HHS Public Access

Author manuscript

Nat Immunol. Author manuscript; available in PMC 2015 April 01.

Published in final edited form as:

Nat Immunol. 2014 October ; 15(10): 947–956. doi:10.1038/ni.2960.

A ThPOK-LRF transcriptional node maintains the integrity and effector potential of post-thymic CD4+ T cells

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Abstract

The transcription factor ThPOK promotes $CD4^+$ T cell differentiation in the thymus. Here, using a mouse strain that allows post-thymic gene deletion, we show that ThPOK maintains $CD4^+$ T lineage integrity and couples effector differentiation to environmental cues after antigenic stimulation. ThPOK preserved the integrity and amplitude of effector responses, and was required for proper T_H1 and T_H2 differentiation *in vivo* by restraining the expression and function of the transcriptional regulator of cytotoxic T cell differentiation, Runx3. The transcription factor LRF contributed in a redundant manner with ThPOK to prevent the *trans*-differentiation of mature $CD4^+$ T cells into $CD8^+$ T cells. As such, the ThPOK-LRF transcriptional module was essential for $CD4^+$ T cell integrity and responses.

$CD4^+$ T cells typically recognize peptide antigens presented by the class II major histocompatibility complex (MHC class II) molecules, and upon activation differentiate into

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Database accession numbers

Microarray data was deposited in the GEO database under accession number GSE57846.

The authors declare no competing financial interests.

Author Contributions

M.V., L.W., Y.B. and R.B designed the research; M.V., L.W., N.B, A.C.C, Y.X, L.C.W, and E.W. performed and analyzed experiments; K.-D.S. and P.E.L constructed and provided *CD2*-Cre mice; R.B. supervised the research; M.V. and R.B wrote the manuscript.

subtypes of effector helper T cells (T_H) expressing specific transcription factors and cytokines[1,2]. Such effector T cell fates include T_H1, which express interferon γ (IFN-γ) and the transcription factors T-bet and Runx3, T_H2, which express interleukin 4 (IL-4) IL-13 and the transcription factor GATA3, or T_H17, which express IL-17 and the transcription factor RORγt. In contrast, MHC class I-restricted T cells express CD8 and typically differentiate into cytotoxic effectors after antigen stimulation[3].

$CD4^+$ and $CD8^+$ T cells differentiate in the thymus from $CD4^+CD8^+$ (double positive, DP) precursors that lack functional potential[4]. Commitment to either lineage involves the mutually exclusive expression of two transcription factors, ThPOK and Runx3[5–8], so that $CD4^+$ T cells express ThPOK but no Runx3, whereas the opposite is true of $CD8^+$ T cells[9]. Current views propose that these factors participate in a dual negative regulatory loop, in which ThPOK represses the genes encoding Runx3 and CD8 subunits (CD8α and CD8β), and Runx3, redundantly with the related molecule Runx1, represses those encoding ThPOK and CD4[10–13]. Such a model accounts for the observation that intrathymic or germline disruption of ThPOK unleashes *Runx3* expression and 'redirects' MHC class II-restricted thymocytes into $CD8^+$ T cells[7].

In addition to controlling CD4 and CD8 expression, ThPOK contributes to thymocyte functional differentiation. Even though effector differentiation is manifest only upon antigen activation, mature thymocytes are functionally 'pre-programmed' as helper or cytotoxic and express genes specific of either fate[9]. Redundantly with the related transcription factor LRF[14], ThPOK is required in the thymus for helper 'pre-programming', as ThPOK and LRF-deficient MHC II-restricted thymocytes fail to express CD40L, a $CD4^+$-lineage specific molecule involved in multiple aspects of $CD4^+$ T cell function[15], and to give rise to functional T_H cells[16].

Although ThPOK remains highly expressed in peripheral $CD4^+$ T cells[7,10–12], little is known about its role in these cells, whether before (naïve T cells) or after (T effector cells) antigen contact. Because T_H1 effector cells co-express ThPOK and Runx3, it remains unclear whether post thymic ThPOK represses *Runx3*. While ThPOK disruption increases the expression of the cytotoxic enzyme Granzyme B and that of IFN-γ in $CD4^+$ T effector cells[10], the biological impact of ThPOK disruption remains to be determined. It has been proposed that ThPOK expression in intestinal $CD4^+$ T cells promotes gut inflammation by sustaining T_H17 responses[17], but whether ThPOK affects $CD4^+$ T effector cell differentiation *in vivo* remains unknown.

In this study, we used a mouse strain expressing the Cre recombinase in post-thymic T cells to inactivate ThPOK in naïve $CD4^+$ T cells, prior to activation and effector differentiation. We show that post-thymic ThPOK restrains the expression of *Runx3* in resting and activated $CD4^+$ T cells and is needed for T_H2, but not for T_H17, effector responses. In addition, even though Runx3 promotes expression of the T_H1 cytokine IFN-γ[18,19], ThPOK was required for T_H1 differentiation and prevented the diversion of T_H1 $CD4^+$ cells to a cytotoxic gene expression program. Last, we demonstrate that ThPOK and LRF redundantly prevented the trans-differentiation of $CD4^+$ into $CD8^+$ T cells. These findings demonstrate that ThPOK is

essential to preserve the functional diversity of $CD4^+$ T cells and the proper matching of $CD4^+$ effector responses to the cytokine environment conditioning effector differentiation.

Results

Post-thymic Thpok inactivation in resting $CD4^+$ T cell

To evaluate the post-thymic functions of ThPOK, we conditionally disrupted *Zbtb7b* (the gene encoding ThPOK, thereafter called *Thpok*) using a Cre recombinase transgene driven by the human *CD2* promoter. Contrary to other *CD2*-driven transgenes, this construct is not active in most DP and CD4SP thymocytes, but is upregulated after thymic development; thus, it ensures efficient deletion in peripheral $CD4^+$ T cells, as shown with a *Rosa26*^{YFP} reporter for Cre activity (Fig. 1a). Deletion was also efficient in $CD8^+$ T cells, and was already substantial in CD8SP thymocytes (Supplementary Fig. 1a), consistent with CD8SP thymocytes taking longer than CD4SP cells to develop from DP thymocytes[20,21]. Consistent with this expression pattern, and unlike thymic (germline or *Cd4*-Cre-mediated) *Thpok* disruption[7,11,12,22], *Thpok*$^{fl/fl}$ *CD2*-Cre mice (hereafter called *Thpok*^{pd} for 'peripheral deleter') had large numbers of CD4SP thymocytes, which expressed a normal amount of ThPOK protein (Fig. 1b,c and Supplementary Fig. 1b). Furthermore, and contrasting with thymic deletion, *Thpok*^{pd} mice had $CD4^+CD8^-$ naïve T cells, most of which expressed *Rosa26*^{YFP} (Fig. 1d and Supplementary Fig. 1c) and little or no ThPOK (Fig. 1c). Thus, this experimental system is suitable to study post-thymic functions of ThPOK. Of note, deletion was incomplete in Foxp3-expressing regulatory T cells[1] (T_{reg})(Supplementary Fig. 1d), whether in the thymus or in the spleen, making this system poorly suited to study ThPOK function in T_{reg} cells.

Thpok^{pd} mice had fewer $CD4^+CD8^-$ T cells than wild-type mice (Supplementary Fig. 1c). However, *Thpok*^{pd} mice had unusual $CD4^{lo}CD8^-$ (hereafter $CD4^{lo}$) and $CD4^+CD8^+$ (peripheral DP, hereafter pDP) cells, which expressed *Rosa26*^{YFP} and were predominantly naive ($CD44^{lo}$; Fig. 1d); as a result, the total number of $CD4^+$ T cells (including $CD4^{lo}$ and pDP) was similar in *Thpok*^{pd} and wild-type mice (Fig. 1e and Supplementary Fig. 1c). Whereas preselection DP thymocytes are TCR^{lo}, pDP cells were TCR^{hi} (data not shown); pDP cells expressed both CD8α and CD8β subunits (Fig. 1f), unlike the small subset of $CD4^+CD8\alpha^+\beta^-$ cells normally found among gut intraepithelial lymphocytes (IELs)[23]. Upon adoptive transfer into lympho-replete recipients, purified *Thpok*^{pd} $CD4^+$ T cells gave rise to pDP and $CD4^{lo}$ peripheral T cells (Fig. 1g). The transferred cells remained naïve ($CD44^{lo}$), confirming that changes in CD4 or CD8 expression occurred without proliferation. Of note, unlike thymic *Thpok* disruption, very few transferred $CD4^+$ T cells became $CD4^-CD8^+$. Thus, post-thymic ThPOK is needed for the proper control of CD4 and CD8 coreceptor gene expression in naïve MHC class II-restricted T cells.

ThPOK represses *Runx3* in thymocytes, so that MHC II-signaled thymocytes that are ThPOK deficient up-regulate *Runx3* to a level characteristic of MHC I-restricted CD8SP thymocytes[12]. To examine if ThPOK represses *Runx3* in peripheral T cells, we generated *Thpok*^{pd} mice carrying a *Runx3*^{dYFP} allele that reports *Runx3* expression[12]. Contrary to *Thpok*$^{+/+}$ $CD4^+$ T cells, about half of naïve *Thpok*^{pd} $CD4^{lo}$ and pDP cells expressed *Runx3* (Fig. 1h); thus, post-thymic ThPOK restrains expression of *Runx3* in naïve $CD4^+$ T cells.

However, most *Thpok*^{pd} CD4^{+}CD8^{-} T cells failed to express *Runx3*^{dYFP}, and immunoblot analyses only found low expression of Runx3 protein in these cells (Supplementary Fig. 1e). Thus, unlike in thymocytes, ThPOK is not required for *Runx3* repression in mature CD4^{+} T cells.

To evaluate the impact of Runx3 de-repression, we generated *Thpok*^{pd} *Cbfb*^{pd} mice, which inactivate ThPOK and the obligatory Runx cofactor Cbfβ post-thymically. We targeted Cbfβ, rather than Runx3, because of the overlapping activities of Runx1 and Runx3 in thymocytes and T cells[24]. Post-thymic disruption of Cbfβ, whether alone or in addition to that of ThPOK, had little impact on thymic development (Supplementary Fig. 1f,g). However, *Thpok*^{pd} *Cbfb*^{pd} mice had no peripheral CD4^{lo} T cells (Fig. 1i,j), suggesting that Runx3 up-regulation mediates *Cd4* repression in *Thpok*^{pd} cells. Of note, there were pDP cells in *Thpok*^{pd} *Cbfb*^{pd} mice; however, the presence of these cells did not imply that Runx activity was dispensable for CD8 re-expression, because Cbfβ inactivation may impair *Cd4* silencing in CD8^{+} T cells[24]. We conclude from these experiments that post-thymic ThPOK protects CD4^{+} T lineage integrity, at least in part by restraining *Runx3* expression.

Conserved T_H17 potential of Thpok-deficient cells

Having shown that ThPOK preserves the differentiation of resting CD4^{+} T cells, we examined its functions during T cell effector differentiation. Because it was recently reported that ThPOK was important for T_H17 differentiation through restraining *Runx3* expression[17], we assessed T_H17 responses in the large intestine lamina propria (liLP) and draining (mesenteric) lymph nodes of mice. Both at steady state or after infection with *Citrobacter rodentium*, an enterobacterium that generates a strong colonic T_H17 response[25], *Thpok*^{pd} mice had a higher frequency of T_H17 cells than wild-type mice (Fig. 2a,b,c). *Thpok*^{pd} IL-17^{+} T cells expressed *Rosa26*^{YFP}, indicating that T_H17 responses did not result from the outgrowth of cells having escaped deletion (Fig. 2b and data not shown). *Thpok*^{pd} IL-17^{+} T cells did not express CD8 (data not shown) and the fraction of IL-17^{+} cells that also made IFN-γ was similar in *Thpok*^{pd} and *Thpok*$^{+/+}$ mice (Fig. 2b). Accordingly, the course of *Citrobacter rodentium* infection was similar in wild type and *Thpok*^{pd} mice (Supplementary Fig. 2a,b). Consistent with these results, IL-17 expression was conserved in *Thpok*^{pd} T effector cells generated *in vitro* in T_H17 polarizing conditions. Although the frequency of IL-17^{+} T cells was modestly increased by ThPOK disruption (Fig. 2d), there was no effect on IL-17 cytokine production assessed by ELISA (Supplementary Fig. 2c), and little or no change in Runx3, IFN-γ or granzyme B expression (Fig. 2d,e and S2d). Altogether, these experiments support the conclusion that T_H17 differentiation of naïve CD4^{+} T cells does not require ThPOK.

Thpok protects T_H2 responses by repressing *Runx3*

We previously showed that the MHC II-restricted CD8^{+} T cells generated following thymic *Thpok* disruption fail to undergo T_H2 differentiation, whether *in vitro* or *in vivo*[16]. In contrast, naïve *Thpok*^{pd} cells made IL-4 when activated *in vitro* in T_H2 polarizing conditions (Fig. 3a). To elucidate the role of ThPOK expression during T_H2 responses *in vivo*, we immunized mice with inactivated *Schistosoma mansoni* eggs[26]. While this typical T_H2 stimulus induces CD4^{+}CD8^{-} T cells expressing IL-4 or the T_H2 regulator GATA3[27] in wild-

type mice, there was almost no T cells expressing GATA3 or IL-4 in *Thpok*^{pd} mice (Fig. 3b). These results suggest ThPOK is important for T_H2 differentiation *in vivo*,

Accordingly, expression of IL-4, assessed by intra-cellular staining or ELISA of IL-4 secretion, and expression of GATA3 were markedly impaired when naive *Thpok*^{pd} CD4$^+$ T cells were activated in non polarizing conditions, which normally favor T_H2 differentiation (Fig. 3c,d and Supplementary Fig. 3a,b). Instead, in these conditions, *Thpok*^{pd} CD4$^+$ T cells made IFN-γ and the cytotoxic enzyme Granzyme B (Fig. 3d, g), and expressed CD8 (both CD8α and CD8β) (Fig. 3e,f), and Eomesodermin (Eomes), a T-bet related transcription factor involved in IFN-γ production in cytotoxic cells[28] (Fig. 3c). Analyses of Runx protein expression showed up-regulation of Runx3 in *Thpok*^{pd} effector cells, which predominated over Runx1 (Fig. 3h), demonstrating that ThPOK is important to restrain Runx3 expression during T cell activation. Impaired production of IL-4 was not restored by co-culture with wild-type CD4$^+$ T cells or by neutralizing IFN-γ activity during activation, indicating that it was intrinsic to ThPOK-deficient CD4$^+$ T cells (Fig. 3i,j). A 10-fold excess of wild-type CD4$^+$ T cells in the culture only modestly improved IL-4 expression by ThPOK-deficient CD4$^+$ T cells activated under T_HN conditions (Fig. 3i). These experiments demonstrate a cell-intrinsic role for ThPOK in IL-4 production and T_H2 differentiation. Disruption of Runx activity (by inactivating Cbfβ) restored IL-4 production in effector cells generated from naïve *Thpok*^{pd} *Cbfb*^{pd} CD4$^+$ T cells activated under T_HN conditions (Fig. 4a), inhibited their expression of IFN-γ and Granzyme B and reversed the switch between GATA3 and Eomes expression (Fig. 4b,c,d and Supplementary Fig. 4a), These results indicate that ThPOK 'protects' T_H2 differentiation by restraining *Runx3* expression and the cytotoxic 'diversion' of activated CD4$^+$ T effector cells.

Because Eomes is downstream of Runx3 in the regulatory circuitry of cytotoxic cells, and also promotes IFN-γ production[29,30], we examined its involvement in the cytotoxic diversion of *Thpok*^{pd} T cells. We used the *CD2*-Cre transgene to generate *Thpok*^{pd} *Eomes*^{pd} mice, in which ThPOK and Eomes were inactivated in mature T cells. As expected, because there is little or no Eomes expression in conventional thymocytes or naïve CD4$^+$ T cells[28], Eomes disruption did not affect steady-state thymic, spleen or LN CD4$^+$ T cell populations (Supplementary Fig. 4b,c). T effector cells generated by activating naïve *Thpok*^{pd} *Eomes*^{pd} in T_HN conditions made less IFN-γ but only marginally more IL-4 and GATA3 than their *Thpok*^{pd} counterparts (Fig. 4e, f and Supplementary Fig. 4a). Thus, Eomes mediates IFN-γ production but not IL-4 repression caused by unrestrained Runx activity in ThPOK-deficient effectors. This agrees with our finding that the impaired T_H2 differentiation of ThPOK-deficient cells was not the consequence of excessive IFN-γ production (Fig. 3j). We conclude from these experiments that ThPOK repression of *Runx3* in CD4$^+$ T cells is essential for T_h2 responses, as it prevents Runx3-mediated activation of a cytotoxic gene expression program and, independently, the repression of IL-4.

ThPOK protects T_H1 differentiation by constraining Runx functions

Because wild-type T_H1 effector cells normally express Runx3[18,19], we investigated if ThPOK also affected T_H1 differentiation. In T cells polarized in T_H1 conditions, ThPOK disruption had little effect on Runx3 or IFN-γ expression (Fig. 5a,b). However, compared to

their *Thpok*$^{+/+}$ counterparts, *Thpok*^{pd} effector cells generated in T_H1 conditions had greater expression of CD8 and granzyme B and they externalized CD107a, a marker of cytotoxic cell degranulation[31] (Fig. 5b). Of note, they retained CD4 expression (Supplementary Fig. 5a).

These observations suggested that a comparison of the transcriptomes of wild-type and ThPOK-deficient T_H1 effector cells would reveal how ThPOK affects gene expression independently of its effect on *Runx3* expression. Thus, we used gene expression arrays to compare the transcriptional profiles of the following effector T cells, all generated in the same T_H1 polarizing conditions:(i) wild-type T_H1 CD4$^+$ T cells, generated from naïve CD4$^+$ T cells, (ii) *Thpok*^{pd} CD4$^+$CD8$^-$ and CD4$^+$CD8$^+$ cells, the two populations generated by activation of naive *Thpok*^{pd} CD4$^+$CD8$^-$ cells (Supplementary Fig. 5a), and (iii) wild-type cytotoxic CD8$^+$ T cells, generated from naïve CD8$^+$ T cells. Except for *Cd8* genes, side by side comparisons showed no divergence between CD4$^+$CD8$^-$ and CD4$^+$CD8$^+$ *Thpok*^{pd} T effector cells generated in these conditions (Fig. 5c, top left, and Supplementary Fig. 5b). When we compared *Thpok*^{pd} CD4$^+$CD8$^+$ to wild-type CD4$^+$ and CD8$^+$ T effector cells, *Thpok*^{pd} CD4$^+$CD8$^+$ had a transcriptional profile that was very similar to that of wild-type CD8$^+$ cytotoxic cells (Fig. 5c, bottom left), whereas they diverged from the wild-type CD4$^+$ T_H1 cells almost to the same extent as wild-type cytotoxic T cells did (Fig. 5c, right two plots). Thus, ThPOK is responsible for the bulk of differential gene expression between T_H1 CD4$^+$ and cytotoxic CD8$^+$ T cells.

Hierarchical clustering distinguished two groups of genes differentially expressed between *Thpok*^{pd} CD4$^+$CD8$^+$ (or CD4$^+$CD8$^-$) and wild-type CD4$^+$ T effector cells (Fig. 5d and Supplementary Fig. 5b). The largest group represented genes up-regulated in each ThPOK-deficient subset in comparison to wild-type CD4$^+$ T cells. In addition to prototypical cytotoxic genes such as perforin or Granzymes A and B, this group included most of the genes preferentially expressed in CD8$^+$ T effector cells but which are not part of the conventional cytotoxic program, such as IL-10, *Zbtb32* (encoding the transcription factor Rog) as well as cell cycle-related genes. The second group contained a smaller gene set that was down-regulated in each ThPOK-deficient subset compared to wild-type CD4$^+$ T cells and comprised genes involved in effector T cell differentiation, such as *Cd40lg* or *Vipr1* (encoding a receptor for the VIP peptide) and molecules involved in cell adhesion (*Amigo2*) or trafficking (*Ccr7*, *S1pr1*). Indeed, *Thpok*^{pd} T effector cells (CD4$^+$CD8$^+$ and CD4$^+$CD8$^-$) had impaired induction of CD40L (Fig. 5e, bottom); of note, this contrasted with the conserved CD40L expression in naïve *Thpok*^{pd} CD4$^+$ T cells (Fig. 5e, top), which express little Runx3.

The repression of the cytotoxic program by ThPOK in T_H1 effector cells supported the conclusion that this factor, in addition to repressing *Runx3*, restrained Runx-mediated activation of cytotoxic genes. Indeed, the cytotoxic gene up-regulation characteristic of ThPOK-deficient effector cells was reverted in *Thpok*^{pd} *Cbfb*^{pd} effector cells derived in T_H1 cultures from naïve CD4$^+$CD8$^-$ T cells (Fig. 5f).

In addition to activating effects, Runx proteins have repressive functions, notably on *Cd4* and *Thpok* during T cell differentiation[24]. While ThPOK can antagonize Runx-mediated

repression[11,32], the physiological relevance of such antagonism has not been established, and was questioned by the continued expression of CD4 in Runx3-expressing ThPOK-deficient T effector cells (Supplementary Fig. 5a). Thus, we next examined if the ThPOK-dependence of helper effector gene expression, such as *Cd40lg*, was explained by ThPOK antagonism of Runx-mediated repression. Indeed, Cbfβ disruption enhanced CD40L expression in *Thpok*^{pd} *Cbfb*^{pd} T_H1 effector cells (Fig. 5g). These observations demonstrate that ThPOK promotes effector gene expression at least in part by antagonizing Runx-mediated repression. We conclude from these experiments that ThPOK is essential to protect helper and restrain cytotoxic gene expression by antagonizing Runx3 functions.

Thpok is important for antigen-induced T cell responses in vivo

We took two approaches to examine how ThPOK expression affects $CD4^+$ T cell responses *in vivo*. First, we evaluated responses to *Toxoplasma gondii*, an intra-cellular parasite generating a massive IFN-γ-mediated $CD4^+$ T_H1 response, during which we tracked T cells reacting against a specific MHC II-presented parasite epitope (AS-15)[33]. At the peak of the acute response, similar frequencies of IFN-γ-producing $CD4^+$ T effector cells were found in both *Thpok*^{pd} and wild-type controls (Supplementary Fig. 6a). ThPOK disruption did not affect the number of AS-15-reacting T cells (Fig. 6a,b) and did not increase parasite load (Supplementary Fig. 6b). However, consistent with the notion that ThPOK prevents cytotoxic gene expression in T_H1 cells, *Thpok*^{pd} effector cells were $CD4^+CD8^+$ and expressed granzyme B and Eomes (Fig. 6a,c and Supplementary Fig. 6c).

We considered the possibility that signals from other cells involved in *T. gondii* responses, including innate immune cells and $CD8^+$ T cells, could help $CD4^+$ T cells overcome ThPOK deficiency. Thus, in a second approach, we examined the cell intrinsic role of ThPOK in a defined, clonotypic $CD4^+$ T cell response. We compared the antigen-driven expansion of ThPOK-deficient and -sufficient cells carrying the ovalbumin-specific OT-II TCR. We purified wild-type or *Thpok*^{pd} $CD4^+CD8^-$ OT-II T cells, and assessed their response after adoptive transfer into ovalbumin-immunized wild-type lympho-replete hosts. We found that the amplification of *Thpok*^{pd} $CD4^+CD8^-$ OT-II T cells was blunted compared to their wild-type counterparts, and that these *Thpok*^{pd} cells re-expressed CD8, while maintaining CD4 (Fig. 6d,e). Thus, even if ThPOK is not required for the generation of antigen-responsive effector T cells, it is important for their proper expansion and differentiation.

Thpok and LRF redundantly maintain $CD4^+$-lineage integrity

The puzzling persistence of CD4 expression in *Thpok*^{pd} effector T cells, despite their high Runx3 expression, contrasted with their Runx-dependent repression of CD40L, or the Runx-dependent repression of CD4 in naïve $CD4^{lo}$ *Thpok*^{pd} cells (Fig. 1h, i). This raised the possibility that the *Cd4* locus was no longer sensitive to Runx-mediated repression in antigen-stimulated T cells. Consistent with this possibility, naïve $CD4^{lo}$ *Thpok*^{pd} cells, in which CD4 repression is Runx-dependent (Fig. 1i) up-regulated CD4 upon activation (Supplementary Fig. 7a). An alternative possibility was that *Cd4* expression in T_H1 effector cells remained responsive to Runx-mediated repression, but was sustained by a redundant factor in the absence of ThPOK. The transcription factor LRF, which has a ThPOK-

redundant role in promoting $CD4^+$ T cell programming in the thymus[16] represented an obvious candidate.

To distinguish between these possibilities, we generated *Thpok*$^{fl/fl}$ *Zbtb7a*$^{fl/fl}$ mice carrying the *CD2*-Cre transgene (hereafter *Thpok*^{pd} *Zbtb7a*^{pd} mice), in which both *Thpok* and *Zbtb7a*, which encodes LRF, are deleted in naïve peripheral T cells. The isolated disruption of LRF did not affect $CD4^+$ T cell expression of CD4 or CD8, or functional differentiation (data not shown and Ref. 16). Compared to *Thpok*^{pd} mice, *Thpok*^{pd} *Zbtb7a*^{pd} mice showed little effect of *Zbtb7a* disruption on thymic development, but they had reduced peripheral $CD4^+CD8^-$ and $CD4^{lo}$ T cell subsets (Supplementary Fig. 7b,c,d), suggesting that ThPOK and LRF redundantly contribute to maintain the $CD4^+$ lineage. Following activation and culture in T_H1 conditions, naive *Thpok*^{pd} *Zbtb7a*^{pd} $CD4^+CD8^-$ T cells gave rise to $CD4^-CD8^+$ effector cells, while most naïve *Thpok*^{pd} $CD4^+CD8^-$ T cells continued to express CD4 (Fig. 7a). Thus, post-thymic inactivation of ThPOK and LRF causes *trans*-differentiation of mature $CD4^+$ into $CD8^+$ T cells. In *Thpok*^{pd} *Zbtb7a*^{pd} T_H1 effector cells obtained from $CD4^+CD8^-$ naïve cells, expression of Runx3 was not increased over that observed in their *Thpok*^{pd} counterparts cells (Supplementary Fig. 7e). Rather, LRF antagonized Runx-mediated *Cd4* repression. In transient transfection experiments that evaluate the activity of a GFP-based reporter construct including *Cd4* cis-regulatory elements (promoter, proximal enhancer and silencer)[24], Runx3 repressed *Cd4* reporter activity, whereas ThPOK, while having no effect *per se*, prevented Runx-mediated repression[32] (Fig. 7b). LRF was as efficient as ThPOK at antagonizing Runx-mediated repression of the *Cd4* reporter (Fig. 7b). Of note, ThPOK and LRF disruption did not prevent T_H17 differentiation *in vitro* (Fig. 7c), indicating that ThPOK and LRF were not required for T_H17 differentiation in post-thymic cells, unlike their function in the thymus[16]. These results suggest that a transcriptional node defined by the overlapping activities of ThPOK and LRF supports CD4 expression in mature T cells.

Thpok expression is sustained independently of Thpok and LRF

Because ThPOK was proposed to protect expression of both *Cd4* and *Thpok* genes[11], the previous findings suggested that ThPOK and LRF would also contribute to sustain *Thpok* expression; that is, that the *Thpok* promoter would be inactive in *Thpok*^{pd} *Zbtb7a*^{pd} *trans*-differentiated $CD4^-CD8^+$ cells. Cre-mediated inactivation of the *Thpok*^{fl} allele excises most of its transcribed sequences, therefore preventing direct gene expression analyses. Thus, we used a *Thpok*^{GFP} BAC reporter[22], genetically independent from the *Thpok* locus, to test this possibility. Inactivation of ThPOK, or both ThPOK and LRF, had little or no effect on reporter expression in naïve $CD4^+$ cells (Fig. 7d). In addition, the reporter was also expressed in *Thpok*^{pd} *Zbtb7a*^{pd} T_H1 effector cells, including those *trans*-differentiating into $CD4^-CD8^+$ cells (Fig. 7e). Thus, while ThPOK and LRF control lineage integrity and effector differentiation of $CD4^+$ T cells, they are dispensable for *Thpok* gene expression.

Discussion

The present study demonstrates that the transcription factor ThPOK, redundantly with its homolog LRF, sustains the phenotypic and functional integrity of the $CD4^+$ lineage.

ThPOK's impact in mature T cells is mediated both by its repression of *Runx3* expression and its antagonism of Runx protein function. Thus, not only do ThPOK and LRF establish functional helper pre-programming in thymocyte[16], they are continuously needed to maintain the integrity of helper T cell responses.

Using a novel mouse strain which targets Cre expression to naïve T cells with greater selectivity than existing lines[34], we demonstrate that the ThPOK-LRF transcriptional 'node' is required to maintain $CD4^+$ lineage integrity beyond the thymic checkpoint that defines $CD4^+$-lineage commitment. The contribution of ThPOK and LRF to CD4 expression is important because CD4 is needed for the survival and fitness of mature $CD4^+$ T cells[35]. Conversely, repression by ThPOK of genes encoding CD8α and CD8β fits with previous reports that ThPOK functionally and physically targets *Cd8* enhancers[36–38]. However, our findings underscore a fundamental difference between $CD4^+$ and $CD8^+$ T cells for coreceptor gene repression. Once established in CD8SP thymocytes by recruitment of the *Cd4* repressor Runx3 to the *Cd4* silencer, *Cd4* silencing is epigenetically maintained independently of Runx3 or the silencer[24,39]. This contrasts with the active repression of *Cd8* in $CD4^+$ T cells.

Our study demonstrates that ThPOK broadly represses cytotoxic gene expression and thereby avoids the 'cytotoxic diversion' of T_H2 effectors. The balance between Runx3 and GATA3 controls T_H1-T_H2 differentiation in $CD4^+$ T cells[18,19,30]. Runx molecules inhibit IL-4 and promote IFN-γ expression presumably both directly and indirectly, by binding to *Il4* and *Ifng* genes and controlling expression of GATA3 and T-bet. GATA3 antagonizes both direct and indirect effects, at least in part through direct binding to Runx3 molecules[30]. By limiting the amount of Runx3 produced during $CD4^+$ T cell activation, ThPOK is critical for T_H2 differentiation. ThPOK disruption results in Runx-dependent Eomes expression, which is needed for IFN-γ production but not to prevent IL-4 expression. This finding supports the conclusions that *Il4* repression and *Ifng* activation are mechanistically independent: the former being principally mediated by Runx3 whereas *Ifng* activation by Runx3 requires Eomes or T-bet, even though Runx3 binds the *Ifng* gene[18,30]

In addition to repressing *Runx3*, ThPOK antagonizes Runx protein activity, the latter function underpinning ThPOK's support for *Cd40lg* expression and its impact on T_H1 differentiation. While such antagonism of Runx function had been identified in cell lines and thymocytes[11,32], our study establishes its physiological relevance by comparing the transcriptome of ThPOK-deficient and -sufficient cells with equivalent expression of Runx proteins. Future experiments will explore whether such mechanisms are important for long term control of intra-cellular pathogens which relies on T_H1 responses, including of *T. gondii*. The central role of $CD4^+$ effector cells for long term *T. gondii* control[41], including providing help to $CD8^+$ T, B, and myeloid cells, notably through CD40L[40], suggests that the function of ThPOK in maintaining the T_H1 circuitry may be essential to coordinate such responses.

ThPOK was reported to 'protect' the colitogenic potential of $CD4^+$ T cells in adoptive cell transfer colitis in alymphoid mice[17], a function assigned to ThPOK promoting the differentiation of colitogenic T_H17 cells. However, both that report[17] and our study found

that ThPOK is not needed for *in vivo* T_H17 responses. Furthermore, ThPOK inhibits, rather than protects, T_H17 differentiation in invariant natural killer (iNK T) cells[42]. We note that ThPOK's control of CD40L in T_H1 effectors provides an alternative rationale for its impact on adoptive transfer colitis. Indeed, T_H1 cells are instrumental in this disease, in which CD40L is critical to induce gut inflammation[43,44].

It was recently proposed that the expression of *Thpok* in $CD4^+$ T cells is subject to regulation, namely that Runx3 up-regulation by environmental signals represses *Thpok* in $CD4^+$ effector cells by activating a *Thpok* silencer[17,38]. However, even though the *Thpok* silencer includes binding sites for Runx molecules[13], such repression did not in fact require Runx3[17]. Nonetheless, given that ThPOK has been reported to protect the *Thpok* gene from repression by Runx molecules[11], we expected that inactivation of ThPOK, or ThPOK and LRF, would facilitate *Thpok* repression by Runx3. Contrary to this prediction, the *Thpok* locus remained transcriptionally active in Runx3-expressing ThPOK-LRF double-deficient cells, challenging the idea that Runx3 expression initiates *Thpok* silencing in effector T cells. Rather, such silencing, if physiologically relevant, could be caused by signals targeting other repressors of *Thpok* expression[45,46].

In primate T cells, CD4 serves as receptor for the human immunodeficiency virus (HIV) and its simian homologs (SIV)[47]. Notably, it was found that antigen-experienced MHC II-restricted cells convert from a $CD4^+CD8^-$ to a $CD4^-CD8\alpha^{lo}\beta^-$ phenotype in African green and Patas monkeys[48,49]. Although such conversion is associated with reduced expression of helper genes, it is believed to be beneficial by protecting the helper T cell population from SIV infection and the ensuing immunodeficiency. The present study provides a conceptual framework to investigate the mechanisms of this conversion, in which the association of functional changes with a concerted switch of CD4 and CD8 expression suggests the possible involvement of Runx molecules and their control by ThPOK or related factors.

In summary, our findings emphasize the inherent instability of the $CD4^+$ lineage, that ThPOK, together with LRF, continuously protects from $CD8^+$ *trans*-differentiation. They indicate that changes in expression or activity of these factors have broad consequences on T helper functions, making them potential targets for physiological control and therapeutic intervention.

Online Methods

Mice

CD2-cre mice were generated as previously described by inserting a Cre cDNA into a *CD2*-based transgenic cassette[8]. Mice carrying floxed alleles for *Zbtb7b*[10], *Zbtb7a*[50] (gift from P.P. Pandolfi), *Eomes*[51] (gift from S. Reiner), *Cbfb*[19] (purchased from the Jackson laboratory) or $Runx3^{dYFP}$ (gift from D. Littman) and $Thpok^{GFP}$ reporters[12,22] were all previously described. $Rosa26^{YFP}$ (Ref. 52) and OT-II (Ref. 53) mice were purchased from Jackson Laboratories. CD45.1 and CD45.2 C57BL/6 mice were obtained from the National Cancer Institute Animal Production Facility. All transgenic mice were maintained were heterozygous for the transgene. Mice were housed in specific pathogen-free facilities and

analyzed between 6 and 16 weeks of age. Animal procedures were approved by the NCI Animal Care and Use Committee.

Antibodies

Antibodies for the following specificities were purchased either from Affymetrix eBiosciences or BD Pharmingen: CD4 (RM4.4 or GK1.5), CD8α (53-6-7), CD8β (53-5.8), CD24 (M1/89), TCRβ (H57-597), CD40L (MR1), CD107a (1D4B), GATA3 (TWAJ), Tbet (4B10), eomes (Dan11mag), IL4 (11B11), CD45.2 (104), Va2 (B20.1), IFN-g (XMG1.2), IL17a,(eBio17B7) CD44 (IM7). Antibodies specific for Granzyme B (GB12) were purchased from Invitrogen. TGME49 Class II I-A^b tetramers loaded with the AS-15 peptide, and control Clip peptide-loaded I-A^b tetramers were obtained from the NIH tetramer facility.

Cell preparation and staining

Lymph node, spleen, thymus and intestinal lymphocytes were prepared and stained as previously described[10,22,54]. Flow cytometry data were 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 forward scatter height by width gating. Purification of lymphocytes by cell sorting was performed on a FACSAria (BD Biosciences). Analyses of intracellular cytokines and transcription factors was performed as previously described[10,16].

In vitro analysis of gene and protein expression

Analysis of CD40L expression was performed as previously described[16]. Effector T cells were generated from sorted $CD44^{lo}$ (naïve) T cells from spleen or LN as previously described[10], except that activating and blocking antibodies were purchased from BioXCell. Transfection of RLM-11 cells was performed as previously described[32]. Analysis of ThPOK and Runx protein expression was performed by Western blot as previously described using an antiserum against ThPOK[22] and anti-Runx antibody (EPR3099, Epitomics). Membranes were subsequently stripped and blotted with anti β-actin (AC-15, Sigma) to control for lane loading. Quantification of cytokine production by ELISA was performed on culture supernatants 5 days after activation without additional restimulation, using mouse DuoSet ELISA kits (R&D Systems) and following the manufacturer's instruction.

Adoptive transfer and immunization

For analyses of coreceptor expression in naïve cells, sorted $CD45.2^+$ $CD44^{lo}$ $CD4^+CD8^-$ T cells (2×10^6) from spleen and or lymph nodes of *Thpok*$^{+/+}$ or *Thpok*^{pd} mice were injected intravenously into CD45.1 recipients, which were analyzed 14 days later. For OVA-specific responses, 30,000 sorted $CD45.2^+$ $CD44^{lo}$ $CD4^+CD8^-$ T cells from spleen and or lymph nodes of OT-II TCR transgenic *Thpok*$^{+/+}$ or *Thpok*^{pd} mice were injected into CD45.1 recipients, followed by immunization with 100ug OVA emulsified in complete Freund's adjuvant administered by intraperitoneal (i.p.) injection 18 hours after adoptive transfer. Mice were analyzed 4–6 days post-immunization.

Schistosoma mansoni infections were performed essentially as previously described[26]. Briefly, mice were injected i.p. with 2500 inactivated (freeze-thaw) eggs, then boosted 8

days later with a second dose of 2500 inactivated egg dose. Eggs were stored at −70°C before use. Mice were analyzed 12–14 days after the initial injection.

Granule exocytosis assay

In vitro activated T cells from 5 day cultures were re-stimulated on anti-CD3 coated plates, for 4 hours at 37°C, 5% CO_2 in the presence of PE-labeled anti-CD107a. Cells were harvested, washed and surface stained before flow cytometric analysis.

Infections

Infection of mice with *C. rodentium* was performed as previously described.[55] Mice were analyzed between 8 to 12 days post-infection. ME-49 clone C1 of *T. gondii* (kindly provided by Dr M.E. Grigg, NIAID) was obtained by electroporation of the parental ME-49 type II strain (ATCC 50840) with the red fluorescent protein (RFP) and was used for production of tissue cysts in C57BL/6 mice. Mice were infected with 10 cysts by oral gavage. Tissues were harvested between 9 and 14 days post-infection and analyzed as described[56]. Parasite load was evaluated by the frequency of RFP-expressing cells in the spleen of infected animals.

Microarray analyses

Sorted naïve ($CD44^{lo}$) $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells, from wild-type mice, and $CD4^+CD8^-$ cells, from *Thpok*^{pd} mice were activated *in vitro* in T_H1 conditions and sorted again 4 days later into purified $CD4^+CD8^-$ and $CD4^-CD8^+$ (wild-type) and $CD4^+CD8^-$ and $CD4^+CD8^+$ (*Thpok*^{pd}) subsets. Total RNA was extracted from sorted subsets and processed for microarray analyses (Affymetrix Mouse Exon 1.0 ST array) at the NCI microarray facility, following the manufacturer's recommendation. Data is from 3 replicates (except wild-type $CD4^-CD8^+$ cells, for which two samples only were processed) generated from two distinct cell preparations. Data was analyzed with Partek Genomic Suite and deposited in the GEO database under accession number GSE57846.

Statistical analyses

All statistical analyses were performed using Prism software. Bars in graphs indicate average ± SEM. Except where otherwise indicated, comparisons were performed by two-tailed unpaired t-test; F-test was performed to check the assumption of equal variance. Two-tailed paired t-test was used where biologically appropriate (e.g. cytokine assays processed in parallel). Significance levels (*P*-values) are indicated on figures. For statistical comparisons, sample size was always greater than 3 and determined empirically based on pilot analyses. We used neither randomization nor blinding since comparisons involve mice of distinct genotypes; animals were excluded from analyses only on the basis of age or poor health status unrelated to the experiment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank T. Ciucci for experimental assistance and discussions, T.-A. Lewis for expert animal care and genotyping, M. McGinty and Q. Xiao for technical assistance, X. Wu for microarray analyses, D. Littman, P.P. Pandolfi and S. Reiner for mice, M. Grigg for the *T. gondii* strain, J. Grainger, T. Hand and S. Spencer for useful discussions, and J. Ashwell and J. Brenchley for reading the manuscript. Supported by the Intramural Research Programs of the National Cancer Institute, Center for Cancer Research, the National Institute of Allergy and Infectious Diseases, and the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health.

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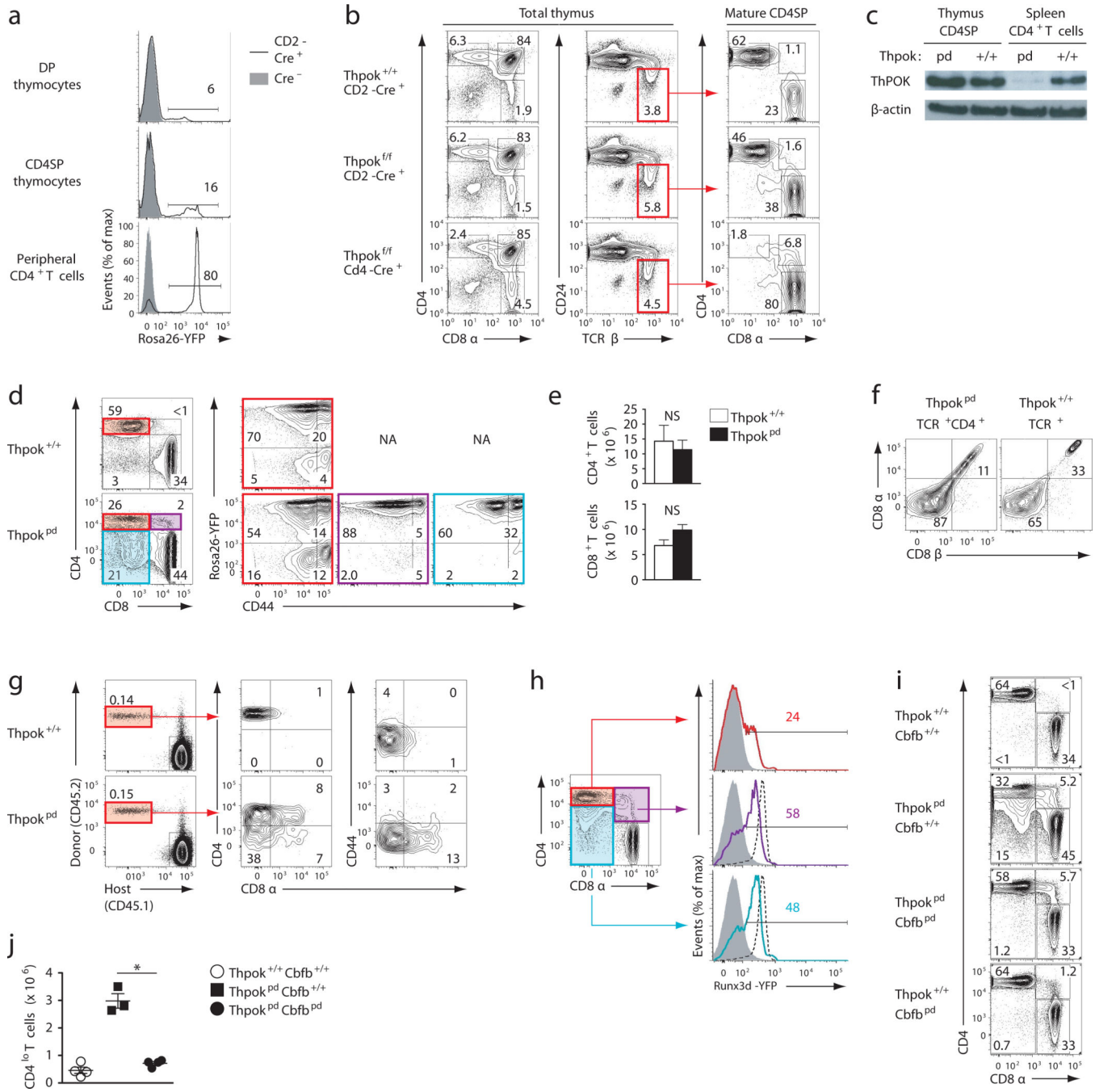

Figure 1. Post thymic ThPOK maintains CD4^{+}-lineage integrity

(a) Histogram overlays of *Rosa26*^{YFP} expression in thymocytes or spleen T cells from *Rosa26*^{YFP} mice carrying (plain line) or not (grey-filled) the *CD2*-Cre transgene. **(b)** Contours plots of CD4 versus CD8 expression on total (left) and TCR^{hi}CD24^{lo} thymocytes (right, defined in middle column) from 6–8 week old *Thpok*^{pd}, *Thpok*$^{+/+}$ and *Thpok*$^{f/f}$ *Cd4*-cre mice. **(c)** Immunoblot analysis of ThPOK protein expression in sorted CD4SP thymocytes or spleen CD4^{+}CD8^{-} T cells from *Thpok*$^{+/+}$ or *Thpok*^{pd} mice. Expression of β-actin serves as a loading control. **(d)** Contour plots of CD4 versus CD8 expression on spleen

T cells (left) define subsets analyzed for CD44 versus *Rosa26*^{YFP} expression (right three columns), in *Thpok*^{pd} or wild-type littermate mice. **(e)** CD4^{+} (including CD4^{lo} and pDP) and CD4^{-}CD8^{+} spleen T cell numbers (mean ± SEM, ×10^{6}) from 6–8 week-old *Thpok*^{pd} (n=7) and *Thpok*$^{+/+}$ littermates (n=6). No significant difference by Student's t-test. **(f)** Contour plots of CD8α versus CD8β expression on indicated spleen T cell subsets. **(g)** (left) Contour plots on gated T cells (TCRβ^{+}B220^{-}) distinguish donor (CD45.2^{+}) from host (CD45.1^{+}) subsets 14 days after transfer of naive CD4^{+} T cells from *Thpok*^{pd} or *Thpok*$^{+/+}$ CD45.2^{+} mice. Center and right plots show donor cell expression of CD8 vs. CD4 or CD44. **(h)** CD4 vs. CD8 expression on gated CD44^{lo} TCRβ^{+} spleen cells from *Runx3*d-YFP *Thpok*^{pd} mice define subsets analyzed for *Runx3d*-YFP expression (left). Histogram plots overlay YFP signal in each defined population from *Thpok*^{pd} *Runx3*^{dYFP} mice (colored lines) over their CD4^{-}CD8^{+} counterparts (dashed), and *Thpok*$^{+/+}$ CD4^{+} splenocytes (solid grey). Cre-negative cells expressing the reporter were excluded from analysis. **(i)** CD4–CD8 expression plots on lymph node T cells of indicated genotype. (**j**) Absolute numbers of CD4^{lo} spleen T cells in mice analyzed in (i). Each symbol represents one mouse. (*: P<0.0002). (**a–i**) All panels are representative of three independent experiments except for (b) (2 experiments) and (g) (2 experiments, each with 4 mice of each genotype).

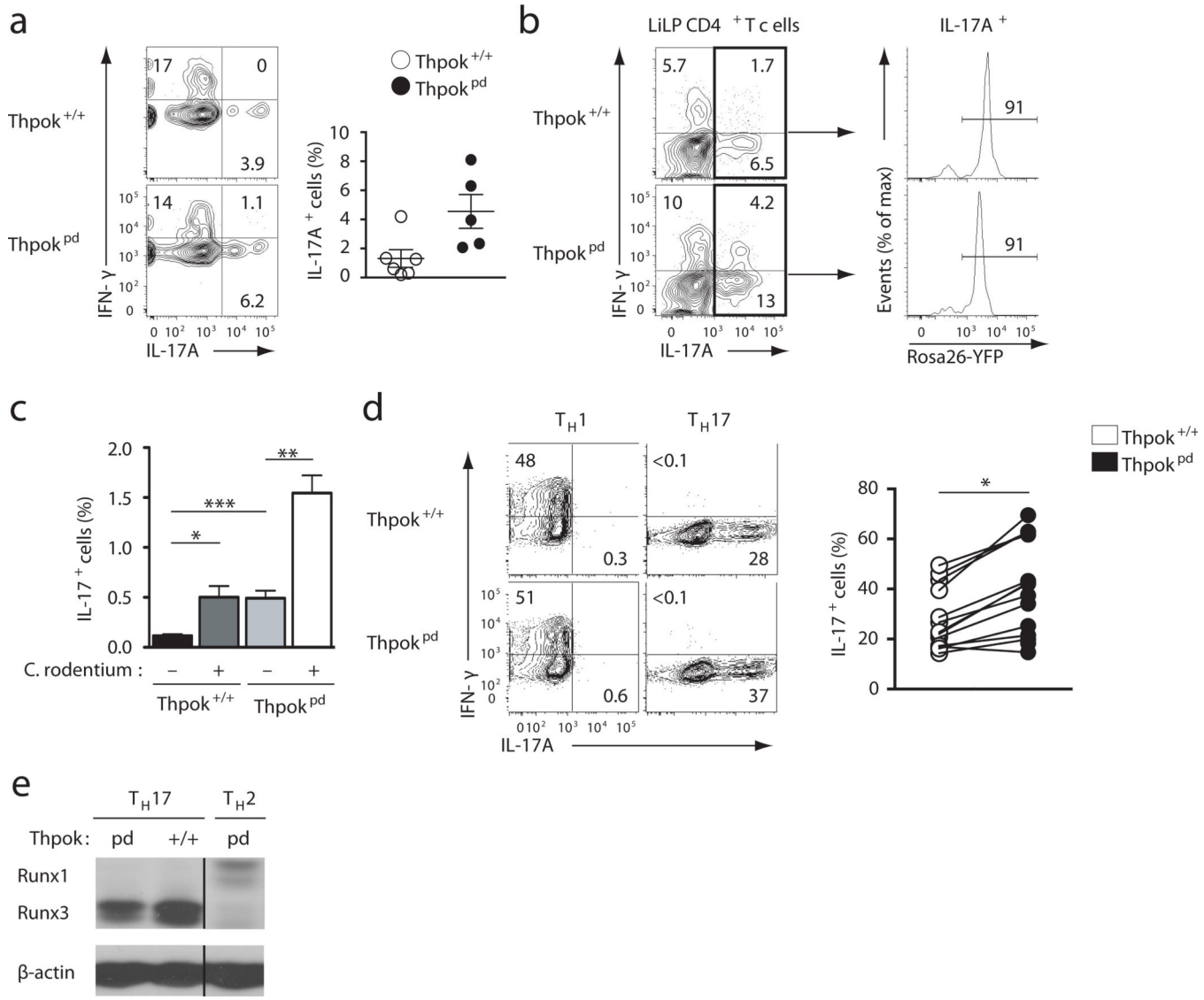

Figure 2. ThPOK is not needed for T_H17 differentiation

(a, b) Contour plots of IFN-γ versus ı IL-17A expression on large intestine lamina propria (liLP) $CD4^+$ $TCR\beta^+$ cells from *Thpok*$^{+/+}$ and *Thpok*^{pd} mice. Data is from unmanipulated mice (**a**, scatter plot on the right summarizes data on 5 mice of each genotype analyzed in 2 experiments), or 11 days after infection with *C. rodentium* (**b,** 2 independent experiments, 6 mice of each genotype per experiment). In (**b**), histograms show YFP expression in Il-17$^+$ $CD4^+$ T cells. **(c)** Bar graph shows percentages (mean ± SEM) of IL 17-producing cells in MLN of mice analyzed in (b)(*: p<0.05, **: p<0.01 ***: p<0.001 per Student's t-test). (**d**) Contour plots (left) show IFN-γ versus IL-17A expression on T_H1 (left) or T_H17 (right) effector cells derived from naïve $CD4^+CD8^-$ T cells after 5 day culture. The graph on the right summarizes 10 such experiments; each symbol represents a distinct culture. Percentages (mean ± SEM) of IL-17 producing cells were 28 ± 3.4 (*Thpok*$^{+/+}$) and 39.7 ± 4.9 (*Thpok*^{pd}). Significance (*: $P<10^{-4}$) was determined by two-tailed paired t-test. **(e)** Immunoblot analysis of Runx protein expression in T_H17 effector cells generated as in (d);

Thpok$^{+/+}$ T_H2 effector cells are shown as a negative control for Runx3. β-actin expression controls for lane loading. Representative of 3 independent experiments.

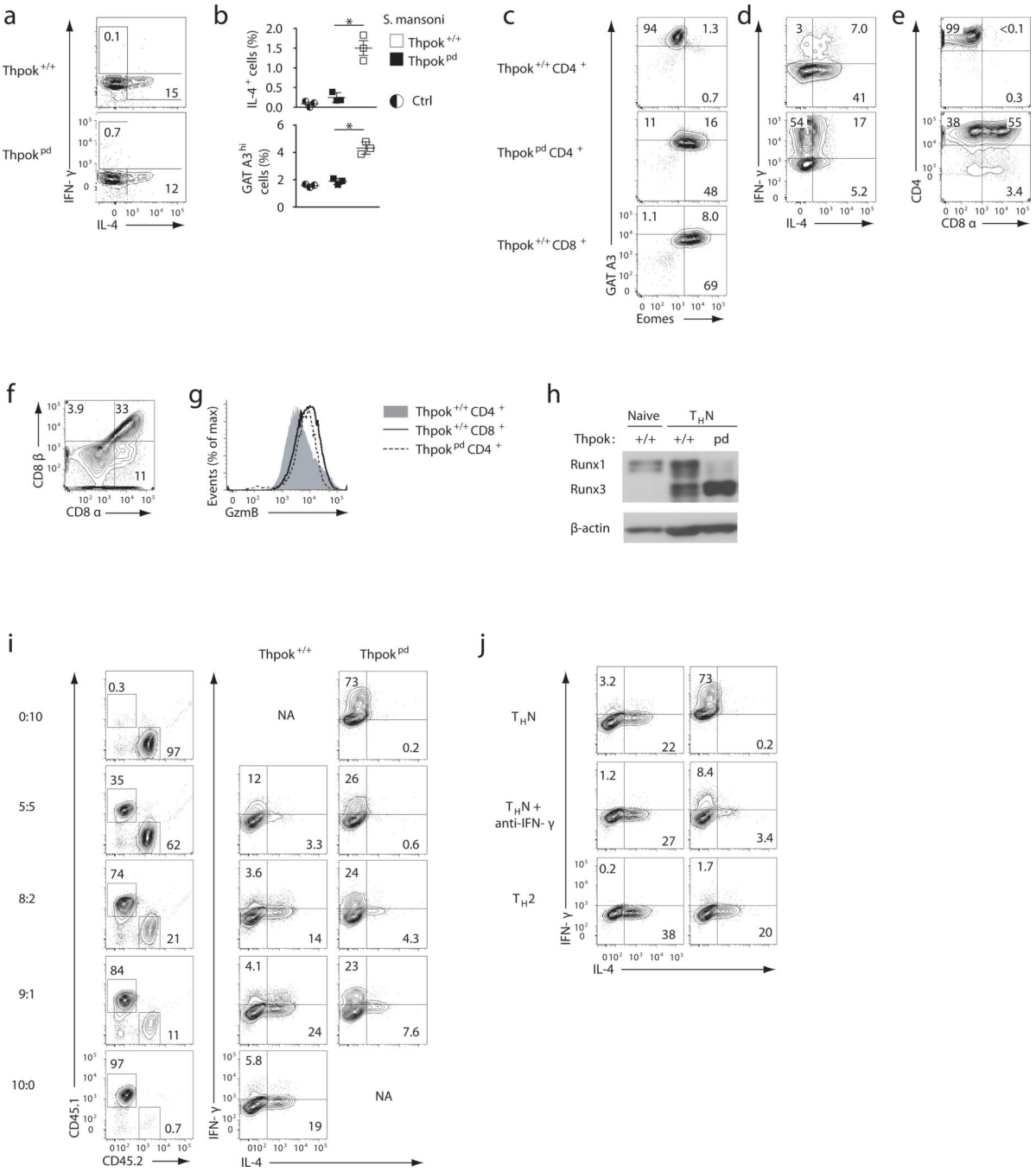

Figure 3. ThPOK 'protects' T_H2 responses

(a) Contour plots of IFN-γ versus IL-4 expression on effector cells derived from naïve *Thpok*^{pd} or *Thpok*$^{+/+}$ CD4$^+$ T cells after 5-day culture under T_H2 conditions. (**b**) Graphs indicate percentage (mean ± SEM) of IL 4- or GATA3-expressing cells among spleen CD44^{hi} CD4$^+$ T cells from *Thpok*$^{+/+}$ or *Thpok*^{pd} mice 12 days post-immunization with *Schistosoma* eggs or in non-immunized controls (Ctrl). Each symbol represents one mouse. Significance determined by t-test (*: p <0.01). **(c–f)** Contour plots assess effector cells derived from naïve CD4$^+$CD8$^-$ *Thpok*$^{+/+}$ or *Thpok*^{pd} T cells after 5-day culture in non-

polarizing (T_HN) for expression of GATA3 versus Eomes (**c**), IL-4 versus IFN-γ (**d**), CD4 versus CD8α (**e**) and CD8α vs. CD8β (**f**). Wild-type $CD8^+$ effectors derived in the same conditions are shown as a control in (**c**). **(g)** Granzyme B expression in effector T cells derived as in (c). **(h)** Immunoblot analysis of Runx protein expression in *ex vivo* naïve spleen $CD4^+$ T (*Thpok*$^{+/+}$, left lane) or *Thpok*$^{+/+}$ and *Thpok*^{pd} effector cells derived as in (c) (T_HN). β-actin expression control for loading. (**i**) Contour plots (right two columns) assess IL-4 versus IFN-γ expression in CD45.1 *Thpok*$^{+/+}$ or CD45.2 *Thpok*^{pd} effector cells, derived from sorted $CD4^+CD8^-$ cells seeded in T_HN co-cultures at ratios indicated on the left. The leftmost column indicates the percentages of each genotype at the end of the culture. (**j**) Contour plots of IL-4 vs. IFN-γ expression in *Thpok*^{pd} and *Thpok*$^{+/+}$ effectors generated in T_HN (top) or T_H2 (bottom) cultures, or in T_HN cultures supplemented by anti IFN-γ (20 μg/ml, middle row). **(a–j)** Profiles are representative of 3 (a, b), 5 (c–g), 2 (h–j) independent experiments.

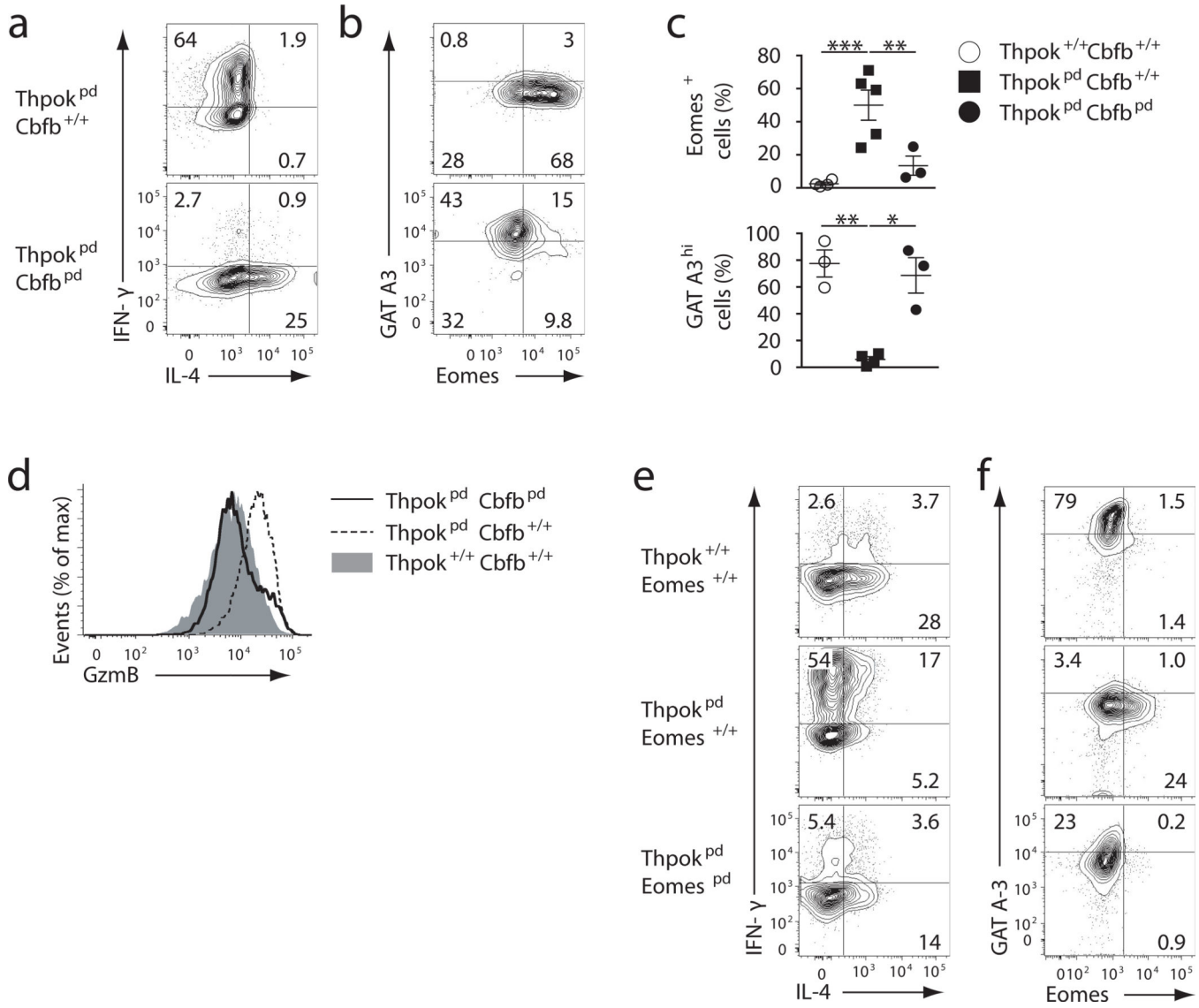

Figure 4. ThPOK 'protects' T_H2 differentiation by restraining Runx functions

(a, b) Contour plots assess IL-4 versus IFN-γ (**a**) and GATA3 versus Eomes expression (**b**) in effector cells derived from naive *Thpok*^{pd} *Cbfb*$^{+/+}$and *Thpok*^{pd} *Cbfb*^{pd} CD4^{+} T cells after 5-day culture in T_HN conditions. (**c**) Percentage of Eomes- and GATA3-expressing cells among effectors of the indicated genotype cultured as in (b). Each symbol represents a distinct mouse. Significance was determined by t-test (*: P<0.05; **: P<0.005; ***: P<0.0005). **(d)** Granzyme B expression in cells prepared as in (a, b). **(e, f)** Contour plots show expression of IL-4 versus IFN-γ (**e**) and of GATA3 versus Eomes (**f**) on effector cells derived in the same conditions as in (a). (**a–f**) Representative of 3 experiments for each panel.

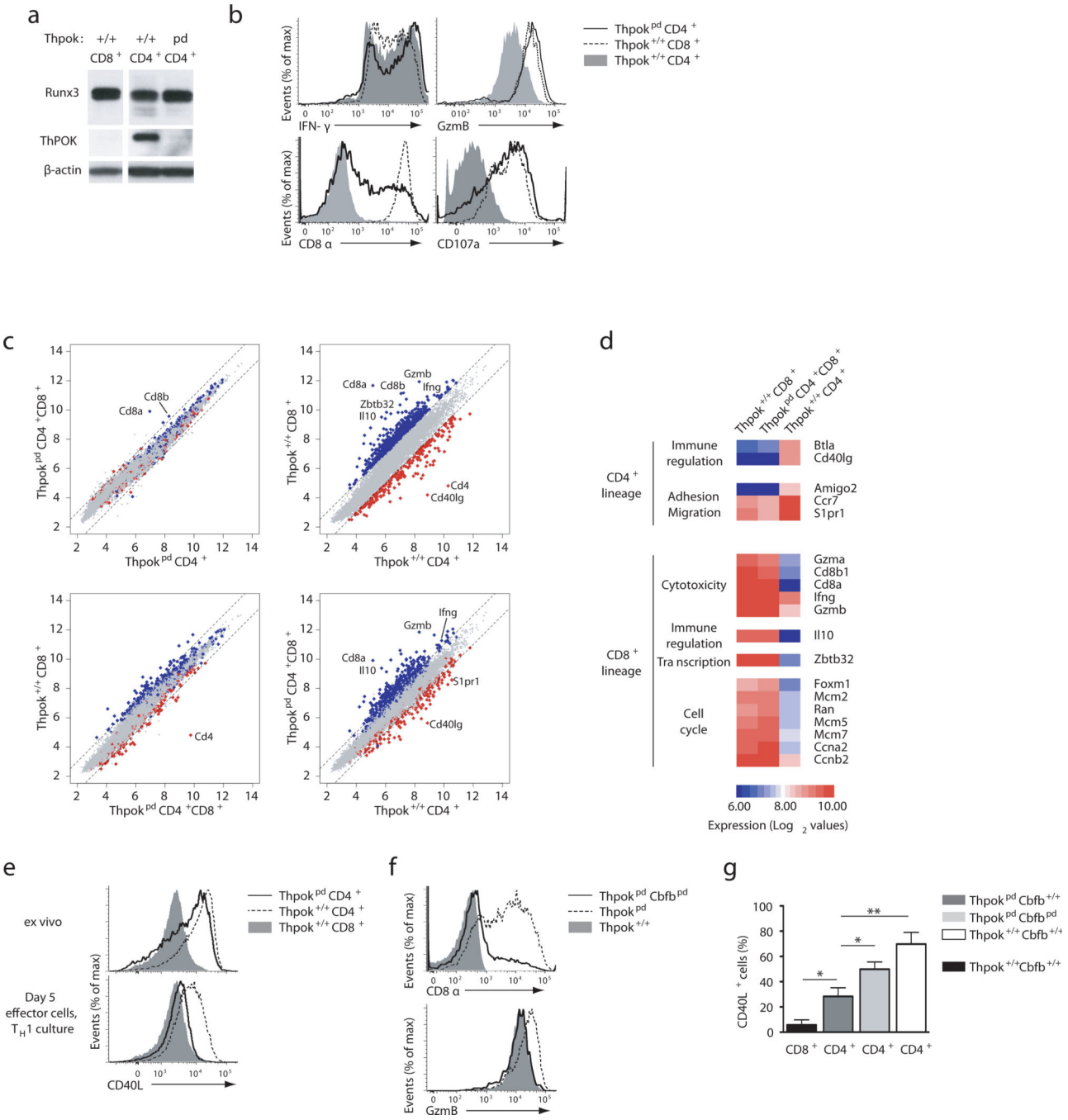

Figure 5. ThPOK antagonism of Runx activity is required for proper T_H1 differentiation

(a) Runx protein expression in effector T cells derived from spleen $CD4^+$ or $CD8^+$ *Thpok*^{pd} or *Thpok*$^{+/+}$ littermates cultured under T_H1 conditions. The membrane was reprobed for ThPOK expression and β-actin control. **(b)** Overlaid histograms show expression of intra-cellular Granzyme B and IFN-γ, or surface CD8, and externalization of surface CD107a, in effectors derived under T_H1 conditions from indicated sorted naïve T cells. **(c)** Scatter plots compare gene expression (log_2 values, complete array gene set) in indicated effector cells derived in T_H1 cultures. Dashed lines indicate 2-fold differential gene expression. Genes

with 2-fold or greater expression in wild-type $CD4^+$ vs. wild-type $CD8^+$ cells (defined on top right plot) are shown in red and blue, respectively, in all plots. Relevant genes are indicated. **(d)** Heat map indicates relative expression (red-blue color scale, $\log_2$ values) on select genes defined by 3-fold or greater change in expression in *Thpok*^{pd} $CD4^+CD8^+$ vs. wild-type $CD4^+CD8^-$ effectors. The complete data set is shown in Fig. S5b. **(e)** Overlaid histograms show CD40L expression in sorted $CD4^+CD8^-$ and $CD4^-CD8^+$ $CD44^{lo}$ T cells *ex vivo* (upper panel) and effector cells derived from these same subsets under T_H1 conditions (lower panel). **(f)** Overlaid histograms show CD8 (top) or Granzyme B expression (bottom) in effector cells derived as in (e) from sorted $CD4^+CD8^-$ cells of the indicated genotype. **(g)** Graph summarizes percentages of CD40L-expressing cells (after 3-hour *in vitro* stimulation) on effector T cells of the indicated genotype, derived as in (e); significance was determined by t-test (*: $P<0.05$, **: $P<0.01$). **(e–g)** Representative of 3 (a, b, e, f) and 6 (g) experiments.

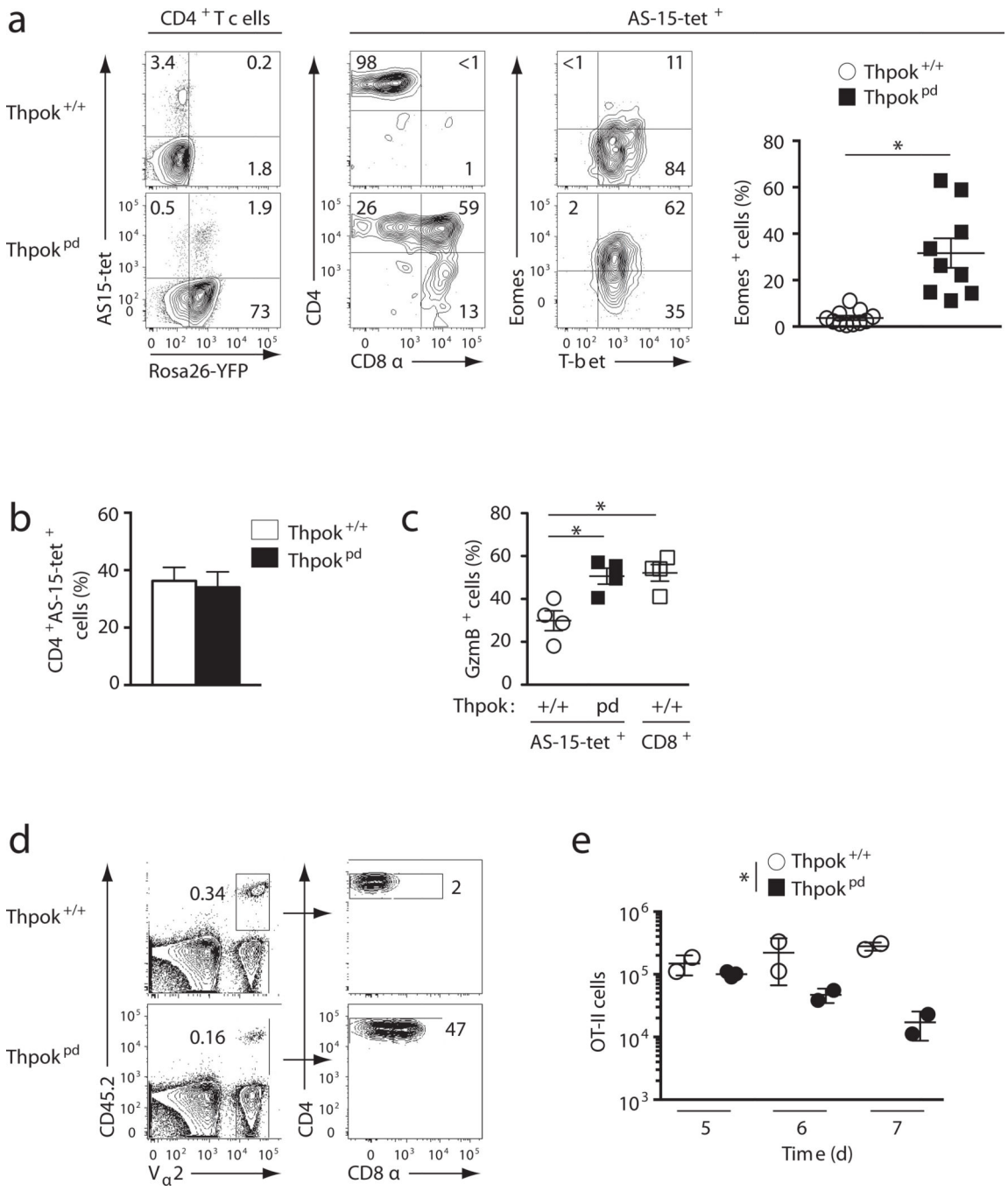

Figure 6. ThPOK is important for CD4$^+$ T cell responses *in vivo*

(a) Contour plots of AS-15-loaded I-A^b tetramer binding versus Rosa26-YFP expression on gated CD4$^+$TCRβ$^+$ spleen cells 10 days post-infection of *Thpok*$^{+/+}$ and *Thpok*^{pd} mice with *T. gondii* (left); expression of CD4 and CD8 (center) or Eomes and T-bet (right) on AS-15-specific cells. The graph on the right summarizes the percentage of Eomes-expressing cells among AS-15-reactive T cells; each symbol represents one mouse and significance was determined by t-test (*: $P<10^{-4}$). Representative of 5 experiments. **(b)** Graph summarizes the number of CD4$^+$ (CD8$^+$ or CD8$^-$) AS-15-specific spleen TCRβ$^+$ cells 9–14 days after

infection with *T. gondii*. Data is from 6 independent experiments. (**c**) Percentage (mean ± SEM) of granzyme B-expressing cells among AS-15-specific $CD4^+TCR\beta^+$ cells as defined in (a) in day 14-infected mice.; open squares show expression in $CD8^+$ T cells from infected $Thpok^{+/+}$ mice. Data is from 2 independent experiments; significance was determined by t-test (*: $P<10^{-4}$). **(d)** Contour plots (left) define $V\alpha2^+$ $CD45.2^+$ donor-derived cells in spleens from $CD45.1^+$ ovalbumin-immunized wild-type hosts, 6 days after adoptive transfer of $CD45.2^+$ OT-II TCR transgenic cells (either $Thpok^{+/+}$ or $Thpok^{pd}$). Donor cells are analyzed for CD4 and CD8 expression (right). Data is representative of 3 experiments. (**e**) Cumulative results of adoptive transfer experiments performed as in (d) and analyzed 5 to 7 days after transfer. Each symbol represents one recipient mouse. The difference between $Thpok^{+/+}$ and $Thpok^{pd}$ responses was significant by 2-way ANOVA (*:$P<0.005$).

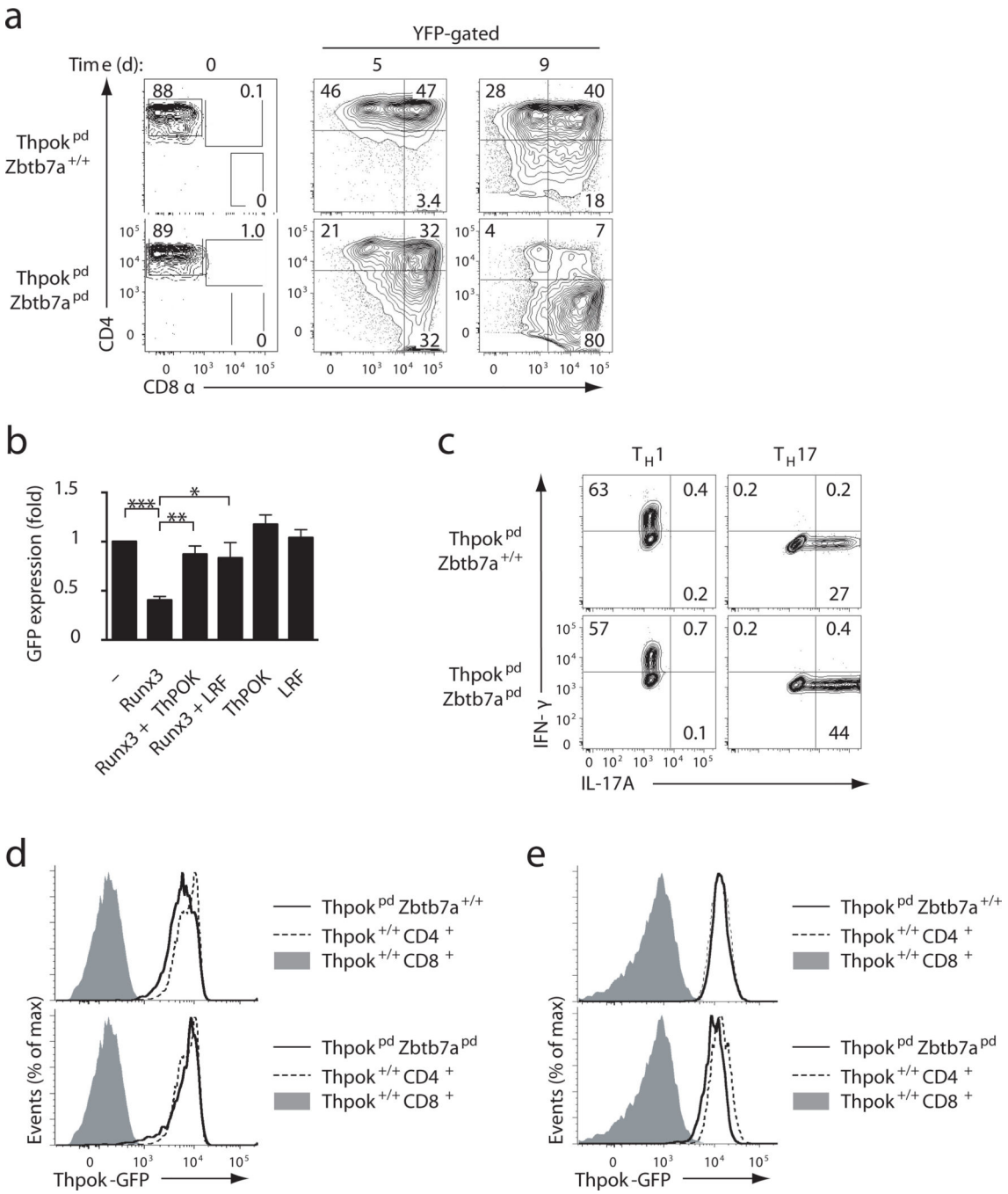

Figure 7. ThPOK and LRF redundantly prevent CD4$^+$ to CD8$^+$ *trans*-differentiation

(a) Contour plots show CD4 versus CD8 expression on *Rosa26-YFP* gated effector cells derived from CD4$^+$CD8$^-$ *Thpok*^{pd}*Zbtb7a*$^{+/+}$ (top) or *Thpok*^{pd}*Zbtb7a*^{pd} (bottom) naïve T cells (sorted populations, left), after 5 day (middle) or 9 day (right) culture under T_H1 conditions. **(b)** Bar graph depicts *Cd4* promoter activity in RLM-11 cells transfected with a GFP-based reporter plasmid for *Cd4* gene expression, and indicated expression vectors. Data shows GFP mean fluorescence activity (mean ± SD from 3 independent experiments), gated on transfected cells and expressed relative to reporter-only control (top bar). Data

significance was analyzed by t-test [P< 0.05(*), <0.01(**), <0.001(***)]. **(c)** Contours plots of IFN-γ versus IL-17A expression (gated on *Rosa26*-YFP^{+} cells) in effector cells derived from CD4^{+}CD8^{-} *Thpok*^{pd}*Zbtb7a*$^{+/+}$ (top) or *Thpok*^{pd}*Zbtb7a*^{pd} (bottom) T cells after 5-day culture under T_H1 (left) or T_H17 conditions (right). **(d, e)** Solid line histograms show GFP expression from the *Thpok*^{GFP} reporter in (**d**) *Thpok*^{pd} *Zbtb7a*$^{+/+}$ (top) or *Thpok*^{pd} *Zbtb7a*^{pd} (bottom) CD44^{lo} CD4^{+}CD8^{-} spleen T cells or (**e**) day 9 CD8-expressing effector cells derived as in (a) from naïve *Thpok*^{pd} *Zbtb7a*$^{+/+}$ (top) or *Thpok*^{pd} *Zbtb7a*^{pd} (bottom) CD4^{+}CD8^{-}T cells. In both panels, plain line histograms are overlaid on CD4^{+}CD8^{-} (dashed line) or CD4^{-}CD8^{+} (solid grey) counterparts from *Thpok*$^{+/+}$*Zbtb7a*$^{+/+}$ mice. Data is representative of 3 (a–c) or 2 (d, e) independent experiments.