Bcl-6 directly represses the gene program of the glycolysis pathway

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Despite the increasing knowledge of the molecular events that induce the glycolysis pathway in effector T cells, very little is known about the transcriptional mechanisms that dampen the glycolysis program in quiescent cell populations such as memory T cells. Here we found that the transcription factor Bcl-6 directly repressed genes encoding molecules involved in the glycolysis pathway, including Slc2a1, Slc2a3, Pkm and Hk2, in type 1 helper T cells (Tfh1 cells) exposed to low concentrations of interleukin 2 (IL-2). Thus, Bcl-6 had a role opposing the IL-2-sensitive glycolytic transcriptional program that the transcription factors c-Myc and HIF-1α promote in effector T cells. Additionally, the Tfh1 lineage–specifying factor T-bet functionally antagonized the Bcl-6-dependent repression of genes encoding molecules in the glycolysis pathway, which links the molecular balance of these two factors to regulation of the metabolic gene program.

The transcription repressor Bcl-6 is necessary for the development of various types of cells of the immune system, including germinal center B cells and CD4+ follicular helper T cells (Tfh cells)1–3. Bcl-6 has also been linked to promoting the formation of memory cells from both CD4+ T cells and CD8+ T cells4–8. In the development of both Tfh cells and memory cells, an important role for Bcl-6 is to inhibit the expression of Blimp-1, a transcription regulator required for the terminal differentiation of effector cell populations2,6,9,10. Although this is one critical activity for Bcl-6 in several populations of cells of the immune system, it has remained unclear which additional gene pathways are regulated by Bcl-6 to promote the functional characteristics of different T cell populations11.

An important functional difference between effector cell populations and memory cell populations is their metabolic states12–14. Studies of CD8+ T cells have shown that genes encoding molecules in the glycolysis pathway are induced in effector cells, which results in a switch to aerobic glycolysis for energy production15–17. This is thought to be essential for the rapid proliferative burst of activated T cells and for the promotion of aspects of effector cell functions15,18. In contrast, the glycolysis pathway is downregulated in CD8+ memory T cells19, which causes the cells to use mitochondrial fatty acid oxidation as a predominant form of cellular metabolism, resulting in a shift to a catabolic state16,20. Notably, experimental inhibition of glycolysis promotes the formation of memory CD8+ T cells, while artificial activation of the glycolysis pathway causes the cells to ‘preferentially’ adopt an effector state14,21. Such observations suggest that the use of different metabolic pathways in T cells actively contributes to differentiation outcomes.

The transcription-regulatory events that induce genes encoding glycolytic molecules for the promotion of effector cell differentiation have been elucidated13,19. Signaling via the T cell antigen receptor and costimulation via the coreceptor CD28 activate the transcription factor c-Myc, which is required for initial upregulation of the expression of glucose transporters and rate-limiting glycolysis enzymes15. Additionally, signaling via interleukin 2 (IL-2) promotes the sustained expression of genes encoding glycolytic molecules in effector CD8+ T cells, with the hypoxia factor HIF-1α being required for this activity17. In contrast to the expanding knowledge of the events that induce the glycolysis pathway in effector T cells, little information is available about the transcription-regulatory events that downregulate or, alternatively, prevent the expression of these genes to enhance the formation of memory cells.

Research has indicated that the gene-expression programs of Tfh1 cells and memory T cells have several common features, which suggests a close relationship between these two populations22. One similarity between Tfh cells and memory T cells is the role of Bcl-6 in determining aspects of each gene program4,23. It appears that the abundance of Bcl-6 is partially responsible for defining the unique characteristics for the two gene programs, with the largest amounts of Bcl-6 promoting Tfh1 differentiation and moderate Bcl-6 expression being needed for memory cell development5,7,24. One environmental signal that regulates Bcl-6 expression in T cells is IL-2. Strong IL-2 signaling inhibits Bcl-6 expression, whereas low amounts of environmental IL-2 promote Bcl-6 expression7,9,25–27. Since IL-2 signaling also regulates the metabolic state of CD8+ T cells17, there is an inverse correlation between the ability of IL-2 signaling to functionally

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Received 2 May; accepted 5 August; published online 7 September 2014; doi:10.1038/ni.2985
regulate Bcl-6 expression and the expression of genes encoding glycolytic molecules.

Here we discovered that Bcl-6 repressed the IL-2-sensitive expression of genes encoding glucose transporters and rate-limiting enzymes involved in glycolysis. By comparison of published microarray data sets, we observed an overlap in the identity of genes that were reciprocally regulated by HIF-1α versus Bcl-6, including numerous genes encoding molecules in the glycolysis pathway. The expression of the genes in this overlapping subset was also sensitive to IL-2 signaling. This led us to hypothesize that Bcl-6 might serve as a key repressor for the genes encoding molecules in the glycolytic pathway that are sensitive to IL-2 signaling and are expressed differently in effector and memory T cell states. We found that Bcl-6 directly repressed many genes encoding molecules in the glycolysis pathway and effectively functioned in direct opposition to the gene programs activated by HIF-1α and c-Myc. Additionally, the type I helper T cell (T H1 cell) lineage–specifying transcription factor T-bet functionally inhibited the ability of Bcl-6 to repress genes encoding molecules involved in glycolysis. This suggests that the molecular balance of T-bet and Bcl-6 influences expression of the metabolic gene program.

RESULTS

Overlap of genes regulated by Bcl-6 and by HIF-1α

To start to address the question of which regulatory pathways Bcl-6 represses to promote the normal differentiation and activity of unique cells of the immune system, we used the web tool GEO2R to compare the gene-expression patterns of wild-type and Bcl-6-deficient bone marrow–derived myeloid cells from a published microarray study. This analysis revealed that many genes encoding molecules involved in glycolysis, including rate-limiting enzymes and glucose transporters, were upregulated in Bcl-6-deficient cells (Supplementary Table 1). These findings suggested a role for Bcl-6 in functionally repressing genes encoding components of the glycolysis pathway, at least in some circumstances.

There was a reciprocal expression pattern for Bcl-6 and HIF-1α in T cells responding to IL-2. Bcl-6 expression was inhibited in the presence of high concentrations of IL-2 (refs. 9,25,26) (Figs. 1 and 2), whereas HIF-1α expression is enhanced by IL-2 signaling. We therefore hypothesized that Bcl-6 might have a role opposing that of HIF-1α in the IL-2-sensitive regulation of target genes encoding glycolytic molecules. To begin to investigate this possibility, we compared the genes from the GEO2R analysis of Bcl-6 with genes identified as IL-2 sensitive and HIF-1α-dependent in a microarray study that analyzed wild-type and HIF-1α-deficient effector CD8+ T cells. There was substantial overlap in the genes functionally activated by HIF-1α in CD8+ T cells and the genes functionally repressed by Bcl-6 in myeloid cells (Supplementary Table 1). The subset of genes regulated by both HIF-1α and Bcl-6 included genes encoding molecules of the glycolytic pathway, such as Slc2a3 and Slc2a1 (which encode the glucose transporters Glut3 and Glut1), and HK2 and Aldo (Supplementary Table 1). It also included genes encoding important hydrolases that modify proteins (such as Pld2), as well as those encoding members of the EGL-9 family of hypoxia-inducible factors (such as Egln1) and prolyl 4-hydroxylases (such as P4ha1) (Supplementary Table 1). This overlap suggested that Bcl-6 might functionally oppose the IL-2-sensitive, HIF-1α-dependent gene program.

IL-2 regulates genes encoding glycolytic proteins

We next hypothesized that environmental IL-2 conditions might serve as a conserved stimulus in Th1 cells and CD8+ type 1 cytotoxic T cells (T C1 cells) that functionally regulates the expression of the overlapping subset of genes regulated by HIF-1α and Bcl-6. Consistent with published results obtained with CD8+ T cells, various genes in the glycolysis pathway were ‘preferentially’ expressed in CD8+ T C1 cells in the presence of a high environmental concentration of IL-2 rather than a low environmental concentration of IL-2 (Fig. 1 and Supplementary Figs. 1 and 2a). These included Slc2a3 and Slc2a1, as well as genes encoding enzymes important in the glycolytic pathway, including Aldoa, Aldoc, Pkm, HK2 and Grhpr. Indeed, there was global induction of genes encoding the key components that regulate the glycolysis pathway and associated pathways in conditions of a high environmental concentration of IL-2, whereas their expression was much lower in conditions of a low concentration of IL-2 (Fig. 1 and Supplementary Figs. 1 and 2a). Several other genes in the overlapping subset of genes regulated by HIF-1α and Bcl-6 followed the same IL-2-sensitive gene-expression pattern as those encoding molecules in the glycolytic pathway in CD8+ T C1 cells (Fig. 1 and Supplementary Fig. 2a). Similar to the results obtained with CD8+ T C1 cells, genes encoding molecules involved in the glycolytic pathway and associated pathways were ‘preferentially’ expressed in CD4+ Th1 cells exposed to a high environmental concentration of IL-2 rather than in those exposed to a low environmental concentration of IL-2 (Fig. 2 and Supplementary Figs. 2b and 3). Notably, Bcl-6 expression inversely correlated with the expression of the glycolytic-pathway genes in both Th1 cells and T C1 cells, with Bcl-6 expression robustly induced in a low environmental IL-2 concentration (Figs. 1 and 2).

Figure 1 IL-2 signaling regulates the expression of genes encoding molecules in the glycolysis pathway in CD8+ T C1 cells. Quantitative RTPCR analysis of the abundance of transcripts from various genes (vertical axes) in primary CD8+ T C1 cells cultured in T C1-polarizing conditions (IL-12 and antibody to IL-4 (anti-IL-4)) and exposed to either a high environmental concentration of IL-2 (250 U/ml) (High IL-2) or a low environmental concentration of IL-2 (10 U/ml) (Low IL-2); results were normalized to those of the control gene Rps18 (which encodes ribosomal protein S18) and are presented relative to those obtained with a high concentration of IL-2, set as 1. **P < 0.01 and ***P < 0.001 (unpaired Student’s t-test). Data are representative of at least three or four independent experiments (error bars, s.e.m.).
Bcl-6 regulates genes encoding glycolytic molecules

The inverse correlation between the expression of Bcl-6 and that of genes encoding molecules involved in the glycolytic pathway in CD4+ T11 cells led us to investigate whether Bcl-6 might be involved in the direct repression of this gene program. To start to test this hypothesis, we cloned promoter-reporter constructs for genes encoding molecules involved in the glycolytic pathway and additional genes from the overlapping subset of genes regulated by HIF-1α. Notably, there was a reduction in the activity of the promoters of Slc2a3, Slc2a1 and Tpi1, as well as those of Plod2 and P4ha2, in response to Bcl-6 expression (Fig. 3a and Supplementary Fig. 4a). In control experiments, Bcl-6 expression alone did not repress the activity of the promoter-reporter vector or several other promoter-reporter constructs (Supplementary Fig. 4b), which demonstrated that the vector itself did not contain any cryptic Bcl-6-binding sites. These data suggested that Bcl-6 was able to repress the promoter activity of a subset of genes encoding molecules involved in glycolysis and the IL-2-sensitive regulatory pathways controlled by HIF-1α.

We next transfected a control or Bcl-6 expression vector into primary T11 cells that we differentiated in a high environmental concentration of IL-2 and analyzed the endogenous expression of genes encoding molecules in the glycolysis pathway. This experimental system assessed whether increasing Bcl-6 expression alone was sufficient to repress these genes in conditions in which HIF-1α and c-Myc would otherwise strongly promote their expression. Many genes encoding molecules in the glycolysis pathway, including Hk2 and Pkm, which encode rate-limiting enzymes, were repressed by the expression of Bcl-6 in primary T11 cells maintained in conditions of high environmental concentrations of IL-2 (Fig. 3b and Supplementary Fig. 5). These data suggested that Bcl-6 expression dominantly repressed genes encoding molecules involved in the glycolysis pathway even when the cellular conditions were favorable for their expression.

If Bcl-6 participated in the direct repression of target genes encoding molecules in the glycolytic pathway in the context of T cells, then there would be an inverse correlation between the expression of those genes and the association of Bcl-6 with those loci. To examine this prediction, we used chromatin immunoprecipitation followed by quantitative RT-PCR analysis of the abundance of transcripts from various genes (vertical axes) in combination with either a Bcl-6 expression vector (Bcl-6) or an empty vector control (Ctrl); results were normalized to those of a cotransfected renilla luciferase control plasmid and are presented (in relative light units (RLU)) relative to those of cells transfected with the control vector, set as 1. (b) Quantitative RT-PCR analysis of transcript abundance in primary CD4+ T cells cultured in T11 conditions with a high concentration of IL-2 and transfected with a control or Bcl-6 expression vector (as in a); results were normalized to those of Rps18 and are presented relative to those of cells transfected with the control vector, set as 1. (c) ChIP-PCR analysis of T11-ligated cells maintained in the presence of a high or low concentration of IL-2 (key), assessed by immunoprecipitation of chromatin with anti-Bcl-6 or a nonspecific immunoglobulin G (IgG) control antibody followed by quantitative PCR analysis of various promoter regions (vertical axes) with promoter-specific primers; results were normalized to those of a standardized aliquot of the input chromatin, followed by subtraction of the results obtained with the IgG control antibody (nonspecific background). *P < 0.05, **P < 0.01 and ***P < 0.001 (unpaired Student’s t-test). Data are representative of at least three (a,b) or two (c) independent experiments (error bars, s.e.m.).
Bcl-6 binds to glycolytic molecule–encoding loci

ChIP followed by high-throughput sequencing (ChIP-seq) has been used to examine the genomic localization of Bcl-6 in B cells and the T_{H}1 subset of helper T cells to define the mechanisms that Bcl-6 uses to repress target gene expression. Such comprehensive data sets have provided extensive information about the genomic localization of Bcl-6 and its corepressor complexes in various cellular settings. We next compared our results (Fig. 3 and Supplementary Fig. 4c) with published ChIP-seq data sets of the binding pattern of Bcl-6 in other lymphocyte subsets. We visualized the data from the published ChIP-seq studies with the UCSC Genome Browser and focused on the Bcl-6 peaks in proximity to the genes encoding molecules in the glycolysis pathway (Fig. 4 and Supplementary Fig. 6). Notably, we identified Bcl-6 peaks in the regulatory regions of SLC2A3, SLC2A1, and HK2 in B cells (Fig. 4 and Supplementary Fig. 6). Additionally, Slc2a1 and Pkm were identified in the published list of genes with IL-2-sensitive overlapping ChIP-seq peaks for Bcl-6 and the STAT family of transcription factors in T_{H}9 cells. Together these data suggested that Bcl-6 associated with the loci of genes encoding molecules involved in the glycolysis pathway in both T cells and B cells in several different settings.

Given the large number of genes functionally repressed by overexpressed Bcl-6 in primary T_{H}1 cells, we next assessed how widespread the association of Bcl-6 was with the loci of genes that were functionally repressed in the Bcl-6-overexpression experiments. The ChIP-seq data sets of B cells revealed Bcl-6 peaks at most of the genes that were repressed by Bcl-6 expression in the primary T_{H}1 cell experiments, including HK2, TPI1, Aldoa, Pfkl, PfkM, Pck2, and Grhpr (Fig. 4 and Supplementary Fig. 6). Many of the Bcl-6 peaks also contained overlapping peaks for the corepressor BCOR and, less often, peaks for the corepressor SMRT and c-Myc. Overlap of c-Myc versus that of Bcl-6, and the overlapping subset of genes identified by microarray analysis as being regulated by Bcl-6 and HIF-1α, suggested that the Bcl-6-dependent gene program might oppose the gene program directly regulated by HIF-1α and c-Myc. To assess this possibility, we first used ChIP to analyze primary T_{H}1 cells exposed to a high or low environmental concentration of IL-2 and examined the association of HIF-1α and c-Myc with a subset of the Bcl-6-repressed promoters. We found enrichment for the binding of c-Myc and HIF-1α at the Slc2a1, Pld2, and P4ha2 promoters in T_{H}1 cells maintained in a high environmental concentration of IL-2, which correlated with the expression of these genes (Fig. 5). In contrast, the binding of c-Myc and HIF-1α with these promoters was reduced in a low environmental concentration of IL-2 (Fig. 5). We also found enrichment for binding of c-Myc at the Slc2a3 and Tpi1 promoters in T_{H}1 cells exposed to conditions of a high environmental concentration of IL-2 (Fig. 5) and correlation with the expression of these genes (Fig. 5). In contrast, the association of Bcl-6 was with the loci of genes encoding molecules in the glycolytic pathway.

HIF-1α and c-Myc bind to glycolytic molecule–encoding loci

The inverse correlation between the IL-2-sensitive expression of HIF-1α and c-Myc versus that of Bcl-6, and the overlapping subset of genes identified by microarray analysis as being regulated by Bcl-6 and HIF-1α, suggested that the Bcl-6-dependent gene program might oppose the gene program directly regulated by HIF-1α and c-Myc. To assess this possibility, we first used ChIP to analyze primary T_{H}1 cells exposed to a high or low environmental concentration of IL-2 and examined the association of HIF-1α and c-Myc with a subset of the Bcl-6-repressed promoters. We found enrichment for the binding of c-Myc and HIF-1α at the Slc2a1, Pld2, and P4ha2 promoters in T_{H}1 cells maintained in a high environmental concentration of IL-2, which correlated with the expression of these genes (Fig. 5). In contrast, the binding of c-Myc and HIF-1α with these promoters was reduced in a low environmental concentration of IL-2 (Fig. 5). We also found enrichment for binding of c-Myc at the Slc2a3 and Tpi1 promoters in T_{H}1 cells exposed to conditions of a high environmental concentration of IL-2 (Fig. 5) and correlation with the expression of these genes (Fig. 5). In contrast, the association of Bcl-6 was with the loci of genes encoding molecules in the glycolytic pathway.

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**Figure 4** Genomic distribution of Bcl-6, HIF-1α, and c-Myc throughout the loci of genes encoding molecules in the glycolysis pathway. ChIP-seq tracks of BCOR, SMRT, and Bcl-6 (with high expression in the primary T_{H}1 cell experiments, including HK2, TPI1, ALDOA, PFKL, PFKM, PCK2, and GRHPR (Fig. 4 and Supplementary Fig. 6). Many of the Bcl-6 peaks also contained overlapping peaks for the corepressor BCOR and, less often, peaks for the corepressor SMRT and c-Myc. Overlap of c-Myc versus that of Bcl-6, and the overlapping subset of genes identified by microarray analysis as being regulated by Bcl-6 and HIF-1α, suggested that the Bcl-6-dependent gene program might oppose the gene program directly regulated by HIF-1α and c-Myc. To assess this possibility, we first used ChIP to analyze primary T_{H}1 cells exposed to a high or low environmental concentration of IL-2 and examined the association of HIF-1α and c-Myc with a subset of the Bcl-6-repressed promoters. We found enrichment for the binding of c-Myc and HIF-1α at the Slc2a1, Pld2, and P4ha2 promoters in T_{H}1 cells maintained in a high environmental concentration of IL-2, which correlated with the expression of these genes (Fig. 5). In contrast, the binding of c-Myc and HIF-1α with these promoters was reduced in a low environmental concentration of IL-2 (Fig. 5). We also found enrichment for binding of c-Myc at the Slc2a3 and Tpi1 promoters in T_{H}1 cells exposed to conditions of a high environmental concentration of IL-2 (Fig. 5) and correlation with the expression of these genes (Fig. 5). In contrast, the association of Bcl-6 was with the loci of genes encoding molecules in the glycolytic pathway.
Figure 5 The association of c-Myc and HIF-1α inversely correlates with binding of Bcl-6 at the promoters of genes encoding molecules involved in glycolysis. (a,b) ChIP-PCR analysis of T H1-polarized cells maintained in the presence of a high or low concentration of IL-2, assessed by immunoprecipitation of chromatin with anti-c-Myc (a) or anti-HIF-1α (b) or a nonspecific IgG control antibody (a,b) followed by quantitative PCR analysis of various promoter regions (vertical axes) with promoter-specific primers (results normalized and presented as in Fig. 3c). NS, not significant (P = 0.0577 (Tpi1 in a) and P = 0.0981 (Plod2 in b)); *P < 0.05 (unpaired Student’s t-test). Data are representative of three independent experiments (error bars, s.e.m.).

Fig. 4 and Supplementary Fig. 6. There was also a subset of genes that contained only Bcl-6 peaks without any detectable HIF-1α peaks (for example, B4GALT1 and PFKM in Supplementary Fig. 6). Many of these genes had not been identified in the initial overlapping subset of genes regulated by HIF-1α and Bcl-6 but instead were repressed by Bcl-6 in our expanded analysis of the glycolysis pathway and also contained c-Myc peaks (Supplementary Fig. 6). In conjunction with published studies,15,17 these data suggested a role for c-Myc and HIF-1α in directly activating a subset of genes encoding molecules important in the glycolysis pathway and associated pathways, and a repressive role for Bcl-6 in direct opposition to that gene program.

T-bet is required for enhanced glycolysis in T H1 cells

Bcl-6 has emerged as one of the critical factors that functionally regulates memory potential, and the activity of Bcl-6 must be precisely controlled to initiate either the memory cell gene program or the T H1 cell gene program.5,7,8,22 In T H1 cells, formation of the T-bet–Bcl-6 complex masks the Bcl-6 DNA-binding domain because the DNA-binding zinc fingers are also required for the interaction with T-bet.9,29 Thus, T-bet can hold Bcl-6 activity in check in effector T H1 cells because a high ratio of T-bet to Bcl-6 promotes complex formation, which dampens the potential of Bcl-6 to regulate its own target gene program. The relative balance of T-bet and Bcl-6 varies substantially in effector T cells versus memory T cells, and high T-bet expression is important for the development of short-lived effector cells.4,36–38 Collectively, these observations led us to hypothesize that the relative balance of T-bet and Bcl-6 might have a role in defining the state of the glycolysis-pathway gene program.

To address that possibility, we first examined the expression pattern of genes encoding molecules in the glycolytic pathway in wild-type versus T-bet-deficient primary T H1 cells. If T-bet inhibits Bcl-6 from repressing genes encoding molecules involved in glycolysis in effector T H1 cells, then the expression of target genes encoding glycolytic molecules would be inhibited in T-bet-deficient cells because Bcl-6 is no longer held in check by T-bet in this setting.9 Consistent with that prediction, the expression of many such genes was lower in T-bet-deficient cells than in wild-type effector T H1 cells (Fig. 6a and Supplementary Fig. 7). These included genes encoding rate-limiting enzymes, such as Aldoc and Pkm, as well as Slc2a3 and Slc2a1, which encode glucose transporters (Fig. 6a and Supplementary Fig. 7). Consistent with the reduced expression of these genes, there was also less of the permissive modification of histone H3 acetylated at Lys9 present at the promoters of Aldoc, Pkm, Slc2a3 and Slc2a1 in T-bet-deficient cells than in primary wild-type effector T H1 cells (Fig. 6b). We observed similar trends for the expression and histone-modification patterns of additional target genes identified in the microarray comparison of Bcl-6 and HIF-1α (Supplementary Fig. 7).

We next monitored lactate production in the setting of wild-type and T-bet-deficient CD4+ T cells to determine whether the observed gene-expression changes had functional consequences on glycolysis activity. Similar to published findings obtained for CD8+ T cells,17 lactate production was induced more in wild-type T H1 cells in the presence of a high environmental concentration of IL-2 than in the presence of a low concentration of IL-2 (Fig. 6c). Lactate production was significantly lower in T-bet-deficient cells cultured with a high environmental concentration of IL-2 than in their wild-type T H1 counterparts cultured with the same concentration of IL-2 (Fig. 6c). Notably, the concentration of lactate in T-bet-deficient cells exposed to a high environmental concentration of IL-2 was similar to that in wild-type cells exposed to a low concentration of IL-2 (Fig. 6c). These data suggested that in T H1 cells, T-bet expression was required for...
the IL-2-dependent increase in glycolysis activity. Consistent with the observation that the robust induction of Bcl-6 expression in conditions of a low environmental concentration of IL-2 is sufficient to bypass T-bet-mediated control, lactate production in wild-type and T-bet-deficient cells exposed to a low environmental concentration of IL-2 was similar (Fig. 6c). Together these data supported the hypothesis that T-bet was needed to inhibit the modest amounts of Bcl-6 expressed in effector T(H)1 cells to prevent Bcl-6 from dominantly repressing the expression of genes encoding molecules in the glycolysis pathway and that this activity was functionally important for promoting glycolysis in effector T(H)1 cells. In contrast, conditions of a low environmental concentration of IL-2 substantially enhanced Bcl-6 expression, which overcame T-bet-mediated control in T(H)1 cells, allowing Bcl-6 to downregulate glycolysis and create a metabolic state more compatible with the formation of memory cells.

T-bet restrains Bcl-6 at glycolysis–encoding genes

We next wanted to more directly address whether the role of T-bet in promoting the expression of genes encoding molecules in the glycolysis pathway might be mediated through its ability to functionally regulate Bcl-6 activity. To address this question, we needed to separate the mechanistic activity that T-bet uses to modulate Bcl-6 activity from all other aspects of the transcription-regulatory potential of T-bet. To accomplish this goal, we created a construct that contains only the carboxy-terminal domain (amino acids 300–530) of T-bet (T-bet(300–530)), which is the domain shown to be required for the physical interaction of T-bet with Bcl-6 (ref. 9). Notably, this truncated protein lacked the centrally located T-box DNA-binding domain as well as an amino-terminal domain needed for the transactivation potential of T-bet.

We first used coimmunoprecipitation analysis to demonstrate that the carboxy-terminal domain of T-bet alone was sufficient to mediate the interaction with Bcl-6 (Fig. 7a). We next assessed whether expression of T-bet(300–530) was sufficient to inhibit Bcl-6 from repressing its direct DNA-binding elements. To address this, we first did a series of experiments with a promoter-reporter construct that contains three Bcl-6 DNA-binding elements upstream of a minimal promoter (3×-Bcl-6)9. Repression of the 3×-Bcl-6 promoter-reporter construct is completely dependent on the Bcl-6 DNA-binding sites9. We transfected the 3×-Bcl-6 promoter-reporter construct into the A20 mouse B cell lymphoma line, together with either a control vector or one expressing T-bet(300–530). Notably, there was enhancement in 3×-Bcl-6 promoter-reporter activity in the presence of T-bet(300–530) (Fig. 7b). In contrast, the expression of another domain of T-bet (amino acids 120–331) that does not interact with Bcl-6 (T-bet(120–331)) had no effect on the activity of the 3×-Bcl-6 promoter-reporter construct (Fig. 7b). In additional control experiments addressing specificity, T-bet(300–530) had no activity when Bcl-6 expression was diminished by stimulation (Supplementary Fig. 8b,c). Together these data supported the interpretation that the physical interaction between T-bet and Bcl-6 inhibited Bcl-6 from repressing its own DNA-binding sites and that this experimental system recapitulated the principles of the T-bet-dependent control of Bcl-6 without introducing the DNA-binding-dependent transcriptional activity of T-bet into the cell.

To test the hypothesis that T-bet has the ability to functionally control the activity of Bcl-6 at genes encoding molecules important in glycolysis, we transfected promoter-reporter constructs for several such genes, together with empty vector or a vector expressing T-bet(300–530) or, as a control, T-bet(120–331). The expression of T-bet(300–530) enhanced the promoter-reporter activity of target genes encoding glycolytic molecules, including Slc2a3, Slc2a1, Pkm, Aldoc and Tpi1, but expression of the control T-bet(120–331) did not (Fig. 7c and Supplementary Fig. 8d). T-bet(300–530) also enhanced the promoter activity of Pld2 and P4ha2 (Fig. 7c and Supplementary Fig. 8d). In control experiments, overexpression of Bcl-6 in conjunction with T-bet(300–530) restored the Bcl-6-dependent repression
of the promoter-reporter constructs (Fig. 7d). Thus, the interaction between T-bet and Bcl-6 functionally inhibited the Bcl-6-dependent repression of the promoter activity of target genes encoding molecules of the glycolysis pathway.

Finally, we used this experimental system to assess whether the molecular balance of T-bet and Bcl-6 might influence the endogenous expression of genes encoding molecules important in glycolysis. Similar to the results obtained with the promoter-reporter constructs, expression of T-bet(300–530) was sufficient to modestly enhance the endogenous expression of a subset of these genes (Fig. 7e and Supplementary Figs. 8e and 9). For these experiments, we used human Ramos B lymphoblastoid cells and mouse A20 B cells, which have constitutive expression of Bcl-6; this provided support for the possibility that this mechanism might be conserved in different settings. Collectively, these data indicated that the interaction between T-bet and Bcl-6 functionally controlled the ability of Bcl-6 to repress a subset of target genes encoding molecules important for glycolysis, which linked the molecular balance of T-bet and Bcl-6 to the regulation of the glycolysis-pathway gene program.

DISCUSSION

Here we identified a role for Bcl-6 in repressing genes encoding molecules important in the glycolysis pathway and associated pathways. IL-2 signaling regulates several key transcription factors required for the activation and differentiation of T cells4,39,40. IL-2 signaling promotes the activity of c-Myc and HIF-1α, whereas it inhibits Bcl-6 expression, which creates reciprocal expression patterns for these factors in effector cell populations versus memory cell populations3,15,17,27. The close correlation between the expression of Bcl-6 and the repression of genes encoding molecules in the glycolysis pathway in the presence of a low environmental concentration of IL-2 led us to hypothesize that Bcl-6 might be one of the critical regulatory proteins that inhibits glycolysis in T cells. Indeed, Bcl-6 associated with the loci of many genes encoding molecules in the glycolysis pathway, including rate-limiting enzymes such as those encoded by Pkm and Hk2, and this correlated with their repression in T FH cells in an IL-2-sensitive manner. These results, in conjunction with the GEO2R microarray analysis of Bcl6−/− cells, the Bcl-6-overexpression studies of primary T H1 cells and the promoter-reporter experiments, indicated that Bcl-6 was an IL-2-sensitive factor that directly repressed genes encoding molecules in the glycolysis pathway. Finally, T-bet was required for the IL-2-dependent induction of glycolysis in effector T H1 cells, as T-bet inhibited the ability of Bcl-6 to dominantly repress target genes encoding glycolytic molecules. Therefore, T-bet indirectly served to promote the expression of genes encoding molecules in the glycolysis pathway in effector T FH1 cells by inhibiting Bcl-6 activity.

Identifying the types of genes that Bcl-6 regulates to serve its role in lineage-commitment ‘decisions’ has been somewhat enigmatic because Bcl-6 is a transcription repressor, and it has been difficult to envision the pathways that are directly regulated by Bcl-6 to promote a specific cellular state. In commitment to the T FH cell and B cell fate, at least one of its direct target genes is Prdm1 (which encodes the transcription repressor Blimp-1)2,9,41,42. However, the reciprocal regulation of Bcl-6 and Blimp-1 does not explain many of the altered cellular characteristics related to Bcl-6 expression. The finding of a role for Bcl-6 in regulating glycolysis in T cells has revealed a previously unknown cellular process that Bcl-6 controls that is probably important for defining effector states versus memory states in T cells. In this context, the metabolic state of cells of the immune system has a profound effect on their functional ability12,13,21,43, and experimentally manipulating the ability of the cell to use the glycolysis pathway for energy production can alter the effector fate–versus–memory fate ‘decision’14,21. Specifically, inhibiting glycolysis promotes the formation of memory cells21. Therefore, if the glycolysis program in effector cells can be turned off in response to changing environmental cues during the course of an immune response, such as conditions of waning IL-2 concentrations, this may initiate the transition of an effector cell into a memory cell. Our study has demonstrated that Bcl-6 dominantly repressed the glycolytic gene program, even in the presence of HIF-1α and c-Myc. This suggests a hierarchy in the competing regulatory pathways involved in the differentiation ‘decisions’ associated with effector and memory potential. This leads to the speculation that if Bcl-6 expression is induced in the effector cell population during the course of an immune response, the dominant nature of the Bcl-6-dependent repression of the glycolysis pathway might alter the metabolic state of the cell to promote memory formation.

Another notable aspect of the identification of a role for Bcl-6 in the IL-2-sensitive repression of genes encoding molecules in the glycolysis pathway is that Bcl-6 is also the lineage-specifying factor for T FH differentiation. It has been proposed that the gene-expression programs of CD8+ memory T cells and CD4+ T FH cells are related22. The data now available in the field suggest that a gradient of Bcl-6 expression serves to define whether a memory program or a T FH program will be initiated. That is consistent with the findings that a similar composition of signaling pathways and regulatory factors contribute to the differentiation potential of both cell populations but that there may be different thresholds for these events that are necessary for generating each unique cell population4,6,44,45. It will be informative to determine in future experiments whether the abundance of Bcl-6 promotes the expression of unique subsets of target genes that are more predominant in the memory phenotype or T FH phenotype or if instead target specificity is related to cofactor availability. In this context, it will be important to determine how the Bcl-6-dependent regulation of the glycolytic-pathway gene program fits into the potential for each of these cellular states.

We speculate that the findings of our study might also relate to the oncogenic potential of Bcl-6. Highly proliferating cancer cells are prone to express a glycolytic gene program, which is similar to the expression profile of the proliferating effector cells of the immune response12,26. The oncogenic potential of Bcl-6 at first glance appears to be incompatible with both its known role in the development of memory T cell and its newly identified role in the inhibition of the glycolysis-pathway gene program. However, studies examining the survival requirements of leukemia cancer stem cells have found that Bcl-6 expression in these cells, which constitute a relatively quiescent population, is important for their survival, and loss of Bcl-6 expression increases the proliferative capacity of the stem cells47. Such data suggest that the oncogenic potential of Bcl-6 in cancer stem cells may be similar to its role in long-lived memory cell populations. In the context of these more quiescent cell populations, inhibiting the glycolysis-pathway gene program may be an advantageous activity that Bcl-6 uses to promote long-term survival.

Finally, we note that creating a gradient of Bcl-6 activity in T H1 cells can also be achieved by modulating the relative molecular balance of T-bet and Bcl-6. This molecular balance is sensitive to environmental signals such as IL-2; this allows flexibility in aspects of the T H1 cell and T FH cell–like gene-expression patterns8. Here we have extended those findings to demonstrate that the IL-2-dependent induction of glycolysis required T-bet, most probably because the relative molecular balance of T-bet and Bcl-6 modulated the ability of Bcl-6 to dominantly repress genes encoding molecules involved in glycolysis. This creates a scenario in which the high expression of
T-bet in effector T<sub>H</sub>1 cells serves multiple purposes, which include promoting the expression of genes specific to the T<sub>H</sub>1 lineage as well as ensuring that genes encoding molecules in the glycolysis pathway have abundant expression. We note that there is a gradient of T-bet expression in effector and memory T cell populations. As such, the study has highlighted the importance of T-bet expression in the regulation of metabolism, and has provided evidence of the involvement of lineage-specifying transcription factors in modulating the expression of the metabolic gene program.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank D. Rawlings (University of Washington) for human Ramos B cells; the preclinical repository of the National Cancer Institute for IL-2 and anti-IL-4; M. Wijaranakula for technical assistance; D. Chisolm for critical reading of the manuscript; and A. Ballesteros-Tato, B. Leon and T. Dadaí-Abel for advice and assistance. Supported by the National Institute of Allergy and Infectious Diseases (AI061061 to A.S.W.) and the American Cancer Society (RSG-09-045-01-DDC to A.S.W.).

AUTHOR CONTRIBUTIONS

K.J.O. and A.S.W. conceived of and designed the study, performed experiments, analyzed data and wrote the manuscript; and K.A.R., S.E.G., K.P.H., P.W.M. and V.K. performed experiments and analyzed data sets.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Primary cells and cell culture. The MagCellect kit (R&D Systems) was used for the isolation of primary CD4+ or CD8+ T cells from the spleen and lymph nodes of wild-type C57BL/6 or Tbx21−/− mice, as published49,50. The primary cells were incubated with plate-bound anti-CD3 (145-2C11) and anti-CD28 (37.51; BD Pharmingen) in T12- and T13-polarizing conditions (anti-IL-4 (5 μg/ml; NIH) and IL-12 (5 ng/ml; R&D Systems)). Cells were split on day 3 and were cultured for an additional 2 or 3 d in conditions of a high concentration (250 units/ml) or low concentration (10 units/ml) of IL-2 (NIH) as described49. Experiments with mice were performed with approval from the Institutional Animal Care and Use Committee of the University of Washington, Virginia Tech and the University of Alabama at Birmingham.

Mouse EL4 T cells (TIB-39; American Type Culture Collection), A20 B cells or human Ramos B cells (provided by D. Rawlings) were cultured in RPMI medium supplemented with 10% FBS and penicillin-streptomycin. The Lonza nucleofection system with program 0-17 and solution V was used for transfection experiments as published49. For studies analyzing endogenous gene expression, Ramos B cells were transfected with an expression construct for the carboxy-terminal domain of T-bet (amino acids 300–530) or an empty vector control, then cells were stimulated with anti-CD40 (553787; BD Pharmingen) at 6–8 h after transfection and were harvested 48 h after transfection. For transfection of primary CD4+ T cells, the 4D nucleofection system from Lonza was used. Cells were transfected according to the protocol supplied by the manufacturer. Primary CD4+ T cells exposed to a high environmental concentration of IL-2 were resuspended in solution P3 and cells were transfected by nucleofection with program DN-100. Then, primary cells were allowed to recover for 24 h in conditions of a high environmental concentration of IL-2 before gene-expression analysis.

Commmunoprecipitation. The coimmunoprecipitation assay was performed as published49. Anti-V5 (AB9116; Abcam) was used for immunoprecipitation. For immunoblot analysis to monitor the coimmunoprecipitation of endogenous proteins, anti-Bcl-6 (K112-91; 561520, BD Pharmingen) was used.

Quantitative RT-PCR. The First Strand Superscript II Synthesis kit (Invitrogen) was used for preparation of cDNA. For quantitative PCR analysis, 20 ng of each cDNA template was amplified with gene-specific primers and qPCR Sybr Green Mix (Bio-Rad). For the experiments in Supplemental Figures 1, 2, 3 and 9, PrimerPCR custom plates (Bio-Rad) were used. All results were first normalized to those of the Rps18 control and are presented as a ratio to results obtained in the appropriate comparison condition.

ChIP assay. ChIP assays were performed as published49,50,51. Chromatin from primary polarized CD4+ T12 cells maintained in variable IL-2 conditions was precipitated with anti-Bcl-6 (C-19; Santa Cruz Biotechnology), anti-c-Myc (N-262; Santa Cruz Biotechnology), anti-HIF-1α (NB100-134; Novus Biologicals) or antibody to histone H3 acetylated at Lys9 (AB4441; Abcam). The purified DNA was analyzed by quantitative PCR with the appropriate primers. Results for the experimental samples were first normalized to those of a standardized input DNA control, then results obtained with the IgG control antibody were subtracted to account for nonspecific background, with this value presented in figures as the percent input of each sample.

Promoter-reporter assays. The 3×-Bcl-6 reporter construct has been described49. Slc2a3, Slc2a1, P浩a2, Plad2 and Tpi1 reporter constructs were prepared by cloning of the promoters of each gene into the pGL3-basic luciferase reporter construct (Promega). EL4 or A20 cells were cotransfected with the appropriate expression vectors along with the promoter-reporter constructs, as well as a CMV-renilla control plasmid to normalize for transfection efficiency. Samples were harvested 16–24 h after transfection and then were analyzed with the Dual-Luciferase Reporter system (Promega).

Quantification of intracellular lactate. Wild-type or Tbx21−/− cells were isolated, cultured and harvested as described above. After collection, cell pellets (5 × 106 cells per assay) were washed with 1× PBS for removal of residual medium, then were resuspended in 200 μl Lactate Assay Buffer (K607-100; BioVision) and were lysed by 20–25 passes through a 22-gauge needle. Lysed samples were centrifuged at 16,100g for 5 min for removal of cell debris before concentration and removal of lactate dehydrogenase via 10-kilodalton spin columns (1997-25; BioVision). Intracellular lactate concentrations were measured with a Colorimetric/Fluorometric Lactate Assay Kit (K607-100; BioVision) according to the manufacturer’s instructions.

GEO2R and genomic analysis. The difference in the expression of genes in untreated Bcl-6-deficient versus that in wild-type cells was determined by GEO2R analysis29 examining microarray expression data from a published study (GEO accession code, GSE24813)29. The Bcl-6 expression status in the two populations was used to confirm the identity of the Bcl-6-deficient sample. Genes that were functionally repressed by Bcl-6 were identified by analysis of the log, value for the difference in expression in Bcl-6-deficient cells versus wild-type cells and the P values for those differences. Those genes were then compared with genes identified in a published study as having different expression in wild-type cells versus HIF-1α-deficient cells17.

Published ChIP-seq data for Bcl-6 (GEO accession codes, GSE29282 and GSE46663)30,31, BCOR and SMRT (GEO accession code, GSE29282)30 and HIF-1α (GEO accession code, GSE39089)32 were uploaded onto the UCSC Genome Browser for visualization. BED files containing peak calls were used when provided with the GEO database file; otherwise, the Wiggle files were uploaded into the UCSC genome browser (hg18 (human genome version 18) reference). The c-Myc ChIP-seq peaks were derived from ChIP-seq data sets of the ENCODE consortium48 generated with human umbilical vein endothelial cells or GM12878 human lymphoblastoid cells (ChIP-seq data sets by the Vighy Iyer laboratory at the University of Texas at Austin or Michael Snyder laboratory from Yale University). The distribution of the Bcl-6, BCOR, SMRT, HIF-1α and c-Myc peaks surrounding the loci for select genes encoding molecules in the glycolysis pathway and associated pathways were then analyzed.

Statistical analysis. For statistical analysis, an unpaired Student’s t test was performed with GraphPad Prism online software. Experiments and analyses were performed in an unblinded fashion.