Chapter 7
Roles of Krüppel-like Factors in Lymphocytes

Kensuke Takada, Kristin A. Hogquist, and Stephen C. Jameson

Abstract Several family members of Krüppel-like factors (KLFs) are found in lymphocytes, and their expression is tightly regulated during development and differentiation. The related factors KLF2 and KLF4 have been suggested to promote lymphocyte "quiescence" by inducing withdrawal of cells from the cell cycle. Although the physiological role of KLF2 in cell cycle control in lymphocytes is currently unclear, it is potentially due to redundancy between related KLFs. On the other hand, there is growing evidence that individual KLFs regulate migration of lymphocytes (and other cells) during normal homeostasis of the immune system and in inflammatory situations. In addition to KLF2 and KLF4, the roles of KLF10 and KL13 in lymphocytes are briefly discussed.

Introduction

The immune system provides defense against diverse threats, including pathogenic microorganisms and cancer. Immunity depends on a complex process mediated by various cellular and humoral factors. Lymphocytes are especially important in the adaptive immune response, in which antigen-specific responses mediated by T cells and B cells can efficiently eliminate pathogens and tumors and lead to lifelong immunity. During the steady state, naïve lymphocytes with a wide range of specificities are maintained in a resting, "quiescent" state and recirculate between secondary lymphoid organs and the blood, the migration being dictated by various chemokines and adhesion molecules. It is within secondary lymphoid organs that encounters with foreign antigens occur, leading to activation and rapid proliferation of specific T and B cells. Upon successful elimination of the antigen, these specific

K.Takada (✉), K.A.Hogquist, and S.C. Jameson
Department of Laboratory Medicine and Pathology, Center for Immunology,
University of Minnesota, 312 Church Street SE, Minneapolis, MN 55455, USA

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T and B cells are maintained in the memory pool, yielding enhanced responses to the subsequent reencounter with the same antigen. Broadly, T cells are comprised of helper and cytolytic cells, the former having various functions including supporting B cell responses and production of cytokines, and the latter being responsible for killing infected or transformed cells. Activated B cells produce antibodies specific for their target antigen which can, for example, neutralize viruses.

Lymphocyte development, migration, activation, and subsequent differentiation are tightly controlled by a number of transcription factors, including some Krüppel-like factor (KLF) family members (Kuo and Leiden 1999). KLFs are a subfamily of the zinc-finger transcription factors and bind to GC-rich DNA elements through a highly conserved DNA-binding domain containing three $cysteine_2$/$histidine_2$-type zinc finger motifs located at the carboxyl terminus (Haldar et al. 2007; Kaczynski et al. 2003; Suzuki et al. 2005; Turner and Crossley, 1999). The mammalian KLF family is composed of 17 family members (Haldar et al. 2007), and distinct functions within the family can be attributed to differences in the expression profile and the structure of N-terminal domains involved in transcriptional repression and activation (Turner and Crossley 1999). Considerable progress has been made over the past decade on understanding the role of KLFs in regulating lymphocyte "quiescence" and "migration."

Regulated Expression of KLF2 and KLF4 in Lymphocytes

KLF2 and KLF4 are both expressed in T and B cells, and the expression of these KLFs changes dynamically with the differentiation state of the lymphocytes, as discussed below.

In the T cell lineage, KLF2 is expressed in mature T cells developing in the thymus (Carlson et al. 2006; Kuo et al. 1997b; McCaughtry et al. 2007; Mick et al. 2004). KLF2 mRNA is not detected in the abundant immature $CD4^+8^+$ thymocyte stage but is strongly upregulated in mature $CD4^+$ and $CD8^+$ thymocytes and is expressed in peripheral naïve T cells. The factors that induce KLF2 expression during T-cell development are unclear. Studies in lymphocyte and endothelial cell lines have suggested that the MEF2 transcription factor promotes KLF2 expression, and MEF2D activity is induced via activation of the mitogen-activated protein kinase–extracellular signal-regulated kinase (MEK5–ERK5) pathway (Parmar et al. 2006; Sohn et al. 2005). Winoto and colleagues have argued that this pathway may be triggered by either the T-cell receptor (TCR) or interleukin-7 receptor (IL-7R), both of which are critical for T-cell development (Sohn et al. 2005). Studies using *ERK5*$^{-/-}$ T cells suggest this factor is not essential for KLF2 expression (our unpublished data), although the role of MEF2D in regulation of KLF2 expression warrants further investigation. Recent studies have revealed that the Forkhead Box O protein FOXO-1 can drive expression of KLF2 in T cells (Fabre et al. 2008) and of KLF4 in B cells (see below) (Fruman 2004; Sinclair et al. 2008)) and is under the control of phosphoinositide 3-kinase (PI3K) signaling.

Downregulation of KLF2 is also tightly regulated. In mature T cells, stimulation through the TCR leads to rapid, profound loss of KLF2 (Endrizzi and Jameson 2003; Kuo et al. 1997b; Schober et al. 1999). This is mediated through reduced transcription of the *KLF2* gene (Endrizzi and Jameson 2003; Kuo et al. 1997b) but also appears to involve active degradation of KLF2 protein (Endrizzi and Jameson 2003; Kuo et al. 1997b). Lingrel's group showed that KLF2 protein is targeted for ubiquitin-mediated degradation, involving the WW domain-containing protein 1 (WWP1) E3-ubiquitin ligase (Conkright et al. 2001; Zhang et al. 2004), although it is not clear if this same pathway regulates KLF2 in lymphocytes. Reexpression of KLF2 occurs with the transition from effector to memory phase in vitro and in vivo (Bai et al. 2007; Grayson et al. 2001; Schober et al. 1999). Cytokines can affect KLF2 reexpression, as shown by the ability of IL-7 and IL-15 to induce KLF2 expression in activated T cells, whereas IL-12, IL-4, and high-dose IL-2 all prevent KLF2 reexpression (Bai et al. 2007; Endrizzi and Jameson 2003; Schober et al. 1999). The differential effects of IL-2 and IL-15 can be attributed to the fact that high-dose IL-2 (but not IL-15) induces sustained signaling through PI3K (Cornish et al. 2006; Sinclair et al. 2008), which negatively regulates KLF2 expression (Fruman 2004; Sinclair et al. 2008). Indeed, two downstream targets of the PI3K pathway, the mammalian target of rapamycin (mTOR) and Akt, negatively regulate transcriptional regulation of KLF2 (Fabre et al. 2008; Fruman 2004; Sinclair et al. 2008).

KLF2 regulation in B cells has been analyzed less extensively, but microarray studies indicate that KLF2 is upregulated by pre-B cell receptor (BCR) signals during development but transcriptionally silenced following BCR signals in mature B cells (Glynne et al. 2000; Schuh et al. 2008), suggesting potentially similar general regulation pathways.

KLF4 is highly homologous to KLF2, belonging to the same subclass (Kaczynski et al. 2003), and this factor has been more extensively studied in B cells. During development in the bone marrow, KLF4 is expressed in pre-B-I and small pre-B-II stages at higher levels than in other stages including large pre-B-II cells (van Zelm et al. 2005). Mature naïve B cells express high levels of KLF4 compared to pro-B cells and total pre-B cells (Klaewsongkram et al. 2007). Transcription of the *KLF4* gene is under the direct regulation of FOXO transcription factors (including FOXO1 and FOXO3a) and δEF1, another transcription factor that partially shares the target genes with FOXOs (Yusuf et al. 2008). Upon activation, mRNA and protein are both immediately downregulated (Good and Tangye 2007; Kharas et al. 2007; Klaewsongkram et al. 2007; Yusuf et al. 2008) through the PI3K–Akt signaling pathway (Yusuf et al. 2008). Reexpression occurs in human memory B cells, but this level is evidently lower than that in naïve B cells (Good and Tangye, 2007). In contrast to B cells, in-depth analysis of KLF4 regulation in T cells has not been reported.

Overall, there appear to be striking parallels between general expression patterns and regulation mechanisms for KLF2 and KLF4 in T and B lymphocytes, although current studies do not reveal whether there is truly coordinated regulation of both factors in the two cell types.

Function of KLF2 and KLF4 in Lymphocytes: Quiescence

Cellular quiescence is a state where cells exit the cell cycle from the G_1 into the G_0 phase (Yusuf and Fruman, 2003). Naïve lymphocytes are in a quiescent state until an encounter with specific antigens, and they are characterized by small size, lack of spontaneous proliferation, diminished metabolic rate, and resistance to apoptosis. Quiescence is evidently not a default state but, rather, is actively controlled (Kuo et al. 1997b; Yusuf and Fruman 2003; Yusuf et al. 2008). As discussed above, KLF2 and KLF4 are expressed in resting lymphocytes (naïve and memory cells), but both factors are downregulated rapidly after lymphocyte activation, suggesting that they may be quiescence factors, as previously discussed (Kuo et al. 1997b; Yusuf et al. 2008).

Analysis of KLF2-deficient mice initially suggested a key role in T-cell quiescence. Kuo et al. generated chimeras from *KLF2*$^{-/-}$ embryonic stem (ES) cells in recombinant-activating gene *(RAG) 2*$^{-/-}$ blastocysts (to bypass the embryonic lethality associated with KLF2 deficiency) and observed that *KLF2*$^{-/-}$ T cells developed ostensibly normally in the thymus but were reduced by more than 90% in peripheral lymphoid tissues (Kuo et al. 1997b). The few *KLF2*$^{-/-}$ T cells in peripheral sites had an activated phenotype ($CD44^{hi}CD69^{hi}CD62L^{lo}$) and were highly sensitive to Fas ligand-induced apoptosis (Kuo et al. 1997b). Based on these observations, a model was suggested that mature T cells undergo spontaneous activation and Fas-mediated death in the absence of KLF2, implying the requirement of KLF2 for T-cell quiescence (Kuo and Leiden 1999; Kuo et al. 1997a). This concept was reinforced by overexpression of KLF2 in the Jurkat T-cell line, which caused a halt in the autonomous proliferation of Jurkat cells, decreased cell size, and reduced metabolic activity (Buckley et al. 2001; Haaland et al. 2005; Shie et al. 2000; Wu and Lingrel 2004; Yusuf et al. 2008; Zhang et al. 2000). An impaired cell cycle was associated with reduced Myc mRNA levels (Buckley et al. 2001; Haaland et al. 2005), and related studies showed induction of $p21^{WAF1/CIP1}$ with KLF2 expression (Buckley et al. 2001; Shie et al. 2000; Wu and Lingrel 2004; Yusuf et al, 2008; Zhang et al. 2000). This expression pattern fits well with the concept that KLF2 expression could lead to cell cycle withdrawal.

Broadly similar functions have been proposed for KLF4 in the B-cell pool: Forced expression of KLF4 in activated B cells by retroviral transduction leads to cell cycle arrest at the G_1 phase (Yusuf et al. 2008). This is accompanied by increased expression of $p21^{WAF1/CIP1}$ and the decreased expression of Myc and cyclin D2, in accordance with the findings in T cells and nonlymphoid cells that KLF2 or KLF4 controls the expression of these cell cycle regulators (Buckley et al. 2001; Shie et al. 2000; Wu and Lingrel 2004; Yusuf et al. 2008; Zhang et al. 2000). Similarly, ectopic expression of KLF4 (or KLF9) in naïve and memory human B cells reduced the number of proliferating cells (Good and Tangye 2007). Concurrently, enforced expression of KLF4 induces apoptosis in B-cell and T-cell leukemia cells (Kharas et al. 2007; Yasunaga et al. 2004). Similarly, in nonlymphoid cells, KLF4 and KLF6 have been shown to induce $p21^{WAF1/CIP1}$ expression, leading to cell cycle arrest,

as a key component of the tumor suppressive activity of these KLFs (Narla et al. 2001; Rowland and Peeper 2006; Zhang et al. 2000). On the other hand, *KLF4*'s function in the cell cycle may be context-dependent, as it can act as an oncogene in $p21^{WAF1/CIP1}$-deficient cells (Rowland et al. 2005; Rowland and Peeper 2006). Hence, it may be hazardous to assume that expression levels of these KLFs allow prediction of cell cycle control, especially in cancer.

The most compelling data indicating a role for KLF2 and KLF4 in lymphocyte quiescence have come from overexpression studies. Such experiments suggest that these KLFs are sufficient for restraining cell cycle progression in lymphocytes, but it is less clear whether physiological levels of KLF2 and KLF4 have these roles. As is discussed below, the changes in the T-cell pool observed in $KLF2^{-/-}$ animals can be explained by altered thymic egress and T-cell trafficking rather than compromised quiescence regulation. Indeed, we found that activation, proliferation, and differentiation of KLF2-deficient T cells is quite normal, suggesting that the lack of KLF2 regulation does not lead to drastic changes in cell cycle control (our unpublished data). Likewise, whereas ectopic expression of KLF4 strongly restrains the cell cycle in activated B cells, KLF4 deficiency has minimal effects on B-cell development, survival, or functional reactivity (Klaewsongkram et al. 2007; Yusuf et al. 2008). In one report no difference was observed in cell cycle progression of stimulated WT and $KLF4^{-/-}$ B cells (Yusuf et al. 2008), whereas another study (using the same model system) reported activated $KLF4^{-/-}$ B cells had slightly impaired proliferation after BCR stimulation, showing a mild arrest of the G_1 to S phase transition, and decreased cyclin D2 expression (Klaewsongkram et al. 2007). Such data suggest that KLF4 has a modest role in promoting (rather than restraining) the cell cycle.

Hence current data make it difficult to determine whether physiological levels of KLF2 and KLF4 are relevant to the regulation of quiescence. Given the fact that there are overlapping patterns of expression for these two factors, and that they are highly homologous, there is a strong likelihood for some functional redundancy. Lymphocytes deficient in both factors are required to resolve this issue.

Forced expression of KLF2 and KLF4 has also been reported to moderately induce apoptotic death in T and B cells, respectively (Buckley et al. 2001; Yusuf et al. 2008), although once again it is unclear whether this reflects the role of KLF2 and KLF4 at physiological expression levels.

Control of Immune Cell Migration

Mature thymocytes leave the thymus through the blood and populate the peripheral T-cell pool. Similarly, immature B cells exit the bone marrow and undergo final maturation in the periphery. At steady state, naïve B and T cells continually circulate through secondary lymphoid organs (SLOs), blood, and lymph. These cellular dynamics allow immune cells to sample numerous SLOs in their search for foreign antigens. Such trafficking patterns require recognition of various chemokines and

adhesion molecules. For instance, T cells interact with high endothelial venules using CD62L/L-selectin and CCR7 to enter the lymph nodes (von Andrian and Mempel 2003). For entry to the mesenteric lymph nodes and Peyer's patches, β7 integrin is additionally required (von Andrian and Mempel 2003). Egress from lymphoid tissues is also regulated, and the sphingosine-1-phosphate receptor $S1P_1$ plays a critical role in permitting T-cell exit from the thymus (after T-cell development) and from SLOs (during lymphocyte recirculation) (Cyster 2005).

Carlson et al. analyzed *KLF2*$^{-/-}$ fetal liver chimeras (Carlson et al. 2006). Consistent with the finding by Kuo et al. in *KLF2*$^{-/-}$*RAG2*$^{-/-}$ chimeras (Kuo et al. 1997b), there were few T cells in the periphery despite superficially normal thymocyte development. However, in contrast to the previously suggested model in which *KLF2*$^{-/-}$ T cells spontaneously underwent activation-induced apoptosis, *KLF2*$^{-/-}$ thymocytes from fetal liver chimeras survived normally after adoptive transfer and showed an abnormal distribution, being absent in the blood and lymph nodes and accumulating in the spleen. Moreover, intrathymic injection of biotin demonstrated the impaired emigration of *KLF2*$^{-/-}$ thymocytes into the periphery, corresponding to the observation that $CD4^+8^-$ and $CD4^-8^+$ mature thymocytes accumulated in the thymus of *KLF2*$^{-/-}$ fetal liver chimeras. These trafficking abnormalities of *KLF2*$^{-/-}$ T cells were associated with defective expression of CD62L, CCR7, β7 integrin, and $S1P_1$, implying that the expression of these molecules is regulated by KLF2. Indeed, direct activation of promoters of genes encoding $S1P_1$ and CD62L by KLF2 was demonstrated in two studies (Bai et al. 2007; Carlson et al. 2006).

KLF2 has been shown to be reexpressed in the memory stage of T cells (Grayson et al. 2001; Schober et al. 1999). Memory T cells are divided into two major populations depending on their trafficking properties (Sallusto et al. 2004). CD62L and CCR7, whose expressions are affected in KLF2-deficient T cells, are commonly used as markers for this classification, distinguishing $CD62L^-CCR7^-$ effector memory (EM) and $CD62L^+CCR7^+$ central memory (CM) T cells (Carlson et al. 2006; Sallusto et al. 2004). Hence, one might expect a potential role for KLF2 in EM and CM T cell differentiation. $CD4^+$ T cell microarray analysis showed higher expression of KLF2 mRNA in CM cells than in EM cells in both humans (Riou et al. 2007) and the mouse (M.K. Jenkins, personal communication). In contrast, other studies of $CD8^+$ EM and CM T cells did not reveal extensive changes in KLF2 transcripts (Bai et al. 2007; our unpublished observations), although assessment of KLF2 protein expression in these subsets has not been reported.

Sedbza et al. employed conditional gene disruption by Vav-Cre transgene and floxed *KLF2* allele (Sebzda et al. 2008). These investigators replicated the findings reported above but suggested that the phenotype was not due to blocked thymic emigration; rather, it reflected misdirected T-cell trafficking into nonlymphoid organs, such as the liver. This abnormal migration was attributed to the increased expression of multiple chemokine receptors, including CXCR3 and CCR5, with the suggestion that KLF2 might suppress inflammatory chemokine receptor expression in addition to induction of homeostatic homing molecules (Sebzda et al. 2008). However, other studies involving T cell-specific KLF2 deficiency (using CD4-Cre) did not observe trafficking to nonlymphoid sites (our unpublished data).

A potential resolution of this issue arises from the observation that deletion of KLF2 in hematopoietic cells leads to severe alterations in the composition of the T-cell pool, with most T-cell subsets being reduced in number but a population of $CD4^+$ TCRγδ T cells being increased in number and frequency (our unpublished data). Clearly, further studies are required to determine how KLF2 regulates differentiation and trafficking of discreet T-cell subsets.

In the B-cell lineage, the significance of KLF2 for migratory regulation is much less clear. Although $S1P_1$ is also required for naïve B-cell trafficking (Matloubian et al. 2004), $KLF2^{-/-}$ naïve B-cell trafficking is not notably compromised (Carlson et al. 2006; Kuo et al. 1997b; our unpublished observations), indicating distinct regulation of $S1P_1$ in naïve B and T cells. On the other hand, the amount of $S1P_1$ in immunoglobulin G (IgG)-secreting plasma cells and plasmablasts determines whether they reside in the splenic red pulp or migrate to bone marrow through the blood (Kabashima et al. 2006). Expression of $S1P_1$ and KLF2 are both higher In the latter population than in the former population, suggesting a possible role of KLF2 in the regulation of plasma cell positioning and differentiation (Kabashima et al. 2006). In addition, pre-B-cell receptor signals strongly upregulate the expression of KLF2 and $S1P_1$, suggesting a potential role of KLF2 in controlling migration during B-cell precursor development (Schuh et al. 2008).

Hence, at present, the significance of KLF2 in B-cell trafficking and the relevance (if any) of KLF4 in T- or B-cell trafficking await further analysis. Given the similar functions of KLF2 and KLF4 in other systems (e.g., their impact on lymphocyte quiescence discussed above and their role in endothelial development as discussed by Lloyd in Chapter 9), we consider it likely that there is some redundancy between these factors.

KLF13 in T Cells

In contrast to the downregulation of KLF2 and KLF4 in activated lymphocytes, KLF13 expression is strongly induced by T-cell activation. KLF13 mRNA levels are similar in naïve and activated T cells (Song et al. 1999), but protein is abundantly detected only during the late stage of T-cell activation. This is due to translational regulation through 5′-untranslated regions of the transcripts that dictate expression of KLF13 protein (Nikolcheva et al. 2002; Song et al. 1999). Translation of KLF13 is dependent on a translational initiation factor, eIF4F, which mediates recruitment of ribosomes to mRNA; its activity is under the control of mitogen-activated protein kinase (MAPK) signaling through p38 and ERK-1/2 and PI3K-mTOR signaling (Nikolcheva et al. 2002). Furthermore, in activated T cells, KLF13 activity is post-translationally downregulated by phosphorylation (Song et al. 1999). Involvement of PRP4, a MAPK family member, has been demonstrated (Huang et al. 2007).

KLF13 was originally identified as a factor that regulates the expression of CCL5/RANTES, a chemokine produced at the late stage of T-cell activation (Song et al. 1999). CCL5 mediates the migration of a wide range of immune cells, including

T cells, monocytes, eosinophils, basophils, and natural killer (NK) cells (Song et al. 1999). The molecular mechanism of CCL5 expression that is driven by KLF13 was investigated in detail by Krensky and colleagues (Ahn et al. 2007; Song et al. 1999, 2002). Reporter gene assays in the Jurkat T-cell line showed that exogenous KLF13 recognizes the CTCCC sequence in the proximal promoter region of the *CCL5* gene and induces its expression (Song et al. 1999, 2002). Expression of CCL5 following activation was suppressed in primary T cells from KLF13-deficient mice and in normal T cells in which KLF13 was knocked down by small interfering RNA (Ahn et al. 2007; Zhou et al. 2007). Binding of KLF13 to the *CCL5* promoter was also demonstrated, indicating a physiological interaction of KLF13 and *CCL5* gene in activated T cells (Ahn et al. 2007). Factors associated with KLF13 for chromatin remodeling were also identified in activated T cells. Soon after activation, MAPK such as Nemo-like kinase binds to KLF13 at the *CCL5* promoter and phosphorylates the near by histones. This enables p300/cyclic AMP response element protein (CBP) and p300/CBP-associated factor to accetylate the histones at the later stage of activation, which is followed by the recruitment of an ATPase involved in chromatin remodeling, Brahma-related gene 1, to the promoter. These events recruit polymerase II to the adjacent TATA box of *CCL5* promoter, leading to transcriptional activation.

In KLF13-deficient mice, abnormalities are observed in multiple stages of lymphocyte development. They include the partial arrest of transition from $CD4^{+}8^{+}$ to $CD4^{+}8^{-}$ thymocytes and from large to small pre-B-II cells, implying that KLF13 regulates lymphocyte differentiation in the downstream of TCR and (pre-) BCR (Outram et al. 2008). However, the target genes of KLF13 in these processes remain to be identified.

KLF13 has also been reported to promote apoptosis. KLF13-deficient mice exhibit enlarged thymi and spleens because of decreased apoptosis of T cells (Zhou et al. 2007). This involves elevated expression of Bcl-X_L, an antiapoptotic factor. KLF13 can bind to the promoter of *Bcl-X_L* and decrease its activity (Zhou et al. 2007). However, KLF13-deficient mice do not show signs of either tumorigenesis or autoimmunity. Effects of KLF13 on lymphocyte cell cycle regulation have not been extensively investigated.

KLF10 in T Cells

KLF10 is induced by transforming growth factor-β (TGF-β) signaling—KLF10's alternative name, TIEG1, stands for TGF-β induced early gene 1—and has recently been found to play a key role in induction of a regulatory T-cell population (inducible Treg). T-cell stimulation in the presence of TGF-β can induce T-cell expression of the transcription factor Foxp3, which dictates a Treg differentiation pathway. KLF10 is a key component in efficient induction of Foxp3 by this mechanism; and to promote Foxp3 expression KLF10 must be monoubiquitinated by the E3 ligase Itch (Venuprasad et al. 2008). The KLF10 knockout shows defective production of inducible Treg. The ability of KLF10 to promote Foxp3 transcription directly

is in contrast with the typical activity of this factor as a transcriptional repressor (Kaczynski et al. 2003). A role for KLF10 in regulating differentiation or function of other T-cell subsets has not been reported, but it will be of interest to explore its role in the Th17 T cells. Th17 T cells play an important role in autoimmune diseases and in the control of certain extracellular pathogens (Bettelli et al. 2007; Dong 2008). Like Treg, their differentiation requires TGF-β signals; but in the case of Th17 cells, these are accompanied by signals through IL-6R.

Conclusion

Although several KLF family members are expressed in resting lymphocytes, the closely related factors KLF2 and KLF4 have been best studied. Both factors can induce lymphocyte quiescence, but the physiological significance of this is still unclear and is complicated by likely functional redundancy between KLF2 and KLF4. On the other hand, KLF2 appears to have a unique role in controlling migration of T cells via regulating expression of key trafficking molecules. In contrast, the factors KLF10 and KLF13 are important in postactivation T cells, contributing to functional T-cell differentiation. As in other tissues, the compensatory, combinatorial, or opposing roles of distinct KLF family members expressed in lymphocytes must be taken into consideration to understand the role played by these factors in regulating lymphocyte homeostasis and function.

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