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The transcription factors Thpok and LRF are necessary and partly redundant for T helper cell differentiation

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Summary

T helper (Th) cells are critical for defenses against infection and recognize peptides bound to Class II Major Histocompatibility Complex (MHC-II) molecules. Although transcription factors have been identified that direct helper cells into specific effector fates, whether a 'master' regulator controls the developmental program common to all Th cells remains unclear. Here we showed that the two transcription factors Thpok and LRF share this function. Although disruption of both factors did not prevent the generation of MHC II-specific T cells, these cells failed to express Th cell genes or undergo Th cell differentiation *in vivo*. In contrast, T cells lacking Thpok only displayed LRF-dependent functions and contributed to multiple effector responses, both *in vitro* and *in vivo*, with the notable exception of Th2 cell responses that control extra-cellular parasites. These findings identify the Thpok-LRF pair as a core node of Th cell differentiation and function.

Introduction

T helper (Th) cells control the function of most immune cells and are critical for defenses against infections and immune homeostasis (Paul, 2008). They recognize peptides bound to

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Class II Major Histocompatibility Complex (MHC-II) molecules and express the CD4 ‘coreceptor’ for MHC-II, contrasting with cytotoxic T cells which are MHC I-restricted and express the CD8 coreceptor for MHC-I. Unlike the relative uniformity of cytotoxic effector differentiation, the 'helper program’ is multi-faceted. Multiple subtypes of helper effectors have been identified, based on cytokine production patterns; these include cells producing the cytokines IFN-γ (Th1), IL-4, IL-5 and IL-13 (Th2), or IL-17 (Th17), in addition to regulatory T (Treg) cells that negatively control immune responses (Zhu et al., 2010; O'Shea and Paul, 2010). However, despite such diversity, a few core markers characterize helper cells and show little or no expression in $CD8^+$ cells (Xiong and Bosselut, 2011); these include CD4 itself, whose expression is epigenetically silenced in $CD8^+$ T cells (Zou et al., 2001), CD40L, a molecule essential for help to dendritic cells and B cells (Quezada et al., 2004), and the zinc finger transcription factor Thpok (He et al., 2005; Sun et al., 2005).

Which transcriptional programs underpin such functional differences between $CD4^+$ and $CD8^+$ T cells remains unknown. Most of the ‘master regulatory’ transcription factors that direct helper effector cell differentiation, including Gata3 for Th2 cells, T-bet for Th1, and RORγt for Th17, are specifically up-regulated in the corresponding effector subtype, but show little or no expression in naive $CD4^+$ cells (Zhu et al., 2010; O'Shea and Paul, 2010). Pre-programming for helper or cytotoxic function occurs in the thymus, during the divergence of CD4-helper and CD8-cytotoxic lineages, defined by the termination of either coreceptor expression after the $CD4^+CD8^+$ 'double positive' (DP) stage (Corbella et al., 1994; Matechak et al., 1996). Consequently, it is thought that the same transcriptional program directs both functional pre-programming and coreceptor expression. There is evidence that this is the case in the CD8 lineage, as the transcription factor Runx3 both represses *Cd4* and promotes cytotoxic gene expression (Taniuchi et al., 2002; Woolf et al., 2003; Wang et al., 2008a; Cruz-Guilloty et al., 2009). However, which factor(s) pre-program for helper functions has not been determined.

The zinc finger transcription factor Thpok (He et al., 2005; Sun et al., 2005) was proposed to serve such a function, because in its absence MHC II-restricted precursors differentiate into $CD8^+$ T cells instead of their normal $CD4^+$ fate (He et al., 2005). Such ‘redirected’ $CD8^+$ Thpok-deficient cells express the cytotoxic marker perforin and were therefore proposed to undergo cytotoxic differentiation. However, possibly because their expression of the CD8 coreceptor does not match their MHC-II specificity, their helper potential has not been analyzed so far.

The present study started with the striking observation that Thpok-deficient MHC II-restricted cells re-express CD4 upon activation, therefore reconstituting a matched TCR-coreceptor pair for MHC binding and raising the obvious question of their effector potential. We show that, unexpectedly, these cells retain key helper attributes. They contribute to multiple effector responses, both *in vitro* and *in vivo*, with the notable exception of Th2 cell differentiation that was inhibited by their expression of the CD8-differentiating factor Runx3. Such Thpok-independent helper functions require the Thpok-related transcription factor LRF, identifying a two-component control of helper T cell function by transcription factors of the Thpok family.

Results

Expression of Th cell genes in redirected Thpok-deficient cells

We first characterized peripheral T cells in mice lacking *Zbtb7b*, the gene encoding Thpok (Wang et al., 2008b). As previously reported (He et al., 2005), Thpok is required for MHC II-restricted cells to adopt their normal $CD4^+CD8^-$ expression pattern: *Zbtb7b*$^{-/-}$ mice had very few $CD4^+$ T cells (Figure S1A), and most *Zbtb7b*$^{-/-}$ MHC II-restricted cells were $CD4^-CD8^+$ and expressed cytotoxic markers (Figures 1A, S1B and data not shown). Using an MHC-II-tetramer-based assay identifying antigen-specific cells in the naïve repertoire (Moon et al., 2007), we found similar numbers of cells recognizing defined MHC-II-peptide complexes in Thpok-sufficient and -deficient mice (Figure 1B and data not shown). These cells were $CD4^+$ in the former and $CD8^+$ in the latter, and in both cases had low expression of the memory marker CD44, a characteristic of naïve T cells. These findings indicate that, despite their mismatched MHC specificity and coreceptor expression, Thpok-deficient cells have an antigenic repertoire similar to that of their wild-type counterparts.

In addition to these naïve $CD4^-CD8^+$ cells, Thpok-deficient spleens also contained small populations of CD4-expressing cells ($CD4^+CD8^+$ and $CD4^+CD8^-$) (Figure 1A). Unlike their $CD4^-CD8^+$ counterparts, these cells expressed high amounts of CD44, a feature typical of antigen-experienced T cells (data not shown). This raised the possibility that Thpok was dispensable for CD4 expression in antigen-activated cells. Indeed, purified Thpok-deficient 'redirected' $CD8^+$ splenocytes (MHC II-restricted, sorted $CD4^-CD8^+$) re-expressed CD4 when activated though their antigen receptor *in vitro* (Figures 1C and S1C). This was unlike MHC I-restricted $CD8^+$ cells, which epigenetically silence *Cd4* (Zou et al., 2001). Of note, Thpok-deficient cells that re-expressed CD4 nonetheless expressed the transcription factor Runx3, which is normally produced in $CD8^+$ cells and promotes *Cd4* silencing during their differentiation (Taniuchi et al., 2002; Woolf et al., 2003) (Figure 1D). Upon activation, redirected Thpok-deficient cells also expressed CD40L, a CD4-lineage molecule required for help to dendritic cells and B cells and for *in vivo* effector responses (Quezada et al., 2004) (Figure 1E). The distinctive gene expression of redirected cells was associated with the deposition of lysine 4-trimethylated histone H3 (H3K4Me3), a mark characteristic of genes actively transcribed or poised for expression (Barski et al., 2007) at characteristic helper genes; these included *Cd40lg*, the *Zbtb7b* locus itself, *Ctla4*, and *Ly6a*, although not *Cd4* (Figure 1F).

Thpok and LRF promote helper gene expression in vitro

These findings suggested that another transcription factor promoted helper gene expression in Thpok-deficient cells, and we considered the possibility that this factor could be Thpok-related. Of the two genes most closely related to *Zbtb7b*, namely *Zbtb7a* and *Zbtb7c* (Figure S2A), only the former, encoding the transcription factor LRF (Davies et al., 1999), is expressed during T cell differentiation (Maeda et al., 2007) (data from the Immgen database, and data not shown). Intra-cellular staining detected LRF protein in $CD4^+$ and $CD8^+$ SP thymocytes and T cells, and much lower expression in DP thymocytes (Figures 2A, S2B); this pattern contrasted with Thpok whose expression is limited to $CD4^+$ T cells (He et al., 2005; Sun et al., 2005). Given the pleiotropic effects of LRF on mouse development (Maeda

et al., 2007), we used *Cd4-Cre* mediated disruption to inactivate *Zbtb7a* and *Zbtb7b* in T cells. Although *Cd4-Cre* deletion of *Zbtb7a* efficiently disrupted LRF protein expression (Figure S2B), it did not detectably affect $CD4^+$ T cell differentiation and expression of helper genes (Figure S3A–E). As expected, *Cd4-Cre* disruption of *Zbtb7b* phenocopied the T cell developmental defects of germline deletion (data not shown).

We followed the fate of MHC II-restricted precursors double-deficient for Thpok and LRF in *B2m*$^{-/-}$ *Zbtb7b*$^{f/f}$ *Zbtb7a*$^{f/f}$ *Cd4-Cre* mice, hereafter called 'double-deficient', in which β2m disruption prevents MHC-I expression. These cells were redirected into the CD8 lineage and became mature CD8 SP thymocytes and T cells, similar to their Thpok-deficient counterparts, despite efficient LRF disruption (Figures 2B, C and S4A). Their Runx3 expression was similar to that of Thpok-deficient cells (Figure S4B). However, the $CD4^+CD8^+$ subset characteristic of Thpok-deficient animals was absent. While there were $CD4^+CD8^-$ cells in the spleen of double-deficient mice (Figure 2C), these cells were $CD44^{hi}$ and retained floxed *Zbtb7b* alleles (Figures S4C, D), suggesting that they resulted from the proliferation, possibly induced by environmental antigens, of small numbers of precursors that had not undergone deletion. If that interpretation were correct, these cells would not expand in the presence of wild-type competitors. To verify this, we generated mixed bone marrow chimeras by reconstituting lethally irradiated recipients with a mix of double-deficient and wild-type progenitors that can be distinguished by CD45 allelism (Figure 2D, left). While double-deficient cells efficiently contributed to spleen T cell populations, they did not give rise to any $CD4^+CD8^-$ or $CD4^+CD8^+$ cells (Figure 2D, right), supporting the idea that disruption of Thpok and LRF resulted in a complete lack of $CD4^+$ T cell differentiation.

Accordingly, double-deficient splenocytes did not re-express CD4 *in vitro* (Figure 3A), and failed to express CD40L, similar to wild-type (MHC I-restricted) $CD8^+$ cells (Figure 3B, top). These functional defects could be tracked to the thymus, where double-deficient CD8 SP cells expressed little CD40L, contrasting with Thpok-deficient CD8 SP or wild-type CD4 SP thymocytes (Lesley et al., 2006) (Figure 3B, bottom). Thus, expression of Th cell markers in Thpok-deficient cells is LRF-dependent.

LRF contributes to helper differentiation in vivo

To compare the contributions of Thpok and LRF to helper differentiation *in vivo*, we interrogated T cell populations in the small intestine lamina propria (siLP), in which effector and regulatory responses develop in unmanipulated mice as a result of exposure to commensal and food antigens (Agace, 2008). Most wild-type siLP T cells are MHC II-restricted $CD44^{hi}$ effectors, and normally $CD4^+$. In contrast, the siLP of Thpok-deficient mice contained very few $CD4^+$ cells but harbored a large subset of $CD4^+CD8^+$ T cells (Figure 4A). These cells were absent in mice lacking both Thpok and I-A^b (*Zbtb7b*$^{-/-}$ *H2-Ab1*$^{-/-}$), indicating that they were MHC II-restricted (Figure 4B); accordingly, their number was not affected by β2m disruption. The siLP $CD4^+CD8^+$ subset was absent in double-deficient mice (Figure 4C, left), and the number of remaining MHC II-restricted $CD4^-CD8^+$ cells was diminished (Figure 4C, right). As in the spleen, there were $CD4^+CD8^-$cells in the siLP of double-deficient mice, and they did not develop in a competitive setting (Figure

S4E). Thus, Thpok and LRF redundantly promote the differentiation of siLP helper effectors.

Expression of cytokines and effector transcription factors are key attributes of helper cells in the siLP (Zhu et al., 2010; O'Shea and Paul, 2010). While both helper ($CD4^+$) and cytotoxic ($CD8^+$) subsets normally produce IFNγ, cytotoxic cells generally do not express IL-17, a cytokine essential for the integrity of the gut mucosal barrier (Klatt and Brenchley, 2010), or the transcription factor RORγt (Zhou and Littman, 2009) (Figure 4D, left two columns). In addition, only $CD4^+$ cells normally express Foxp3, the key regulator of Treg differentiation (Zheng and Rudensky, 2007; Belkaid and Tarbell, 2009). Expression of these effector genes defined Thpok-deficient $CD4^+CD8^+$ cells as helper cells, as they included subsets expressing IL-17 or RORγt, or Foxp3 (Figure 4D, third column). In contrast, the cytokine production of the few $CD4^-CD8^+$ double-deficient cells was dominated by IFNγ, a cytokine normally produced by cells of either lineage, and they failed to expressed Foxp3 and RORγt (Figure 4D, right column). Of note, the siLP helper compartment was not affected by deleting LRF only (Figure S3D, E). These experiments suggested that Thpok and LRF redundantly control the development of $CD4^+$ helper responses *in vivo*.

To further evaluate the potential of LRF-dependent (Thpok-deficient) helper cells *in vivo*, we assessed their contribution to siLP effector responses in a competitive setting. We generated mixed bone marrow chimeras by reconstituting lethally irradiated $B2m^{-/-}$ recipients with a mix of $B2m^{-/-}$ Thpok-deficient and -sufficient progenitors. Remarkably, Thpok-deficient siLP effectors were only modestly out-competed by wild-type cells, as the ratio of mutant to competitor T cells was similar in the spleen and siLP (Figure 5A, B). Thus, LRF efficiently supports helper responses *in vivo*.

LRF supports Th1 and Treg, but not Th2 cell differentiation

While these analyses showed that LRF supported Th1, Th17 or Treg differentiation, they did not assess whether LRF-dependent cells could actually suppress, or if they could mount Th2 responses against extra-cellular parasites. To address the first issue, we examined if Thpok-deficient Treg cells inhibited the proliferation of wild-type effectors *in vitro*. We did not detect any difference between wild-type and Thpok-deficient Treg cells in these assays, demonstrating that LRF enables *in vitro* suppressive functions (Figure 5C).

Responses to extra-cellular parasites were of particular interest because Runx3, expressed in LRF-dependent helper cells (Figure 1D), inhibits expression of the Th2 cell cytokine IL-4 (Naoe et al., 2007; Djuretic et al., 2007). To examine Th2 cell responses, we challenged Thpok-deficient mice with the intestinal helminth *Heligmosomoides polygyrus* (Behnke et al., 2009), which normally generates a strong IL-4 and IL-13 response by $CD4^+$ Th2 effector cells (Figure 6A). Thpok-deficient mice failed to control the parasite, leading to inflammation, disruption of mucosal architecture, and increased parasite fecundity as assessed by egg counts (Figure 6B). Accordingly, Thpok-deficient siLP T cells predominantly produced IFNγ (Figure 6A). It was possible that inappropriate Runx3 expression inhibited the Th2 differentiation of Thpok-deficient cells; alternatively, it was possible that intrinsic properties of Thpok, not shared by LRF, were required for Th2 cell differentiation, or that LRF was not expressed in Th2-differentiating cells. Excluding the

latter possibility, we found LRF expressed in both wild-type ($CD4^+$) and Thpok-deficient ($CD8^+$) cells cultured in conditions that promote Th2 cell differentiation (Figure S5). We could examine the role of inappropriate *Runx3* expression *in vitro* because Thpok-deficient MHC II-restricted cells produced substantially less IL-4 than their wild-type counterparts, even in 'Th2-differentiating' cultures (Figure 6C). We found that retroviral transduction of a dominant negative version of Runx3 ('Runt'), which inhibits Runx functions (Wang et al., 2008a), restored their Th2 differentiation, increasing IL-4 and reducing IFNγ production (Figure 6D). Thus, Thpok preserves the Th2 potential of helper cells by constraining Runx3 expression.

Thpok-deficient animals mount effective responses to *Leishmania major*

The impaired *H. polygyrus* response raised the possibility that LRF-dependent helper cells, although effective under steady-state conditions, were unable to orchestrate an orderly immune response during an acute infectious challenge. To address this possibility, we examined the responses of Thpok-deficient mice to *Leishmania major*, an intra-cellular parasite normally controlled by MHC II-restricted effectors whose differentiation requires CD40L (Campbell et al., 1996; Kamanaka et al., 1996). We found little or no difference between Thpok-deficient and control animals after *L. major* inoculation. The clinical evolution was similar in both genotypes (Figure 7A), characterized by the appearance and resolution of a small lesion on the injected ear, matching the stereotypical *L. major* response in mice with intact helper T cell functions (Belkaid et al., 2000). The same was true of parasite loads at the end of the observation period (Figure 7B), demonstrating an efficient immune response. At the lesion site, we observed the IFNγ-producing Th1 effectors which control infection in *L. major* resistant strains (Wang et al., 1994). However, while these cells were all $CD4^+$ in control mice, they had the $CD4^+CD8^+$ phenotype characteristic of MHC II-specific responders in Thpok-deficient mice (Figure 7C). $Foxp3^+$ Treg cells, which restrain the Th1 response (Belkaid et al., 2002; Suffia et al., 2006), were also present at the infected ear in Thpok-deficient mice and most of them expressed CD4 (Figure 7C). We conclude that Thpok-deficient cells orchestrate helper responses *in vivo*.

In summary (Figure S6), the present study demonstrates that the transcription factors Thpok and LRF redundantly promote helper differentiation, and that, contrary to the current paradigm, Thpok-deficient MHC II-restricted cells have helper functions.

Discussion

While the emergence of helper and cytotoxic T cell functions is coupled to CD4 and CD8 lineage differentiation in the thymus, what pre-programs thymocytes into either functional fate has long remained unclear. In CD8-lineage cells, the transcription factor Runx3 both represses *Cd4* expression (Taniuchi et al., 2002; Woolf et al., 2003) and promotes cytotoxic gene expression (Cruz-Guilloty et al., 2009). $CD4^+$ cells have so far evaded a similar analysis, in part because they can adopt multiple helper effector fates defined by the expression of specific cytokines or transcription factors (O'Shea and Paul, 2010; Zhu et al., 2010). While the transcriptional circuitries that direct differentiation into each of these fates are increasingly well understood (O'Shea and Paul, 2010; Zhu et al., 2010), whether a

common transcriptional program underpins helper functions has remained unclear. Our study identifies such an activity and demonstrates that it relies on the distinct and partly redundant function of two transcription factors, Thpok and the related protein LRF.

While it was presumed that Thpok would be essential for helper functions because it is required for $CD4^+$ T cell differentiation, this hypothesis had never been evaluated. In fact, we found Thpok dispensable for many features of helper differentiation, as well as for Foxp3 expression and the acquisition of suppressive properties. Rather, the combined and partly redundant activities of Thpok and LRF form a critical node in the transcriptional circuits that enable helper differentiation. Such a role for LRF was unexpected. This transcription factor is involved in early hematopoietic differentiation, mature B cell differentiation, and it is also needed for oncogenic cell transformation (Sakurai et al., 2011; Maeda et al., 2009; Maeda et al., 2007). Distinct mechanisms are involved in these previously reported functions, including repression of the anti-apoptotic protein Bim, of Notch signaling or interference with p53 function, none of which is expected to account for LRF or Thpok function in helper differentiation.

Thymocytes lacking Thpok and Runx activities adopt a CD4-like phenotype (Egawa and Littman, 2008), suggesting that Thpok promotes helper differentiation by repressing *Runx3*. However, we found that cells lacking Thpok but having normal Runx activity undergo helper differentiation. Thus, *Runx3* repression is not required for helper differentiation *per se*; rather, it is necessary to preserve specific effector fates (including Th2) that are inhibited by Runx3. Indeed, in addition to directing cytotoxic differentiation (Cruz-Guilloty et al., 2009), Runx3 inhibits Th2 and promotes Th1 differentiation in $CD4^+$ T cells, notably by repressing IL-4 and promoting IFNγ expression (Djuretic et al., 2007; Naoe et al., 2007). Runx3 is normally expressed in Th1 effectors, but not in resting $CD4^+$ T cells or Th2 effectors, and it is because LRF fails to restrain *Runx3* expression that LRF does not support Th2 differentiation. These observations highlight the importance, for MHC II-restricted cells, to repress the cytotoxic differentiation program directed by Runx3. While it could conceivably be beneficial for these cells to maintain helper-cytotoxic bipotency, we show that restraining Runx3 expression is essential to preserve IL-4 production potential. Future studies will determine if the distinctive functions of Thpok and LRF result from intrinsic differences in their biochemical activities, for instance the recruitment of co-repressor molecules (Beaulieu and Sant'Angelo, 2011), or from their different levels or patterns of expression.

Thpok and LRF do not act by promoting the generation of MHC II-restricted cells in the thymus, but by enabling expression of helper effector genes. It is possible that these factors contribute to induce helper genes in MHC II-restricted thymocytes. Alternatively, Thpok and LRF could serve to maintain helper loci in an active configuration, by protecting them from transcriptional repression or silencing (Wildt et al., 2007; Muroi et al., 2008; Sakaguchi et al., 2010). In this alternative scenario, helper gene expression would be initiated in MHC II-restricted thymocytes independently of Thpok and LRF. Instead, it would rely on a distinct set of transcription factors required early during $CD4^+$ T cell differentiation, including Gata3, Tox, or E proteins E2A and HEB, all of which are notably

needed for Thpok expression (Pai et al, 2003; Jones and Zhuang, 2007; Wang et al., 2008b; Aliahmad and Kaye, 2008; Jones-Mason et al., 2012).

Such a 'maintenance' function for Thpok and LRF would agree with evidence that Thpok is not required in the earliest phases of CD4-lineage differentiation (Egawa and Littman, 2008; Wang et al., 2008b). It would also account for the apparent paradox that LRF, despite being expressed at similar levels in $CD4^+$ and $CD8^+$ cells, does not promote helper functions in the latter. Indeed, only MHC II-signaled thymocytes would initiate expression (or epigenetic opening) of helper loci; LRF or Thpok would then maintain these loci in an active configuration in mature $CD4^+$ T cells. In contrast, such 'priming' of helper loci would not happen in MHC I-restricted thymocytes, preventing LRF from promoting helper gene expression in $CD8^+$ cells. It is also conceivable that additional co-factors, specifically expressed in MHC II-restricted cells, have a permissive effect on Thpok or LRF functions. Future studies will explore these possibilities.

Our study emphasizes that helper gene expression has a strong antigen-induced component. While *Cd40lg* is known to be responsive to antigen stimulation (Roy et al., 1993), there is evidence that this is also the case for *Zbtb7b* and *Cd4* (Wang et al., 2008a; Park et al., 2010; Chong et al., 2010). This raises the possibility that the induction of helper genes in MHC II- but not MHC I-restricted cells is the result of an asymmetry in antigenic signals (in this case self MHC peptide) during CD4–CD8 lineage differentiation in the thymus. This hypothesis fits with the concept that longer or stronger antigen activation signals are required for $CD4^+$ than for $CD8^+$ differentiation (Singer et al., 2008).

Future studies will determine how Thpok and LRF maintain helper loci open. While $CD4^+$ T cells do not normally express *Runx3*, they express other factors that contribute to repress *Cd4* or *Zbtb7b*. These include Runx1, required for $CD4^+$ cell survival, AP-4, Mazr and Bcl11b (Egawa et al., 2007; Egawa and Littman, 2011; Sakaguchi et al., 2010; Kastner et al., 2010). It is possible that Thpok and LRF counteract such repression by binding to helper loci, as has been shown of Thpok for its own gene, *Zbtb7b*, and for *Cd4* (Muroi et al., 2008). An additional, non-mutually exclusive possibility is that, since both Thpok and LRF are thought to serve as transcriptional repressors, they repress so far unknown repressors of helper genes (Wildt et al., 2007).

One unexpected twist of our findings is the dissociation between resting T cell phenotype and effector potential: Thpok-deficient cells exit the thymus and colonize lymphoid organs as $CD4^-CD8^+$, yet they express helper genes upon activation. This demonstrates that functional differentiation of thymocytes can be uncoupled from their lineage choice as defined by coreceptor expression. This observation is reminiscent of the re-expression of CD4 by activated human $CD8^+$ T cells (Flamand et al., 1998; Yang et al., 1998). Conversely, studies in non-human primates have shown that MHC II-restricted cells can adopt a variant $CD4^-CD8^+$ surface phenotype, a mechanism that appears to allow these cells to escape infection by Simian Immunodeficiency Virus (Beaumier et al., 2009). In line with these observations, we considered the possibility that a subset of mouse MHC II-restricted cells could normally fail to sustain Thpok expression and differentiate into 'helper' $CD8^+$

cells. However, analyses in *Zbtb7b*$^{+/+}$ *B2m*$^{-/-}$ mice have so far failed to support that idea (data not shown).

In summary, we have identified a bi-functional transcriptional node that includes the transcription factors Thpok and LRF and promotes helper gene expression in MHC II-restricted cells. It serves to repress CD8-lineage genes, including *Runx3*, a function carried by Thpok, and to maintain helper genes in an open configuration, a function for which Thpok and LRF are partly redundant.

Experimental procedures

Mice

Zbtb7b$^{-/-}$, *Zbtb7b*$^{fl/fl}$, *Runx3*^{tRFP}, and *Zbtb7a*^{fl} mice (Wang et al., 2008b; Wang et al., 2008a; Zamisch et al., 2009; Maeda et al., 2007) were previously described. *H2-Ab1*$^{-/-}$, *B2m*$^{-/-}$ and *Cd4-Cre* animals (Grusby et al., 1991; Zijlstra et al., 1990; Lee et al., 2001) were from Taconic; CD45.1 and CD45.2 C57BL/6 animals were from the National Cancer Institute Animal Production Facility. All transgenic mice were heterozygous for the transgene they carry. Mice were housed in specific pathogen-free facilities and analyzed between 6–16 weeks of age unless described otherwise. Animal procedures were approved by relevant NIH Animal Care and Use Committees.

Bone marrow chimeras

T-depleted (Pan T Dynal kit, Invitrogen) bone marrow was isolated from CD45 disparate animals, mixed at a 1:1 ratio and injected into lethally irradiated (900 rads) recipients heterozygous for CD45.1 and CD45.2.

Antibodies

The following antibodies were from either BD Pharmingen or eBioscience: TCR (H57-597), CD4 (RM4.4 or GK1.5), CD8α (53-6.7), CD24 (M1/69), CD44 (IM7), CD69 (H1.2F3), IFNγ, IL-13, IL-4, CD45.1 (A20); CD45.2 (104), CD8β (53-5.8, used for all siLP staining), CD40L (MR1), Foxp3 (FJK-16S), IL-17A, RORγt (AFKJS9), LRF (13E9). Anti H3K4Me3 was from Millipore (#17–614). The anti-Runx antibody was from Epitomics (# 2593-1). Immunoblotting analyses were performed as described (Wang et al., 2008b).

Cell preparation and staining

Lymph node, thymus, spleen, siLP, and ear cells were prepared and stained according to previously described procedures (Wang et al., 2008b; Wang et al., 2008a; Sun et al., 2007). Flow cytometry data was acquired on LSR II or LSR Fortessa cytometers (BD Biosciences) and analyzed with FlowJo (TreeStar) software. Dead cells and doublets were excluded by DAPI and a combination of forward light scatter height and width gating. Enumeration of I-A^{b}-2W binding cells was performed as described(Moon et al., 2007).

For CD40L expression analyses, splenocytes or LN cells were first purified with a Dynal T negative isolation kit (Invitrogen); thymocytes and purified T cells were then cultured in 10% FCS supplemented RPMI 1640 medium in presence or absence of PMA (15 ng/mL)

and Ionomycin (300 ng/mL) at 37°C for three hours prior to staining. Analyses of intracellular cytokine expression were performed as described (Wang et al., 2008a). Transcription factor expression was detected using the eBioscience Foxp3 Staining Buffer Set (#00-5523) according to the manufacturer's instructions, except for RORγt and LRF staining during which the cells were permeabilized overnight. The BD Cytofix/Cytoperm kit (#554714) was used for intracellular stains in retrovirally infected cells. Dead cells were excluded using Invitrogen's LIVE/DEAD fixable UV kit (#L-23105).

In vitro T cell analyses

Sorted naïve ($CD44^{lo}$) LN and spleen cells were activated and cultured as described (Wang et al., 2008a), and re-stimulated for cytokine expression analyses for six hours before staining. siLP cells were stimulated (PMA/Ionomycin with GolgiStop) in RPMI supplemented with 10% FCS, 1% Pen/Strep/Glu, 1% NEAA, 1% Sodium Pyruvate, 1X 2-mercaptoethanol for 4.5 hours. Retroviral transductions were performed as previously described (Wang et al., 2008a).

For suppression assays, flow-sorted $CD4^+CD8^-CD25^+$ cells from *Zbtb7b*$^{-/-}$ or control *Zbtb7b*$^{+/+}$ animals (2×10^4) were mixed at indicated ratios with CFSE-treated $CD45.1^+$ *Zbtb7b*$^{+/+}$ $CD4^+CD25^-CD44^{lo}$ *ex vivo* T cells and incubated in a 96 well plate for 72 hours in 1mg/mL anti-CD3 and T-depleted irradiated splenocytes.

Infections

Mice were orally infected with 200 *H. polygyrus* infective larvae (L3 stage) prepared as previously described (Wilson et al., 2005) using an oral gavage needle. Infected mice were analyzed 28 days later. Cells were prepared as reported(Sun et al., 2007), with additional shaking in EDTA (2mM) supplemented medium prior to the first stirring incubation.

L. major infections were performed as described (Suffia et al., 2006) with 5×10^4 *L. major* clone V1 metacyclic promastigotes by intradermal ear injection. Skin lesions were measured weekly for eight weeks, and animals were subsequently euthanized. Cells were harvested from the infected ear and parasite loads determined as described (Belkaid et al., 1998).

Chipseq and data analysis

MHC I- and MHC II-restricted CD8 LN T cells were purified from *Zbtb7b*$^{+/+}$ and *B2m*$^{-/-}$ *Zbtb7b*$^{-/-}$, respectively, using the mouse CD8 negative isolation kit (Dynal, Invitrogen, #114.15D, #114.17D). Chipseq was performed on micrococcal nuclease-digested chromatin from 5–10 million cells as described (Wei et al., 2009). 36 bp sequence reads were acquired on an Illumina Genome Analyzer II according to the manufacturer's protocol in the NCI sequencing facilities. CD4 cell data was from (Wei et al., 2009). Analyses of Chipseq data were performed essentially as described (Wei et al., 2009) and using Python scripts written in the laboratory. Similar results were obtained using the chipseq analysis feature of Partek Genomic Suite.

Statistical analyses

Statistical analyses were performed using Prism (GraphPad Software, Inc.). Significance was determined using a two-tailed student t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Thpok-deficient MHC II-restricted T cell have helper functions.
- The transcription factor LRF promotes helper functions of Thpok-deficient cells
- Unlike Thpok, LRF does not support Th2 differentiation.

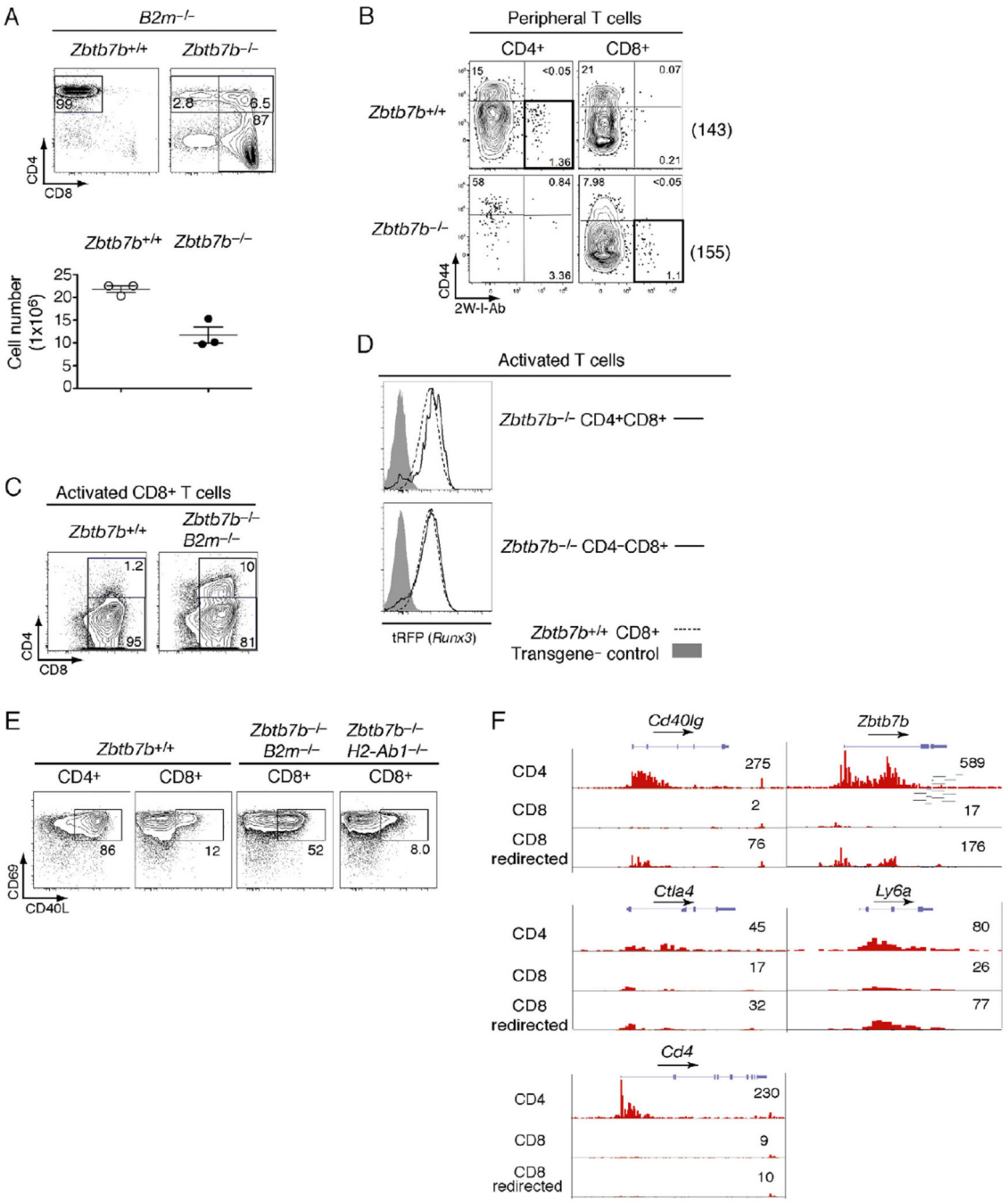

Figure 1. Thpok is dispensable for helper differentiation

(**A**) Contour plots show CD4 vs. CD8 expression on TCRβ$^+$ splenocytes from *Zbtb7b*$^{+/+}$*B2m*$^{-/-}$ and *Zbtb7b*$^{-/-}$ *B2m*$^{-/-}$ mice (top) and numbers of spleen T cells in each strain (bottom). (**B**) Contour plots are gated on CD4$^+$ or CD8$^+$ cells obtained from *Zbtb7b*$^{+/+}$ and *Zbtb7b*$^{-/-}$ mice and enriched on a 2W-I-A^b tetramer column, and show tetramer vs. CD44 staining. Between parenthesis (right) are absolute numbers of tetramer binding cells per mouse in outlined gates. (**C**) Contour plots show CD4 vs. CD8 expression on Th2-activated effectors obtained from sorted naïve (CD44^{lo}) CD4$^-$CD8$^+$ *Zbtb7b*$^{+/+}$ and

Zbtb7b$^{-/-}$ *B2m*$^{-/-}$ LN and splenic cells; the purity of each sorted population is shown in Figure S1C. (**D**) Plain line histograms show tomato-red fluorescent protein (tRFP) signal in activated *Zbtb7b*$^{-/-}$ TCRβ^{+}CD4^{+}CD8^{+} cells (top) and CD8^{+}CD4^{-} cells (bottom) carrying a tRFP BAC transgene (*Runx3*^{tRFP}) that tracks *Runx3* expression (Zamisch et al., 2009). Dashed lines show tRFP fluorescence in activated *Zbtb7b*$^{+/+}$ CD8 cells and grey-filled histograms background fluorescence in the corresponding subset from a 'reporterless' mouse. (**E**) Contour plot show CD69 versus CD40L expression after 3-hour activation of *Zbtb7b*$^{+/+}$, *Zbtb7b*$^{-/-}$*B2m*$^{-/-}$, and *Zbtb7b*$^{-/-}$*H2-Ab1*$^{-/-}$ LN cells; data are gated on TCRβ^{+} CD4^{+} or CD8^{+} cells as indicated. (**F**) Plots depict the distribution of H3K4Me3 along relevant CD4-lineage genes, as obtained from H3K4Me3 Chipseq analyses of wild-type CD4 and CD8 cells (both *Zbtb7b*$^{+/+}$) and redirected *Zbtb7b*$^{-/-}$ MHC II-restricted CD8 cells. Bars represent normalized H3K4Me3 signal in sequential 200 bp windows along each locus; genes are schematically depicted at the top of each graph. The same vertical scale was used for all plots. Numbers indicate cumulative H3K4Me3 signal within the locus. Data are representative of two (B, F) or at least three experiments (A, C–E).

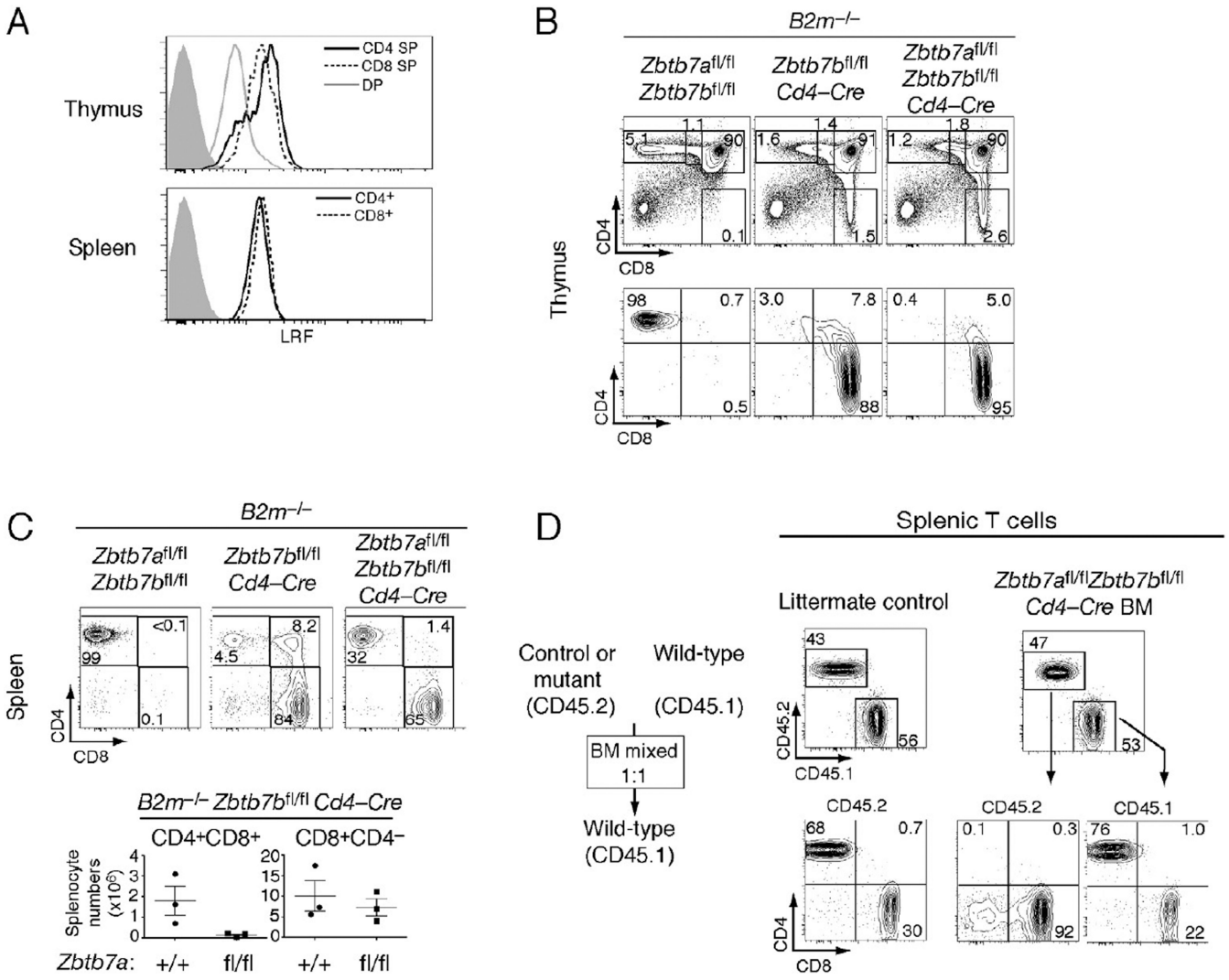

Figure 2. LRF expression and function in T cell development

(**A**) Histogram plots of LRF protein expression in wild-type DP, CD4 SP and CD8 SP thymocytes (top) and CD4$^+$ and CD8$^+$ splenocytes (bottom); grey-filled histograms indicate background fluorescence (no LRF staining) in DP thymocytes. (**B**) CD4 vs. CD8 contour plots on all live (top), and mature (TCRβ^{hi} CD24^{lo}, bottom) thymocytes from control, Thpok-deficient or Thpok and LRF (*Zbtb7b*$^{fl/fl}$ *Zbtb7a*$^{fl/fl}$ *Cd4*-Cre) double-deficient animals. (**C**) (top) Contour plots show CD4 vs. CD8 expression on TCRβ$^+$ splenocytes from the same mice as in (B). Bottom plots show numbers of cells in indicated populations of Thpok- (filled circles) and double-deficient (filled squares) mice. Bars indicate average ± SEM. (**D**) Lethally irradiated CD45.1 wild-type mice were reconstituted with a 1:1 mix of wild-type CD45.1 and either Thpok-LRF double-deficient or control CD45.2 bone marrow (left schematic). Contour plots of CD45.2 vs. CD45.1 on gated T cells (TCRβ$^+$ I-A/I-E$^-$) define donor- vs. competitor-derived populations eight weeks after reconstitution. T cells were further analyzed for CD4 and CD8 expression (bottom). Numbers in boxes represent percent of cells. (A–C) Data are representative of at least three experiments. (D) Data are

representative of two separate transplantation experiments, totaling three sets of double deficient and two sets of control donors (at least three recipients per set).

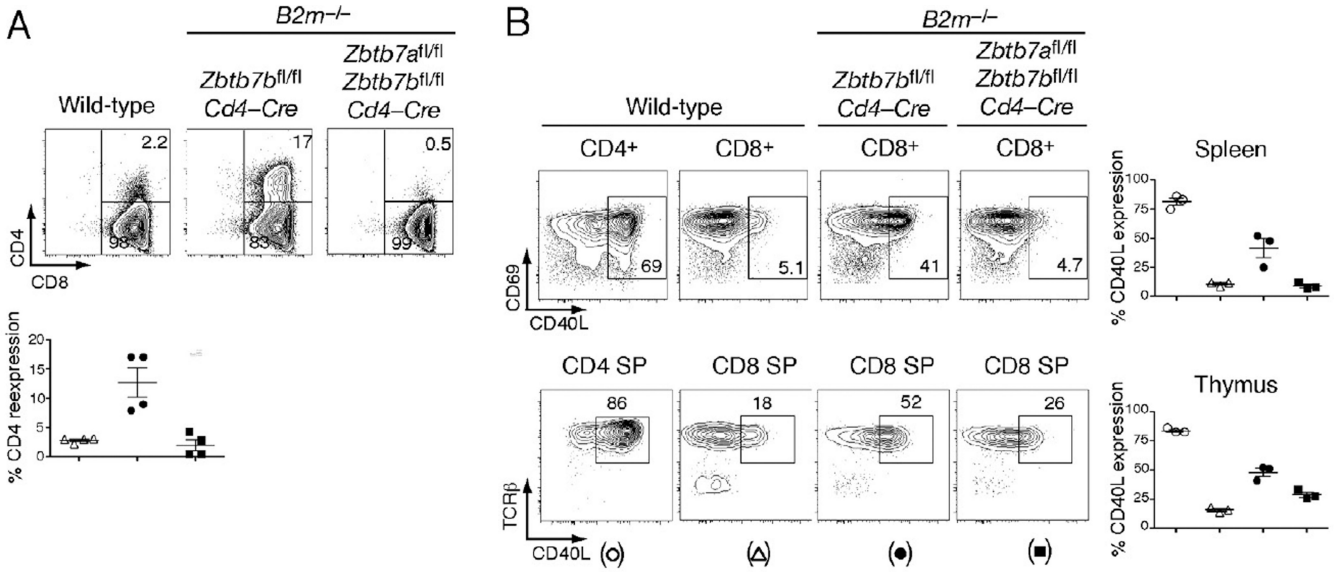

Figure 3. LRF promotes helper gene expression

(**A**) Contour plots of CD4 vs. CD8 expression on effectors obtained four days after activation of sorted naïve ($CD44^{lo}$) $CD4^{-}CD8^{+}$ T cells from the indicated mice; scatter plots on the bottom summarized data from four experiments (triangles: wild-type; circles: Thpok-deficient; squares: double-deficient). (**B**) (top) Contour plots show expression of CD69 vs. CD40L after 3-hour activation of wild type $CD4^{+}$ and $CD8^{+}$ splenocytes, or of $CD8^{+}$ splenocytes from *B2m*$^{-/-}$ mice that were Thpok-deficient or double deficient; (bottom) thymocytes from the same mice were analyzed in the same assay for expression of TCRβ vs. CD40L. Scatter plots on right summarizes all four experiments; symbol code is shown below graphs on the left, and bars depict mean ± SEM.

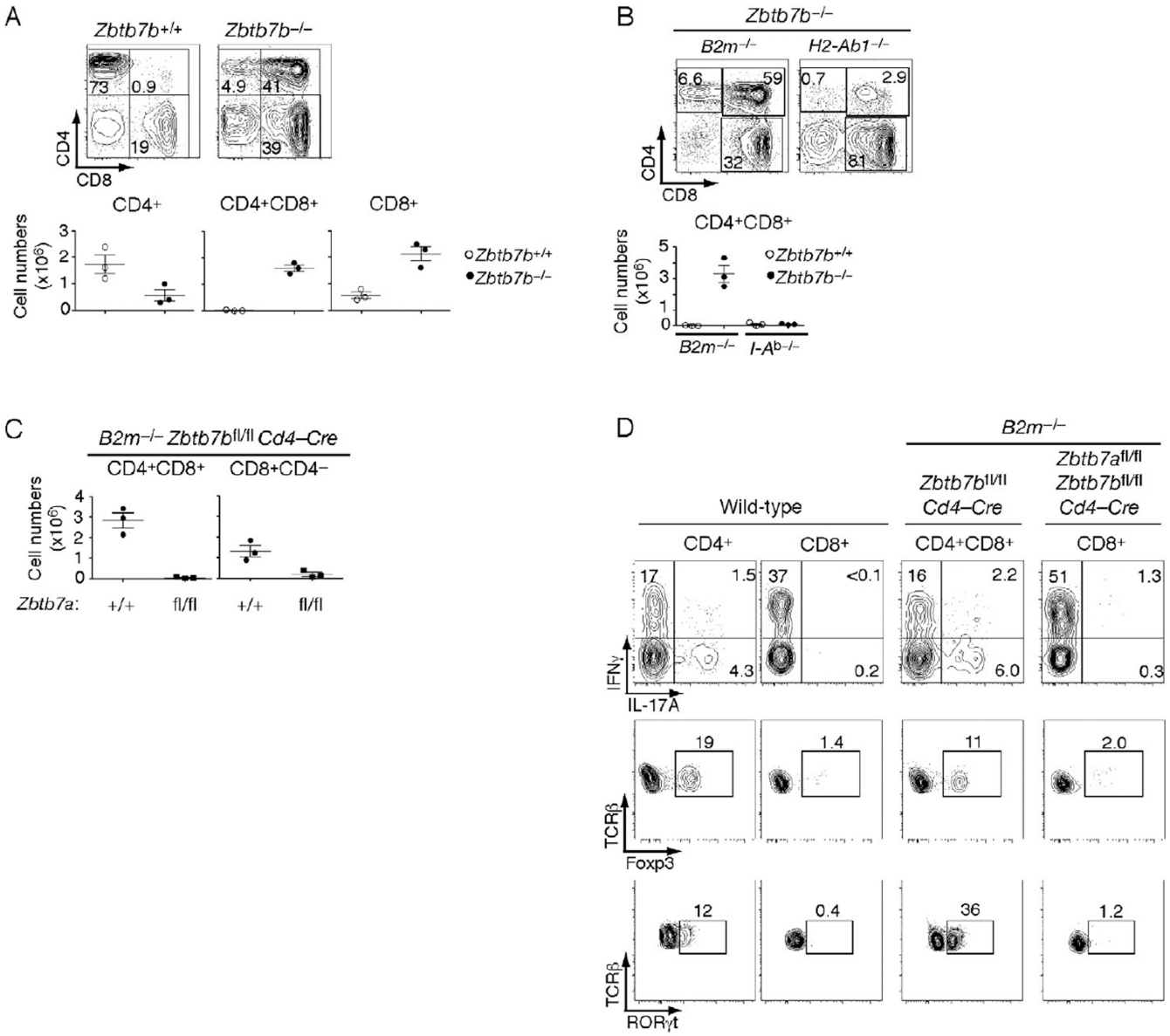

Figure 4. LRF promotes helper effector differentiation

Contour plots show CD4 vs. CD8 expression on TCRβ$^+$ siLP cells from (**A**) *Zbtb7b*$^{+/+}$ or *Zbtb7b*$^{-/-}$ or (**B**) *Zbtb7b*$^{-/-}$ *B2m*$^{-/-}$ or *Zbtb7b*$^{-/-}$ *H2-Ab1*$^{-/-}$ animals. Bottom panels depict numbers of *Zbtb7b*$^{+/+}$ (open circles) and *Zbtb7b*$^{-/-}$ (filled circles) T cells from the indicated subset. (**C**) Numbers of siLP T cells in *B2m*$^{-/-}$ mice that were Thpok-deficient or Thpok and LRF double deficient. Bars (A–C) indicate average ± SEM. (**D**) Contour plots show IFNγ vs. IL-17A (top), TCRβ vs. Foxp3 (middle) or TCRβ vs. RORγt (bottom) on gated TCRβ$^+$ siLP cells from control, Thpok-deficient or Thpok and LRF double-deficient animals. Numbers within or near boxes indicate percent of cells within that box. Data are representative of at least three experiments for each panel.

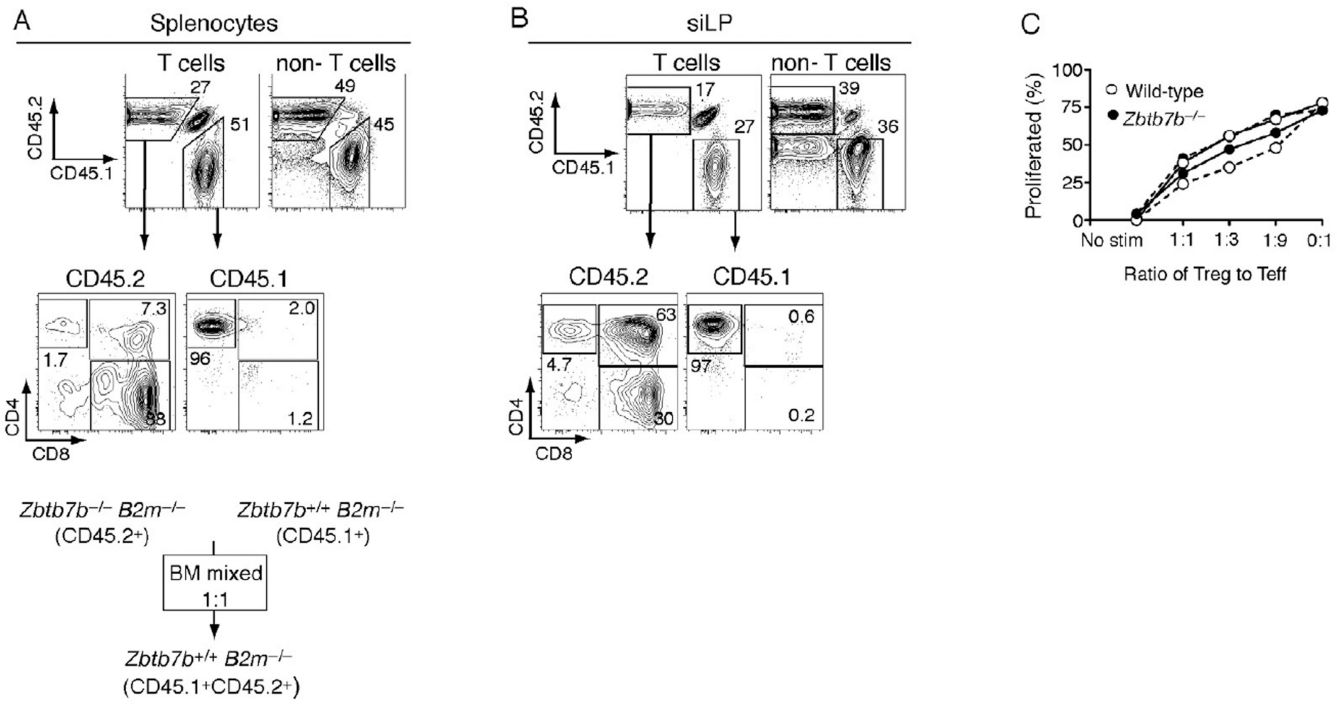

Figure 5. LRF-dependent helpers are functionally fit and have regulatory function

(**A, B**) Lethally irradiated *Zbtb7b*$^{+/+}$*B2m*$^{-/-}$ mice carrying both CD45.1 and CD45.2 alleles were reconstituted with a 1:1 mix of *Zbtb7b*$^{+/+}$*B2m*$^{-/-}$ (CD45.1) and *Zbtb7b*$^{-/-}$*B2m*$^{-/-}$ (CD45.2) bone marrow (A, bottom schematic). Eight weeks after reconstitution, spleen (A) and siLP (B) cells were analyzed by flow cytometry for expression of CD45 alleles, TCRβ, I-A/I-E, CD4 and CD8. Two-parameter plots (top) of CD45.1 vs. CD45.2 on gated T cells (TCRβ^{+} I-A/I-E^{-}) or non-T cells (TCRβ^{-} I-A/I-E^{+}) define donor-derived populations. T cell populations were further analyzed for CD4 and CD8 expression (bottom). Numbers in graphs represent percent of cells in gates. (**C**) Plot shows percentage of proliferating effector cells in a 3-day *in vitro* suppression assay with sorted CD4^{+}CD25^{+} cells from wild-type (open circles) or *Zbtb7b*$^{-/-}$ (filled circles) mice. Plain and dashed lines depict two distinct experiments. In A and B, data are representative of four separate bone marrow transplantation experiments (three experimental and three control animals each), two of which were with *B2m*-sufficient donors.

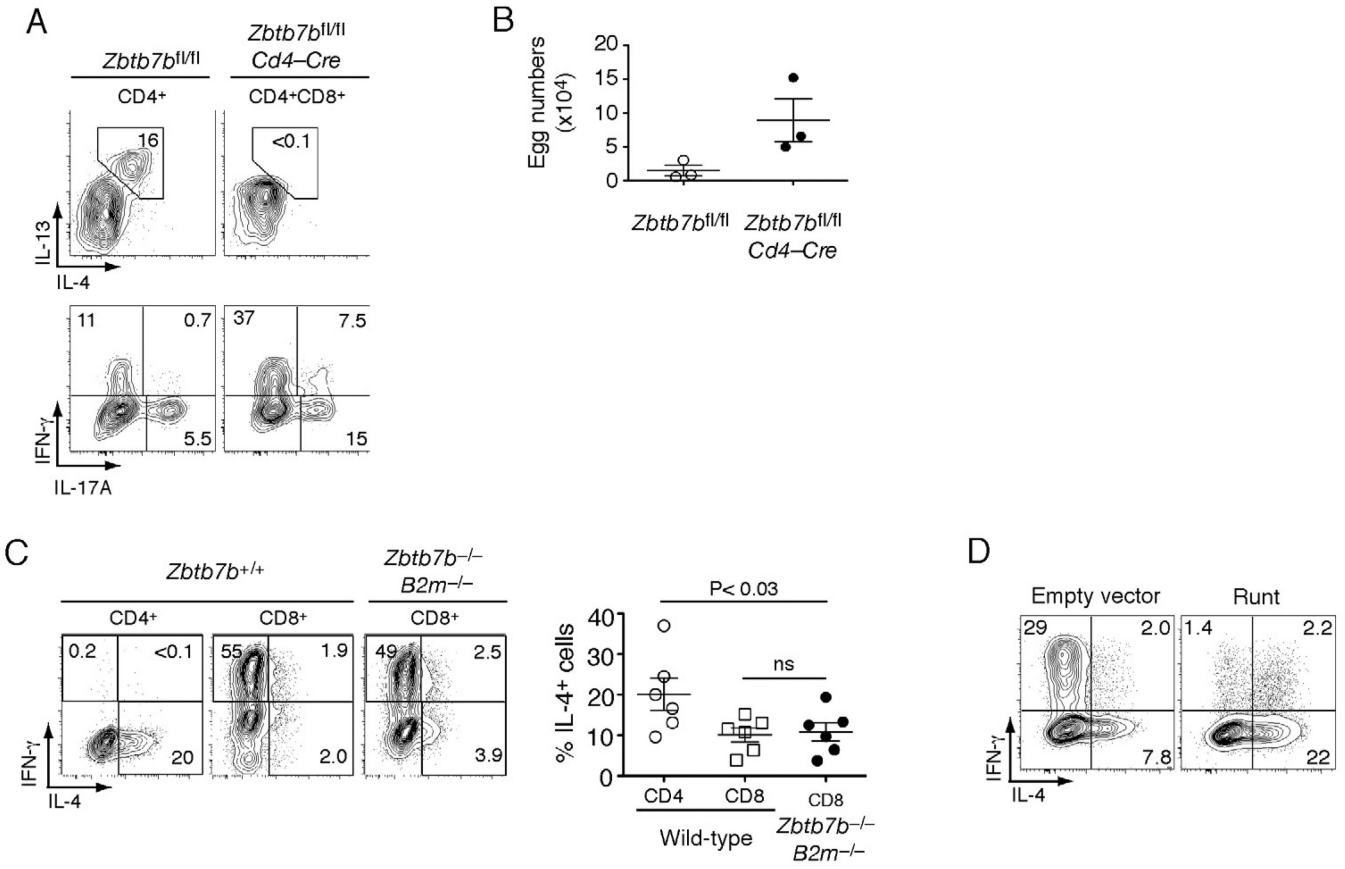

Figure 6. LRF does not support Th2 differentiation

(**A**) Contour plots show IL-4 vs. IL-13 and IFN-γ vs. IL-17A expression in siLP T cells from *H. polygyrus* infected animals. (**B**) Numbers of eggs in animals analyzed in (A). (**C**) Contour plots show IFNγ vs. IL-4 expression on effectors obtained from sorted naïve *Zbtb7b*$^{+/+}$ CD4^{+} (left) or CD8^{+} (middle) LN cells or CD4^{-}CD8^{+} cells from *B2m*$^{-/-}$ *Zbtb7b*$^{-/-}$ mice (right panel) after a four-day culture in Th2 conditions. Plot on right is a summary of those results. (**D**) IFNγ vs. IL-4 expression is shown on activated *Zbtb7b*$^{fl/fl}$ *Cd4-Cre B2m*$^{-/-}$ CD8^{+}CD4^{-} LN cells activated as in (C) and transduced at day 1 with a Runx3 dominant negative (Runt) or control (empty) retrovirus; data are gated on transduced (GFP^{+}) cells. (A, B) Data are representative of two separate infection experiments, one with three *Zbtb7b*$^{fl/fl}$ *Cd4-Cre* and three controls the second with three *Zbtb7b*$^{-/-}$ animals and three controls. (C, D) Data are representative of at least three experiments. Bars indicate average ± SEM. Significance was determined by a paired t test.

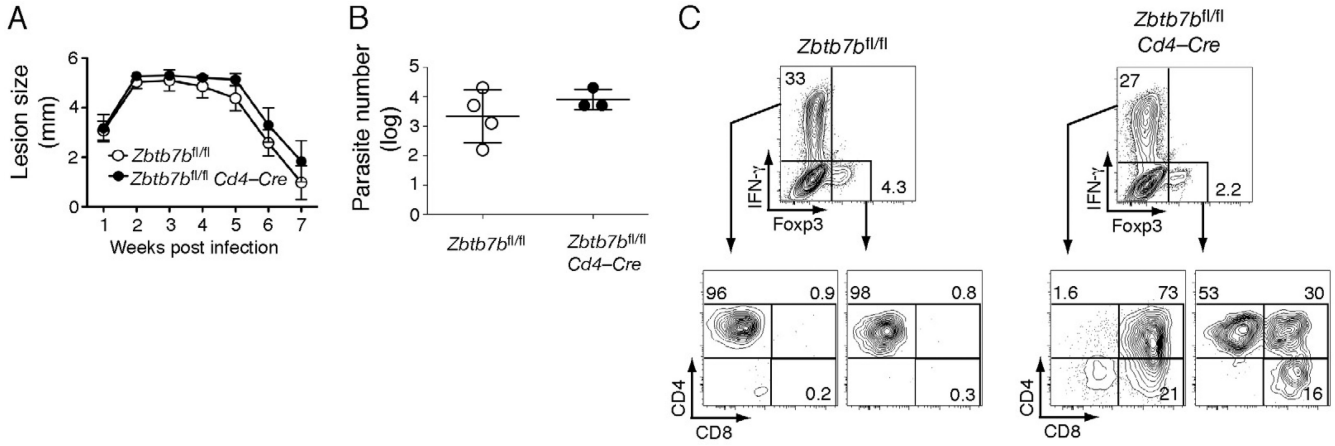

Figure 7. LRF supports helper responses *in vivo*

(**A**) Evolution of ear lesions in *L. major*-infected *Zbtb7b*$^{fl/fl}$ *Cd4-Cre* (filled circles) or *Zbtb7b*$^{fl/fl}$ animals (open circles). (**B**) Numbers of ear parasites in animals in A at eight weeks. (**C**) Top contour plots define IFN-γ and Foxp3-expressing populations in T cells from infected ears, which were analyzed for CD4 and CD8 expression (bottom row). (A–C) Data are representative of two separate experiments, one (shown here) on *Zbtb7b*$^{fl/fl}$ *Cd4-Cre*, the other on *Zbtb7b*$^{-/-}$ and wild-type controls with two animals per group. Bars indicate average ± SEM.