package. Reads were aligned to the mm10 genome build using bowtie2 with the ‘–local’–very-sensitive–local’ parameters15. Reads that mapped to chrM and other blacklisted regions were removed. Samtools16 was used to sort and isolate uniquely mapped reads and bedtools17 was used to remove duplicated reads. Peak calling was performed for each replicate, using MACS2 (ref. 18) on the de-duplicated bam files, with the ‘–nomodel’–shift –100–extend 2000’ keep–dup all parameters. Only overlapping peaks between the two replicates of each condition were kept and then were merged, resulting in a reproducible peak set (one for each condition). Finally, an overall set of 43,317 peaks was generated by combining and merging the peak sets of the four conditions and was used as a reference peak set for the subsequent analysis.

ATAC-seq read counting was performed with the DiffBind package (http://bioconductor.org/packages/release/bioc/vignettes/DiffBind/instd/doc/DiffBind.pdf) and differential accessibility analysis was performed with edger (P < 0.05). For these analyses, duplicated reads were considered, as it has been shown that their removal can reduce the detection power of differential analysis in highly abundant regions19. Only peaks with at least ten reads in at least one of the replicates were kept for counting.

Peaks were assigned according to genes their distance in a gene-centric manner. Therefore, each peak was assigned to its closest gene in a total 10-kb window distance from the transcription start site, whereas peaks located further away were not assigned at all. On the basis of this peak annotation, DEGs between wild-type ST0 and wild-type ST1 were further examined for the assignment of at least one differentially accessible peak between B6 ST0 and wild-type ST1 (BCL-6-dependent regions). Statistical significance of the number of BCL-6-dependent genes was assessed with Monte Carlo simulations (P < 0.05).

Specifically, a total population of expressed genes was generated by considering the expression levels of genes in wild-type cells from the RNA-seq experiment. Random subsets were derived from the total population, equal to the number of upregulated or downregulated genes accordingly and their overlap with the differentially accessible genes was estimated. The expected value, which served as a background, was calculated as the average of all the overlap estimations, after 10,000 resamplings. P value was estimated as the ratio of the number of occurrences that exceeded the experimental overlap estimation to the total population (10,000). Average genomic profiles and the relevant heat maps were generated with the Bioconductor package recoup (https://www.bioconductor.org/packages/release/bioc/html/recoup.html), using the second replicate of all samples after downsampling to the lowest library size. Specifically, the peaks of interest were split into 100 bins of dynamic length and for each bin, a normalized coverage per base pair was estimated, resulting in 100 data points for each peak. The average value of each bin across all peaks was used for generating the coverage profile for each sample.

To determine whether there was an early opening of INKT-related loci in ST0 cells, we compared ATAC-seq from INKT cells and publicly available data for DP thymocytes (GSE107076). Differential accessibility analysis was performed as mentioned before using the overall peak set as reference genome. Average profile plots were generated for all samples after merging of the replicates and downsampling to the lowest library size. Statistical difference between the coverage profiles of two samples was estimated with a two-sample Kolmogorov–Smirnov test. Genome track views were generated with Integrative Genomics Viewer.

Statistics and reproducibility. A standard two-tailed unpaired Student’s t-test was used (unless otherwise stated) to determine the statistical probability of the differences observed between two populations of cells, using the GraphPad Prism software. Detailed information is provided in each figure legend.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

RNA-seq and ATAC-seq data have been deposited to the Gene Expression Omnibus with the accession code GSE134212. Source data for Figs. 2a–2d, 5 and 7 and Extended Data Figs. 1–6 are provided with the paper. The data that support the findings of this study are available from the corresponding authors upon request.

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Author contributions
M.G. designed, performed and analyzed experiments. A.G. performed bioinformatic analysis of RNA-seq and ATAC-seq data and analyzed experiments under the supervision of P.M. and P.H. S.G. assisted with flow cytometry cell sorting and flow cytometry analysis. A.L.D. provided the Bcl6F/F mouse strain. M.S. performed the RNA-seq experiments. B.L.K. obtained funding, performed ATAC-seq experiments, interpreted data and reviewed and edited the manuscript. M.V. conceptualized the project, obtained funding, supervised research, interpreted data, performed and analyzed experiments and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1  |  Bcl6 mRNA expression in ST0 and ST1 cells. **. Flow cytometry plots showing the sorting strategy and purity of ST0 and ST1 cells after sorting. Shown one representative experiment out of three. b, Flow cytometry plots showing expression of PLZF, T-BET and RORγt in ST1 wild-type cells. Graphs show mean percentage and number ± SEM of the indicated populations. n = 7 (PLZFhi, PLZFhiT-BET+, and PLZFloT-BET+), or n = 3 (PLZFintRORγt+) independent experiments. c, Graphs showing the normalized counts of Bcl6 mRNA in ST0 and ST1 cells based on RNA-seq data. n = 3 independent experiments (DESeq, **P < 0.01). d, Flow cytometry plots showing the expression of BCL-6 and CD69 (left) or BCL-6 and EGR-2 (right) in Tetr+TCRβ+CD24+CD44+ iNKT cells. Shown one representative experiment out of four (CD69) or two (EGR-2).
Extended Data Fig. 2 | The iNKT phenotype is cell-intrinsic. 

a, The indicated populations were FACS-sorted from Cd4cre-expressing heterozygous Bcl6 floxed mice and DNA was submitted to PCR genotyping. In ST3 iNKT, CD4 and DP thymocytes only the germline Bcl6 allele was amplified, whereas in DN and tail samples, both the floxed and the germline alleles were amplified. One experiment shown out of one. 
b, Flow cytometry plots showing the percentage of iNKT cells in CD45.1+ (wild-type competitor) and CD45.2+ controls (Bcl6F/F) in the thymus from competitive BM chimeric mice. Graphs show the mean percentage ± SEM of iNKT cells in the indicated chimeric mice. n = 5 independent experiments. 
c, Flow cytometry plots showing the percentage of iNKT cells in CD45.1+ (wild-type competitor) and CD45.2+ (Bcl6ΔΔ) in the spleen from competitive BM chimeric mice. Graphs showing the mean percentage ± SEM of iNKT cells in the indicated chimeric mice. n = 3 independent experiments. 
d, Flow cytometry plots showing the percent of Tetr*TCRβ+ iNKT cells after MACS enrichment in the indicated mice. These experiments were repeated twice with similar results. 
e, f, Flow cytometry plots showing the percent of ST0 (e) and ST1-3 (f) cells in CD45.1+ (competitor) and CD45.2+ control (Bcl6F/F) iNKT cells in competitive bone marrow chimeras. Graphs indicate the mean percentage ± SEM. n = 3 (ST0) or n = 4 (ST1-3) independent experiments. Statistical analysis was performed with two-tailed unpaired t-test. ** P < 0.01.
Extended Data Fig. 3 | Conventional T cell development is normal in the absence of BCL-6. a, FACS plots showing the expression of CD4 and CD8 (upper panel), and TCRβ (lower panel) in total thymocytes from the indicated mouse strains. b, Histograms showing the total number of the indicated thymocyte populations in control and Bcl6Δ/Δ mice. Graphs show the mean ± SEM. n = 5 independent experiments. c, Histograms showing the abundance of Tcrα Vα14-Jα18 and Sh2d1a transcripts in control and Bcl6Δ/Δ sorted DP thymocytes. Graphs show the mean ± SEM. n = 3 independent experiments. d, Histograms showing the expression of CD1D, CD150, and LY108 in DP thymocytes from control and Bcl6Δ/Δ mice. These experiments were repeated three times with identical results. Statistical analysis was performed with two-tailed unpaired t-test.
Extended Data Fig. 4 | BCL-6—deficient spleens lack mature iNKT cells.  

**a**, FACS plots of TetramerTCRβ+ splenocytes showing the percentage of NKT1 (T-BET+PLZF), NKT2 (PLZF-T-BET−) and NKT17 (RORγt+T-BET−) cells in the indicated mouse strains.  

**b**, Cell number and percentage of NKT1, NKT2 and NKT17 cells in control and Bcl6Δ/Δ spleens. Graphs show mean ± SEM. n = 5 (NKT1 and NKT2), or n = 4 (NKT17) independent experiments. Statistical analysis was performed with two-tailed unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001.  

**c**, Histograms showing the fold reduction of Rorc mRNA in Bcl6Δ/Δ ST1 cells compared to wild-type, based on the RNA-seq data. n = 3 independent experiments (DESeq, **P < 0.01).
Extended Data Fig. 5 | Proliferation and apoptosis of immature iNKT cells is independent of BCL-6. **a**, Graphs show the percentage of EDU<sup>+</sup> and Ki67<sup>+</sup> cells in ST0 and ST1 cells in control littermates and Bcl6<sup>Δ/Δ</sup> mice. Data represent mean ± SEM. n = 3 independent experiments. **b**, FACS plots showing expression of IL-7 receptor and LEF1, in control littermates and Bcl6<sup>Δ/Δ</sup> mice. Experiments were repeated three times with identical results. **c**, Percentage of FLICA<sup>+</sup> cells in the indicated iNKT cell stages, in control littermates and Bcl6<sup>Δ/Δ</sup> mice. Graphs show mean ± SEM. n = 3 independent experiments with 3 (control) and 4 (Bcl6<sup>Δ/Δ</sup>) mice. **d**, Flow cytometry plots showing the percent of total iNKT thymocytes, as T etramer<sup>+</sup> TCRβ<sup>+</sup> cells, in 10-days old control littermates and Bcl6<sup>Δ/Δ</sup> mice. **e**, Numbers and percentage of iNKT cells in the indicated mouse strains. Graphs represent mean ± SEM. n = 2 independent experiments with 5 control and 9 Bcl6<sup>Δ/Δ</sup> mice. * P < 0.05, ** P < 0.01, *** P < 0.001. Statistical analysis was performed with two-tailed unpaired t-test (**a, e**), or one-tailed unpaired t-test (**c**).
Extended Data Fig. 6 | PLZF expression is reduced in the absence of BCL-6. a, qPCR analysis showing the transcript levels of Zbtb16 in sorted ST0 and ST1 cells from control and Bcl6Δ/Δ mice. Graphs represent the mean ± SEM. n = 3 independent experiments with 3–5 pooled thymi for each genotype. b, FACS plots showing the expression of PLZF protein in ST0 (upper panel) and ST1 (lower panel) cells from the indicated mouse strains. Graphs show the mean percentage ± SEM of PLZF+ cells in the indicated populations. n = 5 (ST0) and n = 8 (ST1) independent experiments. c, Graph showing the geometric MFI of PLZF expression in PLZF-expressing ST0 cells. Graphs represent the mean ± SEM. n = 4 independent experiments. d, Histograms showing the expression of PLZF in ST2 and ST3 cells in the indicated mouse strains. Graphs show the mean percentage ± SEM of PLZF+ cells. n = 5 independent experiments. e, Histograms showing the expression of CD69 and CD5 in ST0 iNKT cells from the indicated mouse strains. The corresponding graphs show the mean geometric MFI ± SEM. n = 3 independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001. Statistical analysis was performed with one-sample t-test (a), two-tailed unpaired t-test (b, d, and e) or two-tailed paired t-test (c).
Extended Data Fig. 7 | Impaired progression from ST0 to ST1 in the absence of BCL-6. a, GSEA analysis showing enrichment of NKT0-associated genes in ST0 cells, compared to ST1 cells. b, GSEA analysis showing enrichment of NKT17-associated genes in wild-type ST1 cells, compared to Bcl6Δ/Δ ST1 cells. c, GSEA analysis showing enrichment of NKT2-associated genes in Bcl6Δ/Δ ST1 cells, compared to wild-type ST1 cells. n = 3 independent experiments. Normalized enrichment scores (NES) and FDR as implemented by GSEA, based on 1,000 permutations.
Extended Data Fig. 8 | Chromatin accessibility profiles near genes that are developmentally regulated. a, Genome track views of several loci of developmentally-regulated genes in the indicated populations from wild-type mice. b, Heatmaps showing aligned ATAC-seq reads around the center of peaks that are more accessible in ST1 cells (left), or in ST0 cells (right), in the indicated populations. c, Genome track views of Bach2 and Sell genes in wild-type and Bcl6Δ/Δ ST1 cells. d, Genome track views of several loci of developmentally-regulated genes in wild-type and Bcl6Δ/Δ ST0 cells.
Reporting Summary

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Policy information about availability of computer code

Data collection
- FACS Aria III sorter, FACSCanto II, LSR II Fortessa, Illumina NextSeq500, FACS Diva 8.0.2

Data analysis
- Flow cytometry: Flowjo 9.9, Flowjo 10.0
- Statistical analysis: GraphPad Prism 5.0, Bioconductor v3.2 and v3.6
- Pathway analysis: Metascape
- Gene set enrichment analysis: GSEA (Broad Institute)
- RNA-seq analysis: metaseqR, heatmap2, ggplot2, VennDiagram
- ATAC-seq analysis: DiffBind, MACS2, recoup
- NGS analysis: bowtie2, tophat2, samtools, bedtools
- Visualization: Integrative Genomics Viewer 2.4.19

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

RNA-seq and ATAC-seq data have been deposited in public repositories are publicly available upon publication [GSE134212]
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-list.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No statistical method was used to pre-determine sample size. For all experiments, at least 3 animals/genotype were used, based on previous literature. Experiments were repeated at least three times, sometimes with multiple mice each. ATAC-seq experiments were repeated twice, due to limited availability of mice with the desired genotype and limited cell number of the desired populations. |
| Data exclusions | No data were excluded. |
| Replication | All experiments were repeated for reproducibility, as stated in the figure legends. All experiments were reproducible. |
| Randomization | Mice were randomly selected for all experiments, based on their genotype. Littermates were used in all cases. |
| Blinding | No blinding was applied. Blinding was not possible, because the same researchers were performing the experiments and analyzing the results. Experiments were performed by at least two different researchers, throughout the course of the study. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|-------------------------------|----------|
| n/a | n/a |
| ☐ | ☐ | Antibodies |
| ☒ | ☒ | Eukaryotic cell lines |
| ☐ | ☐ | Palaeontology |
| ☐ | ☐ | Animals and other organisms |
| ☐ | ☐ | Human research participants |
| ☒ | ☒ | Clinical data |
| ☐ | ☐ | ChIP-seq |
| ☒ | ☒ | Flow cytometry |
| ☐ | ☐ | MRI-based neuroimaging |

Antibodies

| Antibodies used |
|------------------|
| CD127 (A7R34), Brilliant Violet 421, 1:200, BioLegend (135024) |
| CD4 (GK1.5), FITC, 1:400, ebioscience (11-0041-85) |
| PE, 1:400, ebioscience (12-0041-85) |
| APC, 1:400, ebioscience (17-0041-83) |
| Alexa Fluor 700, 1:400, ebioscience (56-0041-80) |
| Brilliant Violet 421, 1:400, BioLegend (100437) |
| DBx5 (53-6.7), PE-Cyanine7, 1:400, ebioscience (25-0081-82) |
| APC, 1:400, ebioscience (17-0081-81) |
| TCRB (H57-597), FITC, 1:200, ebioscience, (11-5961-82) |
| PE, 1:200, ebioscience, (12-5961-82) |
| APC, 1:200, ebioscience, (17-5961-83) |
| APC-eFluor 780, 1:200, ebioscience, (47-5961-82) |
| CD62L (MEL14), PE-Cyanine7, 1:500, ebioscience (25-0621-82) |
| CD44 (IM7), PE-Cyanine7, 1:400 ebioscience (17-0441-82) |
| Alexa Fluor 700, 1:400 ebioscience (56-0441-80) |
| PE, 1:400, BD Bioscience (553134) |
| CD24 (M1/69) FITC, 1:400, ebioscience (11-0242-82) |
| APC-eFluor 780, 1:400, ebioscience (47-0242-80) |
| Brilliant Violet 421, 1:400, BioLegend (101825) |
| IFN-γ (XMG1.2) PE, 1:100, ebioscience (12-7311-81) |
| IL-4 (11B11) Alexa Fluor 488, 1:100, BD Bioscience (557728) |
| IL-17A (eBio17B7) PE-Cyanine7, 1:200, ebioscience (25-7177-80) |
| CD69 (H1.2F3) FITC, 1:400, ebioscience (11-0691-81) |
Validation

All antibodies were validated by the manufacturer.

| Antibody | RRID   | Catalog Number  | Manufacturer  |
|----------|--------|-----------------|---------------|
| CD127    | AB_11218800 | BioLegend Cat. No. 135024 |
| CD4 (GK1.5) | AB_464892 | eBioscience Cat. No. 11-0041-85 |
| AB_465508 | eBioscience Cat. No. 12-0041-85 |
| AB_469321 | eBioscience Cat. No. 17-0041-83 |
| AB_490011 | eBioscience Cat. No. 56-0041-80 |
| AB_10900241 | BioLegend Cat. No. 100437 |
| CD80    | AB_465984 | eBioscience Cat. No. 25-0081-82 |
| AB_469334 | eBioscience Cat. No. 17-0081-81 |
| TCRP (H5-597) | RRID: AB_465323 | eBioscience Cat. No. 11-5961-82 |
| AB_466066 | eBioscience Cat. No. 12-5961-82 |
| AB_469482 | eBioscience Cat. No. 17-5961-83 |
| AB_1272173 | eBioscience Cat. No. 47-5961-82 |
| CD26L (MEL14) | RRID: AB_469633 | eBioscience Cat. No. 25-0621-82 |
| CD44 (IM7) | RRID: AB_469623 | eBioscience Cat. No. 25-0441-82 |
| AB_490432 | eBioscience Cat. No. 56-0441-80 |
| AB_394649 | BD Biosciences Cat. No. 553134 |
| CD24 (M1/69) | RRID: AB_464988 | eBioscience Cat. No. 11-0242-82 |
| AB_10853190 | eBioscience Cat. No. 47-0242-80 |
| AB_10901159 | BioLegend Cat. No. 101825 |
| IFN-γ (XMG1.2) | RRID: AB_466192 | eBioscience Cat. No. 12-7311-81 |
| IL-4 (11B11) | RRID: AB_396836 | BD Biosciences Cat. No. 557728 |
| IL-17A (eBio17B7) | RRID: AB_10717952 | eBioscience Cat. No. 25-7177-80 |
| CD69 (H1.2F3) | RRID: AB_465118 | eBioscience Cat. No. 11-0691-81 |
| NK1.1 (PK136) | RRID: AB_465318 | eBioscience Cat. No. 11-5941-82 |
| AB_469665 | eBioscience Cat. No. 25-5941-82 |
| AB_469479 | eBioscience Cat. No. 17-5941-82 |
| CD45.1 (A20) | RRID: AB_395043 | BD Biosciences Cat. No. 553775 |
| AB_465629 | eBioscience Cat. No. 25-0453-82 |
| AB_1582228 | eBioscience Cat. No. 47-0453-82 |
| CD45.2 (104) | RRID: AB_465062 | eBioscience Cat. No. 11-0454-85 |
| AB_1186098 | BioLegend Cat. No. 109830 |
| PLZF-R17BD9 | RRID: AB_2738238 | BD Biosciences Cat. No. 563490 |
| RORγt (AFK5.9) | RRID: AB_1834470 | eBioscience Cat. No. 12-6988-82 |
| T-BET (AB10) | RRID: AB_11042699 | eBioscience Cat. No. 25-5825-82 |
| LEF1 (C12A5) | RRID: AB_10949502 | Cell Signaling Technology Cat. No. 8490 |
| PLZF (Mags.217) | RRID: AB_2574445 | eBioscience Cat. No. 53-9320-82 |
| AB_11148894 | eBioscience Cat. No. 12-9320-82 |
| K67 (SolA15) | RRID: AB_11151689 | eBioscience Cat. No. 11-5698-80 |
| BCL-6 (K112-91) | RRID: AB_10717126 | BD Biosciences Cat. No. 561522 |

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

**Laboratory animals**
WT, C4Dcre, B6/F, or C4DcreB6/F mice on C57BL/6J background were used in all experiments. All mice used were either 10 days old, or 3-6 weeks old, and were gender-mixed for each experiment. Mice were housed in a pathogen-free, temperature- and humidity-controlled barrier facility, in a standard 12h light/dark cycle with water and food available ad libitum.

**Wild animals**
This study did not involve wild animals

**Field-collected samples**
This study did not involve samples collected from the field.

**Ethics oversight**
Mice were kept according to the guidelines of The Institutional Animal Care and Use Committee of the University of Chicago and
ChIP-seq

Data deposition

☐ Confirm that both raw and final processed data have been deposited in a public database such as GEO.

☐ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links
May remain private before publication.

For “Initial submission” or “Revised version” documents, provide reviewer access links. For your “Final submission” document, provide a link to the deposited data.

Files in database submission

Provide a list of all files available in the database submission.

Genome browser session
(e.g. UCSC)

Provide a link to an anonymized genome browser session for “Initial submission” and “Revised version” documents only, to enable peer review. Write “no longer applicable” for “Final submission” documents.

Methodology

Replicates

Describe the experimental replicates, specifying number, type and replicate agreement.

Sequencing depth

Describe the sequencing depth for each experiment, providing the total number of reads, length of reads and whether they were paired- or single-end.

Antibodies

Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

Peak calling parameters

Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.

Data quality

Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.

Software

Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

Flow Cytometry

Plots

Confirm that:

☐ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☐ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).

☐ All plots are contour plots with outliers or pseudocolor plots.

☑ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Thymus, spleens and livers were mechanically dissociated in FACSD buffer (HBSS supplemented with 0.25% BSA, 0.01% sodium azide and 25ug/ml DNase), filtered with 70um MESH filters and stained with appropriate antibodies and tetramers. Liver lymphocytes were isolated with 40% Percoll and red blood cells were lysed with water, prior to staining.

Instrument

LSRII Fortessa, FACSCanto II, FACSaria III

Software

FACSDiva 8.0, Flowjo 9.9 and 10.0.6

Cell population abundance

Representative post-sort data are presented in Extended Data Figure 1.

Gating strategy

FSC-A/SSC-A was used to initially gate lymphocytes. Singlets were gated with FSC-A/FSC-H and dead cells were excluded with Propidium iodide staining. Indicated markers were used to define lymphocyte populations.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.
# Magnetic resonance imaging

## Experimental design

| Design type | Indicate task or resting state; event-related or block design. |
|-------------|----------------------------------------------------------------|
| Design specifications | Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials. |
| Behavioral performance measures | State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects). |

## Acquisition

| Imaging type(s) | Specify: functional, structural, diffusion, perfusion. |
|-----------------|-----------------------------------------------------|
| Field strength | Specify in Tesla |
| Sequence & imaging parameters | Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle. |
| Area of acquisition | State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined. |
| Diffusion MRI | □ Used □ Not used |

## Preprocessing

| Preprocessing software | Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.). |
|------------------------|----------------------------------------------------------------------------------------------------------------------------------|
| Normalization | If data were normalized/standardized, describe the approach(es); specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization. |
| Normalization template | Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized. |
| Noise and artifact removal | Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration). |
| Volume censoring | Define your software and/or method and criteria for volume censoring, and state the extent of such censoring. |

## Statistical modeling & inference

| Model type and settings | Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects, drift or auto-correlation). |
|-------------------------|----------------------------------------------------------------------------------------------------------------------------------|
| Effect(s) tested | Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used. |
| Statistic type of analysis | □ Whole brain □ ROI-based □ Both |
| Statistic type for inference (See Eklund et al., 2016) | Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods. |
| Correction | Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo). |

## Models & analysis

| n/a | Involved in the study |
|-----|-----------------------|
| □  | Functional and/or effective connectivity |
| □  | Graph analysis |
| □  | Multivariate modeling or predictive analysis |

| Functional and/or effective connectivity | Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information). |
|-------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------|
| Graph analysis | Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.). |
Multivariate modeling and predictive analysis

Specify independent variables, features extraction and dimension reduction, model training and evaluation metrics.