RNA m6A demethylase ALKBH5 regulates the development of $\gamma\delta$ T cells

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Article

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Abstract

$\gamma\delta$ T cells are abundant T cell population at the mucosa and are important in providing immune surveillance as well as maintaining tissue homeostasis. However, despite $\gamma\delta$ T cells origin in thymus, detailed mechanisms regulating $\gamma\delta$ T cell development remain poorly understood. *N*⁶-methyladenosine (*m*⁶A) represents one of the most common post-transcriptional modifications of mRNA in mammalian cells, but whether it plays a role in $\gamma\delta$ T cell biology is still unclear. Here we show that depletion of *m*⁶A demethylase ALKBH5 in lymphocytes specifically induces an expansion of $\gamma\delta$ T cells, which confers enhanced protection against gastrointestinal *S. typhimurium* infection. Mechanistically, loss of ALKBH5 favors the development of $\gamma\delta$ T cell precursors by increasing the abundance of *m*⁶A RNA modification in thymocytes, which further reduces the expression of several target genes including Notch signaling components *Jagged1* and *Notch2*. As a result, impairment of *Jagged1/Notch2* signaling contributes to enhanced proliferation and differentiation of $\gamma\delta$ T cell precursors, leading to an expanded mature $\gamma\delta$ T cell repertoire. Taken together, our results indicate a checkpoint role of ALKBH5 and *m*⁶A modification in the regulation of $\gamma\delta$ T cell early development.

Introduction

Lymphocytes in the thymus are subdivided into two major populations based on their surface marker of $\alpha\beta$ and $\gamma\delta$ T cell antigen receptors (TCR)¹. $\gamma\delta$ T cells belong to the innate immune lymphocytes, and represent a link between innate and adaptive immunity². Similar to conventional $\alpha\beta$ T cells, $\gamma\delta$ T lymphocytes can produce different chemokines and various cytokines, such as interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), Interleukin-4 (IL-4), IL-17, IL-21, IL-22 and so on, and differentiate into different effector profiles based on their ability to produce either IFN- γ ($\gamma\delta$ T1), IL-17 ($\gamma\delta$ T17) or both IL-4 and IFN- γ ($\gamma\delta$ NKT)^{3,4}. In addition, some $\gamma\delta$ T cells also produce particular cytokines, such as keratinocyte growth factor (KGF), connective tissue growth factor (CTGF) and macrophage colony-stimulating-factor (M-CSF), or antimicrobial peptides^{5,6}. Accordingly, $\gamma\delta$ T cells show great promise to develop novel immunotherapies for mucocutaneous infections, autoimmune diseases and malignancies^{6–8}.

It has been shown that $\alpha\beta$ T and $\gamma\delta$ T cells originate from a common thymic progenitor, which is a CD4/CD8 double-negative (DN) lymphocyte⁹. The decision between $\alpha\beta$ T and $\gamma\delta$ T cells occurs at the DN stage, when pre-commitment-selection and signal strength models can determine $\alpha\beta/\gamma\delta$ lineage commitment¹⁰. Different from $\alpha\beta$ T lymphocytes, $\gamma\delta$ T cells are abundant during the embryonic stage, and the functions of some $\gamma\delta$ T cells are programmed during thymic development^{3,11}. The development and function of $\gamma\delta$ T cells does not only depend on antigen recognition mode, and they have the inherent ability to produce cytokines such as IFN- γ and IL-17. It is well identified that development of these cytokine-producing $\gamma\delta$ T subsets is largely pre-programmed in the thymus. During the development of both the $\alpha\beta$ T and $\gamma\delta$ T lineages, the $\alpha\beta/\gamma\delta$ selection is mainly dependent on Zap70/Syk-mediated signaling, which is regulated by protein tyrosine kinase p56 (*Lck*)^{12–15}. TCR- $\gamma\delta$ signaling strength exerts a critical role in thymic acquisition of $\gamma\delta$ T cell effector fate^{16,17}. Strong TCR- $\gamma\delta$ signaling induces a general IFN- γ -producing $\gamma\delta$ T phenotype^{17–19}, while weak or no TCR- $\gamma\delta$ signaling promotes the development of the $\gamma\delta$ T17 subset^{19–22}. Additionally, Notch signaling pathways play an important role in T cell lineage commitment and function²³. It is now clear that thymocyte progenitors that receive a strong Notch1 signal preferentially enter the $\alpha\beta$ T cell lineage at the expense of $\gamma\delta$ T cells²⁴; however the stages through which Notch signaling, and the underlying developmental checkpoints that regulate murine $\gamma\delta$ T cell lineage commitment events in the thymus, are still unresolved.

*N*⁶-methyladenosine (*m*⁶A) is the most prevalent and abundant mammalian RNA modification. *m*⁶A can modulate almost every aspect of mRNA metabolism, and its comprehensive roles are mediated by specific RNA binding protein complexes including ‘writers’ (mainly METTL3 and METTL14), ‘erasers’ (ALKBH5 and FTO), and ‘readers’^{25,26}. *m*⁶A RNA methylation is involved in a variety of physiological and pathological processes, such as maintenance of pluripotency in embryonic stem cells^{27,28}, sex determination²⁹, energy metabolism³⁰, antiviral immunity³¹, DNA damage response³², tumorigenesis^{33,34}, and anti-tumour immunity³⁵. Our previous work demonstrated that *m*⁶A RNA methylation controls $\alpha\beta$ T cell homeostasis and differentiation³⁶, and sustains Treg suppressive functions³⁷. However, $\gamma\delta$ T cells as another important T cell subset, are also critically in maintaining T cell homeostasis and immunoregulation and whether *m*⁶A RNA modification govern the development and function of $\gamma\delta$ T cells is unclear.

Lck kinase is expressed in the earliest thymic immigrants and in all T cell subsets, but not in B and NK cells^{38,39}. *Lck-Cre* transgenic mice have been widely used to study the involvement of different genes in T cell development and function^{40,41}. Here, using *Alkbh5*^{fl/fl} *Lck-Cre* conditional knockout mice, we identified that loss of ALKBH5 specifically expands the $\gamma\delta$ T cell population, but has little impact on the $\alpha\beta$ T cell compartment; this confers an enhanced immune protection against *S. typhimurium* infection. We observed that loss of ALKBH5 is a specific driver for the development of $\gamma\delta$ T cell progenitors, and is responsible for a significant expansion of $\gamma\delta$ T cells starting at the embryonic stage. Furthermore, we discovered that ALKBH5 deficiency-mediated *m*⁶A RNA modification induces the suppression of *Jagged1/Notch2* signaling environment in which $\gamma\delta$ T precursors can acquire a strengthened capacity to proliferation and differentiation. This study demonstrates that *m*⁶A RNA modification plays a vital role in regulating the early development of murine $\gamma\delta$ T cells, serving as a potential developmental checkpoint in the decision between $\alpha\beta$ T and $\gamma\delta$ T cells.

Results

ALKBH5 deficiency leads to an expanded $\gamma\delta$ T cell population and enhanced protection against *S. typhimurium* infection. Dynamic removal of the *m*⁶A modification on mRNA is a likely mode of regulation of T cell development but its contribution is unknown. Further, ALKBH5 is highly expressed across different subsets of T lymphocytes (Extended Data Fig. 1a), implying it plays important roles in regulating T cell biology. We therefore crossed *Alkbh5*^{fl/fl} mice with *Lck-Cre* transgenic mice to assess the function of ALKBH5 and *m*⁶A RNA modification in developing T cell populations (Extended Data Fig. 1b–d). Interestingly, loss of ALKBH5 resulted in a significant expansion of $\gamma\delta$ T cells in both thymus and peripheral tissues including spleens, peripheral lymph nodes (pLNs), mesenteric lymph nodes (mLNs), intestinal epithelial lymphocyte (IEL) and lamina propria lymphocyte (LPL) of colons (Fig. 1a,b and Extended Data

Fig. 2a,b). On the other hand, $\alpha\beta$ T cells were unchanged between *Alkbh5^{f/f}* wild-type (WT) and *Alkbh5^{f/f} Lck⁺* (KO) mice. To further confirm this observation in $\alpha\beta$ T cells, we examined the frequencies and counts of different $\alpha\beta$ T cell subsets in peripheral lymphoid tissues. Although we observed a slight reduction of CD8 T cells and a slightly skewed naive/memory balance in the spleen and pLN of *Alkbh5^{f/f} Lck⁺* mice (Extended Data Fig. 3), the total populations were comparable (Extended Data Fig. 4). We measured whether deficiency in ALKBH5 affected the composition and proportion of different $\gamma\delta$ T cell subsets. $\gamma\delta$ T cells have previously been categorized into three subpopulations based on CD8 expression: CD8 $\alpha\beta$ ⁺, CD8 $\alpha\alpha$ ⁺ and CD8⁻ cells⁴². Of note, loss of ALKBH5 did not alter the frequencies of these three subpopulations (Extended Data Fig. 5a,b), but the numbers of all subsets were markedly increased due to expansion of total $\gamma\delta$ T cells (Extended Data Fig. 5c). These results suggest that loss of ALKBH5 selectively expands $\gamma\delta$ T cells.

Since $\gamma\delta$ T cells have important roles in controlling mucocutaneous infections^{6,7}, we next performed a *Salmonella typhimurium* (*S. typhimurium*) model of colonic infection to determine the functional consequences of the expanded $\gamma\delta$ T cells. Strikingly, *Alkbh5^{f/f} Lck⁺* mice were better protected from infection than their WT littermates, manifested in significantly reduced weight loss and delayed fatality (Fig. 1c,d). Moreover, *Alkbh5^{f/f} Lck⁺* mice had a lower bacterial burden in their feces (Fig. 1e), caecum, spleen and liver (Fig. 1f) than WT littermate mice, altogether suggesting that *S. typhimurium* infection and dissemination were better controlled in *Alkbh5^{f/f} Lck⁺* mice. To further confirm that the protective phenotype observed in *Alkbh5^{f/f} Lck⁺* mice during infection was mediated by $\gamma\delta$ T cells rather than other lymphocyte populations, we crossed *Alkbh5^{f/f}*, *Alkbh5^{f/f} Lck⁺* mice onto TCR δ deficient (*TCR δ ^{-/-}*) mice for targeted deletion of $\gamma\delta$ T cells. Of note, *Alkbh5^{f/f} TCR δ ^{-/-} Lck⁺* mice showed similar pathological outcomes compared to *Alkbh5^{f/f} TCR δ ^{-/-}* mice upon *S. typhimurium* infection, including comparable weight loss, survival rate, and fecal bacterial burden (Fig. 1g-i). Taken together, these results indicate that expanded $\gamma\delta$ T cells in *Alkbh5^{f/f} Lck⁺* mice lead to a more efficient immune response against *S. typhimurium*.

Loss of ALKBH5 has no impact on IFN- γ and IL-17 production, proliferation and apoptosis of mature $\gamma\delta$ T cells. Similar to $\alpha\beta$ T cells, $\gamma\delta$ T cells can exert their immune functions by secreting pro-inflammatory cytokines such as IL-17A and IFN- γ ¹⁷⁻¹⁹. To further address the phenotype of *S. typhimurium* infection whether it was only attributable to increased number of $\gamma\delta$ T cells or also to their enhanced function, we next assayed the cytokine changes secreted by $\gamma\delta$ T cells in WT and ALKBH5 KO mice. $\gamma\delta$ T cell effector fate is largely dependent on TCR- $\gamma\delta$ signaling strength in the thymus^{16,17}. We confirm no big changes in the percentage of IFN- γ or IL-17-producing $\gamma\delta$ T cells between *Alkbh5^{f/f}* and *Alkbh5^{f/f} Lck⁺* mice in the thymus and peripheral immune tissues (Fig. 2a,b and Extended Data Fig. 6), suggesting that the quality of $\gamma\delta$ T cells and the strength of TCR- $\gamma\delta$ signaling are not affected by ALKBH5 deficiency.

To better understand the quantitative increase of $\gamma\delta$ T cells in *Alkbh5^{f/f} Lck⁺* mice, we tested whether proliferation and apoptosis of mature $\gamma\delta$ T cells had changed in those mice. Surprisingly, we did not observe enhanced proliferation or impaired apoptosis in mature $\gamma\delta$ T cells isolated from the thymus of *Alkbh5^{f/f} Lck⁺* mice (Fig. 2c-f). Together, these findings imply that loss of ALKBH5 neither changes the cytokine profile, nor alters proliferation or apoptosis of mature $\gamma\delta$ T cells.

Depletion of ALKBH5 in thymocytes promotes the expansion of $\gamma\delta$ T cell precursors. To further explore mechanistically how ALKBH5 deficiency facilitates the expansion of $\gamma\delta$ T cells, we next investigated whether loss of ALKBH5 affected the proportion and number of $\gamma\delta$ T progenitors at early developmental stages. Since $\alpha\beta$ T cells and $\gamma\delta$ T cells are both derived from thymic DN cells, we first evaluated these cells isolated from *Alkbh5^{f/f} Lck⁺* mice and WT littermates. Interestingly, *Alkbh5^{f/f} Lck⁺* mice had significantly more DN lymphocytes than WT mice in the thymus (Fig. 3a-c). It is well known that the DN population can be further sub-divided into four subsets according to their expression of surface markers CD44 and CD25: CD44⁺CD25⁻ (DN1) cells, CD44⁺CD25⁺ (DN2) cells, CD44⁻CD25⁺ (DN3) cells and CD44⁻CD25⁻ (DN4) cells⁴³. In *Lck-Cre* transgenic mice, the ALKBH5 deletion is initiated at the DN2 stage and completed by the DN3 stage^{40,44}. In accordance to this, we observed that the frequency of DN3 cells, and the numbers of DN2 and DN3 cells were markedly increased in the absence of ALKBH5 (Fig. 3d-f), suggesting that ALKBH5 is involved in the development of early $\gamma\delta$ T cell precursors.

Until now, few surface markers have been identified on developing $\gamma\delta$ T cells, and most studies have used CD73⁺ TCR $\gamma\delta$ ⁺ DN lymphocytes as $\gamma\delta$ T progenitors that are committed to the $\gamma\delta$ lineage^{18,45}. Human $\gamma\delta$ T cells are usually characterized based on the features of their TCR δ variable region (V δ), while mouse $\gamma\delta$ T cell subsets are distinguished by the V γ chain they bear, most of which are either V γ 1.1 or V γ 2⁴⁶⁻⁴⁸. Therefore, we next analyzed whether loss of ALKBH5 affects CD73-expressing TCR δ ⁺, V γ 1.1⁺ and TCR δ ⁺, V γ 2⁺ progenitors (Fig. 3g). We observed that ALKBH5 deletion slightly affected the frequency of TCR δ ⁺ V γ 1.1⁺ and TCR δ ⁺ V γ 2⁺ cells at the DN stage, as well as their progenitor subsets (Extended Data Fig. 7). Of note, compared to *Alkbh5^{f/f}* mice, *Alkbh5^{f/f} Lck⁺* mice had more immature TCR δ ⁺ V γ 1.1⁺ cells, TCR δ ⁺ V γ 1.1⁺ precursor cells and $\gamma\delta$ NKT V γ 1.1⁺ progenitors at the DN stage (Fig. 3h). Similarly, increased number of immature TCR δ ⁺ V γ 2⁺ cells, TCR δ ⁺ V γ 2⁺ precursor cells and $\gamma\delta$ NKT V γ 2⁺ progenitors at the DN stage (Fig. 3i) were found in the absence of ALKBH5. Finally, we further determined the number of mature $\gamma\delta$ T cell subsets in the thymus, and found highly increased counts in all these subpopulations in *Alkbh5^{f/f} Lck⁺* mice compared to control *Alkbh5^{f/f}* mice (Extended Data Fig. 8), indicating that the increased number of mature $\gamma\delta$ T cells observed above is due to the expansion of $\gamma\delta$ T cell precursors.

Absence of ALKBH5 promotes the proliferation of $\gamma\delta$ T cell precursors and expands $\gamma\delta$ T cell repertoire during embryonic stage. These above findings prompted us to hypothesize that ALKBH5 deficiency might increase the proliferation rate of $\gamma\delta$ T cell progenitors. Therefore, we labelled the cells *in vivo* with BrdU, a thymidine analogue used to identify proliferating cells. As expected, the frequency of BrdU positive $\gamma\delta$ T cell progenitors was significantly higher in *Alkbh5^{f/f} Lck⁺* mice compared to WT littermates (Fig. 4a,b). On the other hand, no obvious changes were observed regarding the apoptotic rate of these $\gamma\delta$ T cell progenitors (Fig. 4c,d), suggesting that $\gamma\delta$ T cell precursors in *Alkbh5^{f/f} Lck⁺* mice were expanded because of increased proliferation rather than reduced cell death.

From a developmental point of view, it has been shown that murine $\gamma\delta$ T cells begin to appear during the middle and late period of embryonic development (~E13.5)¹⁷. We then analyzed the quantity of $\gamma\delta$ T cells in the thymus of *Alkbh5^{f/f} Lck⁺* mice at different fetal stages. Surprisingly, the increased frequency of $\gamma\delta$ T cells was observed in *Alkbh5^{f/f} Lck⁺* mice as early as day E14.5 (Fig. 4e,f), suggesting that ALKBH5 affected the $\gamma\delta$ T cell population during midterm fetal

development. Taken together, these data indicate that loss of ALKBH5 promotes the proliferation of $\gamma\delta$ T cell precursors and leads to expanded $\gamma\delta$ T cell repertoire during embryonic development.

Loss of ALKBH5 results in altered Jagged1/Notch2 signaling pathway in $\gamma\delta$ T cell precursors. To explore the molecular mechanisms underlying the expanded $\gamma\delta$ T cell progenitors in the absence of ALKBH5, we performed RNA-seq on $\gamma\delta$ T cell precursors isolated from thymus in *Alkbh5^{5/f}* and *Alkbh5^{5/f} Lck⁺* cohoused independent littermates. Overall, about 698 genes ($P < 0.05$) were differentially expressed in ALKBH5-deficient $\gamma\delta$ T cell progenitors, including 339 downregulated genes and 359 upregulated genes (Extended Data Fig. 9a). Since m⁶A RNA modification is mainly involved in mRNA decay, we reasoned that removing its 'eraser' ALKBH5 likely would promote the degradation of certain mRNA transcripts with increased m⁶A levels. Among those differentially expressed genes identified from *Alkbh5^{5/f} Lck⁺* mice, Jagged1 was the most markedly downregulated gene (Fig. 5a). Jagged1 is a key ligand for Notch, a pathway which is both necessary and sufficient for T cell lineage commitment²³. In line with down-regulated *Jagged1* gene expression, pathway analysis revealed that Notch signaling was one of the most substantially reduced signaling cascades in the $\gamma\delta$ T cell progenitors isolated from *Alkbh5^{5/f} Lck⁺* mice (Fig. 5b). In depth analysis further showed that Jagged/Notch signaling genes and their target genes were indeed down-regulated, suggesting that Jagged1/Notch2 signaling pathway was functionally suppressed in ALKBH5-deficient $\gamma\delta$ T cell precursors (Fig. 5c and Supplementary Table 1). In addition, pathway analysis implied that most of the genes up-regulated in the absence of ALKBH5 are involved in regulating cell cycle (Extended Data Fig. 9b and Supplementary Table 2), which is also consistent with our flow cytometric analysis on $\gamma\delta$ T cell progenitors mentioned above (Fig. 4a,b). Next, we validated by qPCR that the expression of Jagged1 and Notch2 was down-regulated at both mRNA and protein levels in the $\gamma\delta$ T cell progenitors of *Alkbh5^{5/f} Lck⁺* mice (Fig. 5d,e). Besides, downstream components of Notch signaling such as its effector genes Hey1 and Hes1^{23,49} were also confirmed to be markedly decreased, while cell cycle factor Cdkn1a⁵⁰ was significantly increased in the absence of ALKBH5 (Fig. 5c,f). Taken together, these evidences suggest that ALKBH5 regulates the development of $\gamma\delta$ T cells through Jagged1/Notch2 signaling thereby regulating the proliferation of $\gamma\delta$ T cell precursors.

We next sought to understand whether impaired Jagged1-Notch2 signaling in the absence of ALKBH5 occurs only in $\gamma\delta$ T cell progenitors, but not in mature $\gamma\delta$ T cells. Therefore, we performed RNA-seq on mature $\gamma\delta$ T cells isolated from three independent *Alkbh5^{5/f} Lck⁺* as well as WT littermates. We found much fewer up- and down-regulated genes in *Alkbh5* KO mature $\gamma\delta$ T cells comparing to precursor T cells. Surprisingly, the dysregulation of Jagged1/Notch2 signaling and related gene expression observed in ALKBH5 KO $\gamma\delta$ T cell progenitor cells was absent in ALKBH5 KO $\gamma\delta$ T mature cells (Extended Data Fig. 10 and Supplementary Table 3). Of note, the level of Jagged1 mRNA markedly decreased in WT mature $\gamma\delta$ T cells compared with WT $\gamma\delta$ T progenitors (Supplementary Tables 1,3), indicating that Jagged1 expression in $\gamma\delta$ T cell may have temporospatial characteristics. Taken together, this evidence indicates that ALKBH5 regulates the development of $\gamma\delta$ T cells by influencing the expression of Jagged1 and Notch2 mRNAs, and through this other Notch-related genes in $\gamma\delta$ T cell progenitors.

ALKBH5 deficiency enhances m⁶A RNA modification on Jagged1 and Notch2 mRNAs to decrease their stability and expression. To address mechanistically how ALKBH5 affects Jagged1 and Notch2 mRNA levels, we first confirmed that the m⁶A levels in total mRNA of thymus lymphocytes increased in *Alkbh5^{5/f} Lck⁺* mice than in *Alkbh5^{5/f}* mice (Fig. 6a). Based on our previous m⁶A-seq data from murine T cells³⁶, we found highly enriched and specific m⁶A peaks on *Jagged1* and *Notch2* mRNAs (Supplementary Table 4). m⁶A-RNA immunoprecipitation (RIP) combined with qPCR revealed that *Jagged1* and *Notch2* m⁶A enrichment was markedly increased in *Alkbh5^{5/f} Lck⁺* mice than in the WT littermates (Fig. 6b). RNA m⁶A methylation is understood to mainly affect RNA stability. To further prove that increased m⁶A led to more rapid degradation of *Jagged1* and *Notch2* mRNAs, we performed RNA decay analysis and identified that *Jagged1* transcription was more rapidly degraded in *Alkbh5^{5/f} Lck⁺* mice than the WT littermates (Fig. 6c,d). Although the degradation of *Notch2* transcription was slightly accelerated in the absence of ALKBH5 (Fig. 6e), it was enough to reduce the expression level of *Notch2* mRNA compared to WT littermates (Fig. 6f). These data suggest that ALKBH5 deletion enhances m⁶A modification on *Jagged1* and *Notch2* mRNAs to decrease their stability and expression in $\gamma\delta$ T cells. Collectively, loss of ALKBH5-mediated m⁶A RNA modification increase promotes the development of $\gamma\delta$ T cell progenitors through targeted suppression of Jagged1/Notch2 signaling (Fig. 6g).

Discussion

In this study, we discovered that ALKBH5 in thymic lymphocytes plays a critical role in the cell fate decision between $\alpha\beta$ T and $\gamma\delta$ T cell lineages by targeting the Jagged1/Notch2 signaling pathway. We observed that loss of ALKBH5 results in a marked expansion of $\gamma\delta$ T cells, with a minor effect on $\alpha\beta$ T cells in both the thymus and periphery. It is well demonstrated that $\gamma\delta$ T cells are required to combat invasive bacterial infection, and may have great promise for the development of novel immunotherapies^{6,7}. Surprisingly, expanded $\gamma\delta$ T cells led to a more efficient immune response against *S. typhimurium* infection, while the depletion of $\gamma\delta$ T cells eliminated this the protective role in *Alkbh5^{5/f} Lck⁺* mice, together suggesting that loss of ALKBH5 specifically affects the expansion of $\gamma\delta$ T cells. ALKBH5 deletion had no impact on cytokine profiles, and affected neither proliferation nor apoptosis of mature $\gamma\delta$ T cells; it did however expand the pool of developing $\gamma\delta$ T-cell progenitors and enhanced their proliferative capacity during embryonic development. Previously, the Notch signaling pathway was shown to be necessary and sufficient for T cell lineage commitment²³. However, whether specific Notch receptor–ligand interactions influence the later cell fate decision by early thymocyte progenitors to become $\gamma\delta$ T cell in mice is still unknown^{51,52}. Here, our data demonstrate that loss of ALKBH5-mediated RNA m⁶A levels promotes the proliferation and differentiation of $\gamma\delta$ T precursors through targeting the Notch signaling genes, and shows which specific Notch receptor–ligand interaction controls the development of murine $\gamma\delta$ T-cell progenitors in the thymus.

Our findings raise the intriguing question of how ALKBH5 affects the proportion and number of $\gamma\delta$ T cells in the thymus. Although $\alpha\beta$ and $\gamma\delta$ T cells originate from a common thymic lymphocyte progenitor⁹, $\gamma\delta$ T cell precursors are believed to branch off from the $\alpha\beta$ T cell development pathway before or at the time of TCR- α rearrangement at the DN2 stage⁴⁰. We observed that the frequency of DN3 cells, and the number of DN2 and DN3 cells are significantly increased in the thymus at the absence of ALKBH5. Our data also show that ALKBH5 deficiency results in a significant expansion of $\gamma\delta$ T cell progenitors in the thymus,

including TCR δ^+ V γ 1.1 $^+$, TCR δ^+ V γ 2 $^+$ and $\gamma\delta$ NKT subsets. However, the generation of $\gamma\delta$ T cell subpopulations is developmentally regulated during ontogeny, such that V γ 5 cells develop during the fetal period, V γ 6 cells around birth, V γ 4 cells in the neonatal period, and V γ 1 and V γ 7 cells at the adult stage^{39,53}. Therefore, we do not rule out other subsets of $\gamma\delta$ T cell precursors that may have changed during ontogeny in the absence of ALKBH5. Further studies will address which population of other $\gamma\delta$ T cell precursors are differentially induced by the loss of ALKBH5 during ontogeny.

Notch signaling is involved in the regulation of lymphocyte development and function²³. In mammals, this pathway is composed of 4 Notch receptors (Notch1–Notch4), and five ligands: Jagged1 and Jagged2, and Delta1, Delta3 and Delta4. Recent evidence indicates that Notch receptor–ligand interactions have been implicated in governing cell fate decisions during T-lymphocyte differentiation^{24,51,54,55}. By RNA-seq and m⁶A epigenomic analysis, we confirm that loss of ALKBH5-mediated m⁶A RNA modification directly suppresses Jagged1/Notch2 signaling in $\gamma\delta$ T-cell progenitors, which in turn promotes the development of immature $\gamma\delta$ T cell. Interestingly, murine bone marrow–derived stem cells do not respond to Jagged1 signals, while Jagged1 signals can influence the differentiation of T-cell progenitors along $\gamma\delta$ T-cell lineages during a brief window of their development between the DN1 and DN3 stages of thymic development⁵². Out of curiosity, we compare the expression of Jagged1 in mature and immature $\gamma\delta$ T cells based on RNA-seq data, and were surprised to find that the level of Jagged1 expression in mature $\gamma\delta$ T cells is very low, and significantly decreased compared with $\gamma\delta$ T cell precursors, pointing to a potential role of Jagged1 in $\gamma\delta$ T cell development. Recent studies have shown critical roles of RNA m⁶A ‘eraser’ ALKBH5 in acute myeloid leukemia stem cells by specifically regulating a limited number of genes in leukemia cells^{56,57}. Here, we observe that loss of ALKBH5 only markedly reduces the expression of Jagged1 and Notch2, without detectable changes in any other receptors or ligands. Furthermore, the Notch signaling downstream effectors, such as Hey1 and Hes1 are significantly decreased at the absence of ALKBH5. Taken together, these data indicate that RNA m⁶A modification may serve as one checkpoint in the development of $\gamma\delta$ T cells, by at least controlling Notch signaling strength.

Collectively, we show for the first time that RNA m⁶A modification ‘eraser’ ALKBH5 regulates the development of murine $\gamma\delta$ T cell precursors in the thymus mainly through a specific interaction between Jagged1 and Notch2 (Fig. 6g). Further investigation is needed to characterize the regulation of the m⁶A checkpoint and identify more downstream ‘responders’ that control the development of $\gamma\delta$ T cell precursors.

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Declarations

Competing interests

The authors declare no competing interests.

Author contributions

C.D., H.-B.L. and R.A.F. initiated the study. C.D., H.X. and H.-B.L. designed the experiments. C.D. performed most of the experiments and analyzed data. C.D., H.X., H.-B.L. and R.A.F. wrote and revised the manuscript. R.Q. conducted bioinformatics analysis. Z.Y., H.X. and J.Z. helped to perform the FACS analysis. M.R., A.J., R.J., Z.W., G.Y., M.S., E.S., Z.Y., Q.Z., B.S. and Q.X. contributed to key discussions. H.-B.L., R.A.F. and Q.X. supervised research, coordination, and strategy.

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Methods

Mice. *Alkbh5* conditional knockout mice (*Alkbh5*^{fl/fl}) were generated as previously described with CRISPR–Cas9 technology by insertion of two loxp sites into *Alkbh5* genome loci⁵⁸. The gRNA and donor oligonucleotides used in this study are listed as follows. Left loxp insertion: 5'-tt*g*c*ccgaattttcggttgacatacacctctagctctctcctgctcaggctcagcagccacttaaataaactctgtataatgtatgctatacgaagttagggaatcgctctgagtggttgagacctgagaggactgtattcc3'; Right loxp insertion: 5'-gg***ctgactgccttttttttttgcgtgcaaaccataggaagctgtgtctgaaactcatagcataaactctgtataatgtatgctatacgaagttaggataagactaatgtggaattgtgtacctgcaggcaggttgaag3'.

Briefly, gRNA and ssDNA Ultramer (IDT) donor oligonucleotides were synthesized and injected into C57BL/6N embryos. *Alkbh5*^{fl/fl} mice generation, and conditionally deleted *Alkbh5* in thymic lymphocytes by generating *Alkbh5*^{fl/fl} *Lck-Cre* mice. We further crossed *Alkbh5*^{fl/fl} *Lck-Cre* with TCRdelta deficient (*TCRδ*^{-/-}) mice to get *Alkbh5* and TCRδ double knockout mice. *Lck-Cre* (B6.Cg strain; Stock No: 003802) and *TCRδ*^{-/-} (B6.129P2 strain; Stock No: 002120) mice were obtained from Jackson Laboratory, and have been backcrossed to C57BL/6N mice (Charles River laboratories) for more than ten generations.

We used *Alkbh5*^{fl/fl} without *Lck-Cre* as wild-type controls for *Alkbh5*^{fl/fl} *Lck-Cre* mice. All wild-type and knockout mice used in most of the experiments described here were sex- and age- (6 to 10 weeks old) matched littermates and were co-housed after weaning. Unless they came with special instructions, mice were randomly assigned to different experimental groups and each cage contained animals of all different experimental groups. Both male and female mice were used in experiments. All mice were kept under specific pathogen-free (SPF) conditions, on a 12/12 on/off light cycle, maintained at 72 (+/-2) °F with 70% humidity in the animal facility at Yale University. Animal procedures were approved by the Institutional Animal Care and Use Committee of Yale University (IACUC).

In vivo *S. Typhimurium* infection. *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* (SL1344 strain) was provided by J. Galan and stocked in our lab as previously described⁵⁹. Routinely, 8 to 10 weeks old mice were fasted for 4 hours followed by gavage of streptomycin (20 mg), at day - 1. At day 0 of infection, mice were fasted again for 4 hours and infected with 1 × 10³ CFUs of *S. Typhimurium* per mouse. Bacteria were propagated overnight in LB containing streptomycin (100 µg/ml) before infection, and subcultured in high salt LB medium (0.3M NaCl) with no antibiotics. Bacterial CFU was calculated by using spectrophotometry with OD600nm wavelength. To calculate fecal CFU, feces were collected at day 4 post infection and resuspended in PBS at the ratio of 50 mg feces per ml PBS. After being vortexed for 30 min, bacteria containing supernatants were clarified by centrifugation at 50 g for 10 min. The supernatants were serially diluted and plated on LB streptomycin plates (100 µg/ml) with 10 µl samples each dot. Similarly, caecum, spleen and liver tissues were harvested and dissociated with gentleMacs C Tubes (Miltenyi Biotech, #130-096-334) as previously described⁵⁹. Organs CFU counts were calculated using similar methodology as above. All CFU counts were performed blinded by two experimenters separately. All animal experiments were performed in compliance with Yale Institutional Animal Care and Use Committee protocols.

Colon lymphocytes isolation. Colon tissues was harvested as described and kept in cold RPMI medium until next procedure, when they were turned inside out and incubated in 25 ml dissociation medium (RPMI with 1 mM DTT, 2 mM EDTA and 2% FBS). The tissues were kept stirring at the speed of 500 rpm in 37°C

incubation boxes for 30 minutes. The supernatant which contains the intraepithelial lymphocytes (IEL) was centrifuged and washed with FACS buffer (DPBS with 2% FBS). The remaining colon tissues were cut into small pieces and incubated with 20 ml digestion medium (RPMI with 0.5 mg/ml Dispase, 1 mg/ml Type II Collagenase and 2% FBS). For the digestion procedure, tissues were treated for 45–60 minutes at 37°C, with 600 rpm of stirring speed. When digestion was over, the samples were filtered through 70 µm cell strainer, and rinsed with additional 25 ml of FACS buffer, followed by centrifuging at 500 g for 10 minutes. The cell pellet which contains the lamina propria lymphocytes (LPL) was collected and washed with FACS buffer. Each collected IEL and LPL samples were purified using 40% percoll, and then were strained to remove any undigested material and washed in FACS buffer before processing for flow cytometry analysis.

Flow cytometry. For surface staining, single-cell suspensions were prepared as described and stained for surface markers for 15–30 min at 4 °C. For intracellular cytokine staining, the cells were re-stimulated for 3 h at 37 °C with phorbol 12-myristate 13-acetate (PMA) (Sigma, 50 ng/ml) and ionomycin (Sigma, 1 µg/ml) in the presence of Monensin (Bioscience). After stimulation, cells were washed and stained as the manufacturer described (BD, #554714). For staining of nuclear factors, cells were fixed and stained according to the manufacturer's instructions (eBioscience, #00-5523-00). For BrdU experiment, mice were retro-orbital injected with BrdU at a dose of 100 µg per gram of body weight 2 hours before euthanasia. Then thymocytes were collected and stained as described above. Cells were all re-suspended in PBS, 0.5% FBS, 5 mM EDTA and acquired with an LSRII cytometer (BD Bioscience). Data were analyzed by using FlowJo software (version 9.0 or higher, BD Bioscience). The list of antibodies and reagents used in flow cytometry is shown in Supplementary Table 5.

RNA isolation and qPCR. TRIzol reagent (Invitrogen) was added to total thymic lymphocytes and then processed following the manufacturer's instructions. RNA was further purified using the RNase-Free DNase Set (QIAGEN, #79256). The RNA isolation of γδ T cells or progenitors was performed with RNeasy Micro Kit (QIAGEN, #74034) following the manufacturer's protocol. Maxima H Minus Reverse Transcriptase Kit (ThermoFisher Scientific, #EP0753) was used for cDNA synthesis. Sigma KiCqStart predesigned SYBR green primers, gene-specific 6FAM-MGB probes (ThermoFisher Scientific) and iTaq Universal SYBR Green Supermix or FAM Supermix (Biorad) were used for real time PCR, and β-actin mRNA level was used as internal control to calculated mRNA relative abundance. Primer sequences used for qPCR are as follow: mouse *Alkbh5* (forward, 5'-CGCGGTCAACGACTACC-3'; reverse, 5'-ATGGGCTTGAAGTGAAGTGA-3'), mouse *β-actin* (forward, 5'-AGTGTGACGTTGACATCCGT-3'; reverse, 5'-GCAGCTCAGTAACAGTCCGC-3'), mouse *Myc peak* (forward, 5'-GCTTCGAAACTCTGGTGCAT-3'; reverse, 5'-AATTCCAGCGCATCAGTTCT-3'). Mouse *Jagged1*, *Notch1*, *Notch2*, *Hey1*, *Hes1*, *Cdkn1a* and *β-actin* specific probes (ThermoFisher Scientific, #4453320).

Western blot. Total protein of thymic lymphocytes was extracted with RIPA lysis buffer supplemented with protease inhibitors (ThermoFisher Scientific). Antibodies against ALKBH5 (Proteintech, #16837-1-AP) and β-actin (Cell Signaling Technology, #4970) were used at a 1:1,000 dilution in 5% no-fat milk buffer at 4 °C overnight. Membranes were washed with 0.1% PBST buffer and then incubated in HRP-conjugated secondary antibody (Cell Signaling Technology, #7074) at room temperature for 1 hour. After extensive wash, membranes were incubated in TLC chemiluminescence reagent (Biorad) and exposed to X-ray film.

m⁶A dot blot. m⁶A dot blot was conducted as previously described³¹. Briefly, total RNA extracted from total thymic γδ T cells, were purified with the Dynabeads mRNA Purification Kit (Invitrogen, #61006) according to the manufacturer's instructions. Then, purified mRNA was further mixed with gly sample loading dye (Invitrogen, #AM8551) and denatured at 65 °C for 20 min, and put on ice immediately. Denatured samples were dropped onto the Hybond-N+ membrane, dried naturally and performed UV crosslinking for 2 minutes. 0.1% PBST wash buffer was used to wash the membranes, which were then blocked with 5% non-fat milk for 1 hour. Later, the membranes were probed with anti-m⁶A antibody (Synaptic Systems, #202003) at a dilution of 1:1000 in 4 °C for overnight with gentle shaking. 0.1% PBST buffer washed the membranes three times, 10 min each time. After that, an HRP-conjugated secondary antibody (Cell Signaling Technology, #7074) was added to the membranes and incubated at room temperature for 1 hour. After extensive wash, the signal was detected by enhanced chemiluminescence with pico ECL using Chemidoc MP (Biorad).

RNA-seq library preparation and data processing. Thymic mature γδ T cells (CD45.2⁺CD3⁺TCRγ/δ⁺) and γδ T progenitors (CD45.2⁺CD4⁻CD8⁻TCRγ/δ⁺CD73⁺) were sorted by FACS Aria II (BD Bioscience). Total RNAs were isolated by RNeasy Micro Kit (QIAGEN, #74034) following the manufacturer's protocol. mRNA for RNA-seq analysis was purified using polyA+ selection and RNA-seq libraries were constructed by Yale Center for Genome Analysis (YCGA) using SMARTer® Stranded Total RNA-Seq Kit-Pico Input Mammalian (Clontech). The libraries were sequenced on Illumina HiSeq X 4000 platform (paired-end 150-bp read length) by Geneseq Technology Inc. Each sample obtained 50 million reads. STAR was used to align the raw sequencing reads to mouse genome mm10 with default parameters after trimming adapter sequences with cutadapt^{60,61}. Subsequently, we counted reads in features with htseq-count and used DESeq2 for identifying differentially expressed genes between the WT and KO samples with the same number of biological replicates⁶². Visualizations using volcano-plots and heatmaps were produced with R. Gene Ontology (GO) enrichment analysis of biological process was performed by Reactome signaling pathway analysis based on differentially expressed genes.

m⁶A-RIP-qPCR. m⁶A-RIP-qPCR analysis was performed as described previously³¹. Briefly, total thymic γδ T cells were isolated using mouse TCRγ/δ + T Cell Isolation Kit (Miltenyi Biotec, #130-092-125) and processed according to the manufacturer's protocol. The purified poly(A)+ mRNA was incubated with anti-m⁶A antibody (Synaptic System, #202003), or rabbit IgG in RIP wash buffer (150 mM NaCl, 10 mM Tris, 0.1% NP40, PH 7.4, supplemented with RNase inhibitor) for 2 hours at 4 °C. Then, Protein A beads (ThermoFisher Scientific, #21348) were added and incubated in 4 °C for 2 hours. After extensive wash with RIP buffer, immunoprecipitated beads-m⁶A antibody-mRNA complex was competitively eluted by m⁶A nucleotide by using TRIzol reagent (Invitrogen), and then purified by 75% ethanol precipitation. Maxima H Minus Reverse Transcriptase Kit (ThermoFisher Scientific, #EP0753) was used for cDNA synthesis. Sigma KiCqStart predesigned SYBR green primers, gene-specific 6FAM-MGB probes (ThermoFisher Scientific) and iTaq Universal SYBR Green Supermix or FAM Supermix (Biorad) were used for real time PCR. *β-actin* as m⁶A negative control, *Myc peak* as m⁶A positive control³⁶.

RNA decay analysis. RNA decay analysis was conducted as previously described⁶³. Briefly, total thymic γδ T cells were isolated using mouse TCRγ/δ + T Cell Isolation Kit (Miltenyi Biotec, #130-092-125) and processed following the manufacturer's instruction. Then, cells were plated on 96-well plates with 5 × 10⁴

cells per well. After treatment with actinomycin D (5 µg/ml) (Sigma-Aldrich, #A1410) for 0, 2 hours and 4 hours, cells were collected and subjected to RNA extraction. Total RNAs isolation and qPCR conducted for mRNA levels as described above.

Statistical analysis. Statistical analysis was calculated in GraphPad Prism 8 series software. Paired or unpaired Student's *t*-test and two-tailed Mann–Whitney *U* test were used for measurement data of two groups analysis. All general statistical analysis was calculated with a confidence interval of 95%. *P* values ≤ 0.05 were considered as statistically significant. Survival curves were compared by the log-rank test using GraphPad Prism 8. Data are represented as mean ± s.e.m. or mean ± s.d. as indicated in the figures.

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Figures

Figure 1

ALKBH5 deficiency leads to expanded γδ T cell population and enhanced protection against *S. typhimurium* infection. a, Representative dot plots showing the balance of αβ/γδ T cells isolated from indicated organs of *Alkbh5^{fl/fl} Lck⁺* and *Alkbh5^{fl/fl}* mice. b, Statistical analysis of cell numbers in (a) is reported (WT, n = 7; KO, n = 6). Unpaired t test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean ± s.d.). c, Weight loss of *Alkbh5^{fl/fl}* (n = 8) or *Alkbh5^{fl/fl} Lck⁺* (n = 9) mice infected with *S. typhimurium*. Unpaired t test was used for statistical analysis. Data represent one out of three independent experiments (mean ± s.e.m.). d, Survival curve for *S. typhimurium*-infected *Alkbh5^{fl/fl}* (n = 12) or *Alkbh5^{fl/fl} Lck⁺* (n = 8) mice.

mice. Log rank test was used for analysis. Data represent one out of three independent experiments. e,f, *S. typhimurium* Colony-forming Units (CFUs)/g of (e) feces, (f) caecum, spleen, and liver from *Alkbh5^{fl/fl}* (n = 7) or *Alkbh5^{fl/fl} Lck⁺* (n = 7) mice, 4 days post-infection. Mann-Whitney test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean $\pm$ s.d.). g, Weight loss of *Alkbh5^{fl/fl} TCR $\delta^{-/-}$* (n = 11) or *Alkbh5^{fl/fl} TCR $\delta^{-/-}$ Lck⁺* (n = 9) mice infected with *S. typhimurium*. Unpaired t test was used for statistical analysis. Data represent one out of three independent experiments (mean $\pm$ s.e.m.). h, Survival curve for *S. typhimurium*-infected *Alkbh5^{fl/fl} TCR $\delta^{-/-}$* (n = 11) or *Alkbh5^{fl/fl} TCR $\delta^{-/-}$ Lck⁺* (n = 9) mice. Log rank test was used for analysis. Data represent one out of three independent experiments. i, *S. typhimurium* CFU/g of feces from *Alkbh5^{fl/fl} TCR $\delta^{-/-}$* (n = 11) or *Alkbh5^{fl/fl} TCR $\delta^{-/-}$ Lck⁺* (n = 9) mice, 4 days post-infection. Mann-Whitney test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean $\pm$ s.d.). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, n.s., not significant.

Figure 1

ALKBH5 deficiency leads to expanded $\gamma\delta$ T cell population and enhanced protection against *S. typhimurium* infection. **a**, Representative dot plots showing the balance of $\alpha\beta/\gamma\delta$ T cells isolated from indicated organs of *Alkbh5^{fl/fl}* Lck⁺ and *Alkbh5^{fl/fl}* mice. **b**, Statistical analysis of cell numbers in (a) is reported (WT, n = 7; KO, n = 6). Unpaired t test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean $\pm$ s.d.). **c**, Weight loss of *Alkbh5^{fl/fl}* (n = 8) or *Alkbh5^{fl/fl} Lck⁺* (n = 9) mice infected with *S. typhimurium*. Unpaired t test was used for statistical analysis. Data represent one out of three independent experiments (mean $\pm$ s.e.m.). **d**, Survival curve for *S. typhimurium*-infected *Alkbh5^{fl/fl}* (n = 12) or *Alkbh5^{fl/fl} Lck⁺* (n = 8) mice. Log rank test was used for analysis. Data represent one out of three independent experiments. **e,f**, *S. typhimurium* Colony-forming Units (CFUs)/g of (e) feces, (f) caecum, spleen, and liver from *Alkbh5^{fl/fl}* (n = 7) or *Alkbh5^{fl/fl} Lck⁺* (n = 7) mice, 4 days post-infection. Mann-Whitney test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean $\pm$ s.d.). **g**, Weight loss of *Alkbh5^{fl/fl} TCR $\delta^{-/-}$* (n = 11) or *Alkbh5^{fl/fl} TCR $\delta^{-/-}$ Lck⁺* (n = 9) mice infected with *S. typhimurium*. Unpaired t test was used for statistical analysis. Data represent one out of three independent experiments (mean $\pm$ s.e.m.). **h**, Survival curve for *S. typhimurium*-infected *Alkbh5^{fl/fl} TCR $\delta^{-/-}$* (n = 11) or *Alkbh5^{fl/fl} TCR $\delta^{-/-}$ Lck⁺* (n = 9) mice. Log rank test was used for analysis. Data represent one out of three independent experiments. **i**, *S. typhimurium* CFU/g of feces from *Alkbh5^{fl/fl} TCR $\delta^{-/-}$* (n = 11) or *Alkbh5^{fl/fl} TCR $\delta^{-/-}$ Lck⁺* (n = 9) mice, 4 days post-infection. Mann-Whitney test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean $\pm$ s.d.). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, n.s., not significant.

Figure 2

Loss of ALKBH5 has no impact on IFN- γ and IL-17 production, proliferation and apoptosis of mature $\gamma\delta$ T cells. **a**, Flow cytometric analysis of IFN- γ and IL-17A production in mature $\gamma\delta$ T cells isolated from the thymus, spleen, pLN and mLN of *Alkbh5*^{ff}/f Lck⁺ and *Alkbh5*^{ff}/f mice. **b**, Statistical analysis of the frequencies for IFN- γ /IL-17A-producing mature $\gamma\delta$ T cells isolated from the thymus, spleen, pLN and mLN of *Alkbh5*^{ff}/f Lck⁺ ($n = 7$) and *Alkbh5*^{ff}/f ($n = 7$) mice. Unpaired t test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean $\pm$ s.d.). **c**, Flow cytometric analysis of Ki-67 expression in mature $\gamma\delta$ T cells isolated from the thymus of *Alkbh5*^{ff}/f Lck⁺ and *Alkbh5*^{ff}/f mice. **d**, Statistical analysis of Ki-67+ $\gamma\delta$ T mature cells in thymus in (c) is reported (WT, $n = 6$; KO, $n = 6$). Unpaired t test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean $\pm$ s.d.). **e**, Flow cytometric analysis of thymic mature $\gamma\delta$ T cells undergoing apoptosis of *Alkbh5*^{ff}/f Lck⁺ and *Alkbh5*^{ff}/f mice. **f**, Frequency of apoptotic cells (Annexin V+) in (e) is reported (WT, $n = 6$; KO, $n = 8$). Unpaired t test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean $\pm$ s.d.). n.s., not significant.

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Figure 3

Depletion of ALKBH5 in thymocytes promotes the expansion of γδ T cell precursors. a, Representative dot plots showing CD4⁺, CD8⁺, double positive (DP) and double negative (DN) populations isolated from thymus of *Alkbh5*^{fl/fl} Lck⁺ and *Alkbh5*^{fl/fl} mice. b, Statistical analysis of frequencies for each population in (a) is reported (WT, n = 7; KO, n = 6). Unpaired t test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean ± s.d.). c, Statistical analysis of cell number for DN population in (a) is reported (WT, n = 7; KO, n = 6). Unpaired t test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean ± s.d.). d, Representative dot plots showing thymic lymphocytes at different DN stages. Cells were isolated from the thymus of either *Alkbh5*^{fl/fl} Lck⁺ or *Alkbh5*^{fl/fl} mice. e, Statistical analysis of frequencies for each DN sub-population in (d) is reported (WT, n = 7; KO, n = 6). Unpaired t test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean ± s.d.). f, Statistical analysis of cell number for cells at DN2 and DN3 stages in (d) is reported (WT, n = 7; KO, n = 6). Unpaired t test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean ± s.d.). g, Gating strategies for flow cytometric analysis of different subsets of γδ T cell precursors in thymus. Basically, immature TCRvγ1.1⁺ γδ T cells were identified as CD45⁺, CD4⁻, CD8⁻, TCRγδ⁺ and TCRvγ1.1⁺ cells. TCRvγ1.1⁺ γδ T precursor cells were further identified as CD73⁺, TCRvγ1.1⁺ γδ T cells. NKTCRvγ1.1 precursor cells were further identified as CD73⁺, CD24⁻, NK1.1⁺, TCRvγ1.1⁺ γδ T cells. Similarly, immature TCRvγ2⁺ γδ T cells (CD45⁺, CD4⁻, CD8⁻, TCRγδ⁺ and TCRvγ2⁺), TCRvγ2⁺ γδ T precursor cells (CD73⁺, TCRvγ2⁺ γδ T cells) and NKTCRvγ2 precursor cells (CD73⁺, CD24⁻, NK1.1⁺, TCRvγ2⁺ γδ T cells) were identified. h,i, Statistical analysis of cell numbers for immature TCRvγ1.1⁺ (h) and immature TCRvγ2⁺ (i) γδ T cells at DN stage, as well as their precursor subsets are reported (WT, n = 6; KO, n = 6). Unpaired t test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean ± s.d.). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, n.s., not significant.

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Figure 4

Absence of ALKBH5 promotes the proliferation of $\gamma\delta$ T cell precursors and expands $\gamma\delta$ T cell repertoire during embryonic stage. **a**, Flow cytometric analysis of thymic $\gamma\delta$ T cell precursors after in vivo BrdU labelling from *Alkbh5^{f/f} Lck⁺* and *Alkbh5^{f/f}* mice. **b**, Frequency of BrdU+ $\gamma\delta$ T cell precursors in (a) is reported (WT, n = 6; KO, n = 7). Unpaired t test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean $\pm$ s.d.). **c**, Flow cytometric analysis of thymic $\gamma\delta$ T cell precursors undergoing apoptosis from *Alkbh5^{f/f} Lck⁺* and *Alkbh5^{f/f}* mice. **d**, Frequency of apoptotic cells (Annexin V+) in (c) is reported (WT, n = 6; KO, n = 8). Unpaired t test was used for statistical analysis. Each dot represents one mouse. Data represent one out of three independent experiments (mean $\pm$ s.d.). **e**, Flow cytometric analysis of thymic $\gamma\delta$ T cells at different developmental stages from *Alkbh5^{f/f} Lck⁺* and *Alkbh5^{f/f}* mice. **f**, Statistical analysis of the frequencies for thymic $\gamma\delta$ T cells in (e). Unpaired t test was used for statistical analysis. Each dot represents one mouse (WT, n = 5; KO, n = 7 for E13.5, E14.5 and E15.5; WT, n = 3; KO, n = 4 for E18.5). Data represent two independent experiments combined (mean $\pm$ s.d.). ***P < 0.001, ****P < 0.0001, n.s., not significant.

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Figure 5

Loss of ALKBH5 results in altered Jagged1/Notch2 signaling pathway in $\gamma\delta$ T cell precursors. a, Volcano plot of the differentially expressed genes between $\gamma\delta$ T cell precursors isolated from Alkbh5f/f Lck+ mice and Alkbh5f/f mice. The most significantly down-regulated gene Jagged1, was marked in red. Data represent three independent experiments combined. b, Gene Ontology (GO) enrichment analysis of biological process for down-regulated genes in ALKBH5-deficient $\gamma\delta$ T cell precursors. c, Heatmap showing Jagged/Notch signaling-related genes across different samples. Data represent three independent experiments combined. d, Quantitative PCR (qPCR) analysis of Jagged1, Notch1 and Notch2 mRNAs expression in $\gamma\delta$ T cell precursors isolated from Alkbh5f/f Lck+ (n = 3) and Alkbh5f/f (n = 3) mice. Unpaired t test was used for statistical analysis. Data represent one out of three independent experiments (mean $\pm$ s.d.). e, Flow cytometric analysis of Jagged1 and Notch2 protein level in $\gamma\delta$ T cell precursors isolated from Alkbh5f/f Lck+ (n = 4) and Alkbh5f/f (n = 5) mice. Data represent one out of three independent experiments. f, qPCR analysis of Notch signaling downstream genes Hey1, Hes1 and Cdkn1a in $\gamma\delta$ T cell precursors isolated from Alkbh5f/f Lck+ (n = 3) and Alkbh5f/f (n = 3) mice. Unpaired t test was used for statistical analysis. Data represent one out of three independent experiments (mean $\pm$ s.d.). *P < 0.05, **P < 0.01, ***P < 0.001, n.s., not significant.

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Figure 6

ALKBH5 deficiency enhances m6A RNA modification on Jagged1 and Notch2 mRNAs to decrease their stability and expression. a, Western blot of ALKBH5 protein (top) and dot blot of m6A levels (200 ng or 400 ng total RNA; middle) in thymocytes isolated from *Alkbh5^{fl/fl} Lck⁺* and *Alkbh5^{fl/fl}* mice (n = 3–4 per group). Data represent one out of three independent experiments. b, m6A-RIP-qPCR analysis showing m6A enrichment of Jagged1 and Notch2 mRNAs in total thymic $\gamma\delta$ T cells from *Alkbh5^{fl/fl} Lck⁺* and *Alkbh5^{fl/fl}* cohoused three independent littermates. Results are presented relative to those obtained with immunoglobulin G (IgG). β -actin, m6A negative control; Myc peak, m6A positive control. Paired t test was used for statistical analysis. n = 3–5 mice each group for one time independent experiment. Data represent three independent experiments combined (mean $\pm$ s.d.). c, RNA decay analysis showing Jagged1 mRNA degradation in total thymic $\gamma\delta$ T cells treated with actinomycin D for 2 hours and 4 hours from *Alkbh5^{fl/fl} Lck⁺* and *Alkbh5^{fl/fl}* cohoused three independent littermates. The residual RNAs were normalized to 0 hour. Paired t test was used for statistical analysis. n = 3–5 mice each group for one time independent experiment. Data represent three independent experiments combined (mean $\pm$ s.d.). d, qPCR analysis of Jagged1 mRNA levels, as in (c). Paired t test was used for statistical analysis. n = 3–5 mice each group for one time independent experiment. Data represent three independent experiments combined (mean $\pm$ s.d.). e, RNA decay analysis showing Notch2 mRNA degradation in total thymic $\gamma\delta$ T cells treated with actinomycin D for 2 hours and 4 hours from *Alkbh5^{fl/fl} Lck⁺* and *Alkbh5^{fl/fl}* cohoused three independent littermates. The residual RNAs were normalized to 0 hour. Paired t test was used for statistical analysis. n = 3–5 mice each group for one time independent experiment. Data represent three independent experiments combined (mean $\pm$ s.d.). f, qPCR analysis of Notch2 mRNA levels, as in (e). Paired t test was used for statistical analysis. n = 3–5 mice each group for one time independent experiment. Data represent three independent experiments combined (mean $\pm$ s.d.). g, Working model summarizing how ALKBH5 and m6A RNA modification regulate the development of $\gamma\delta$ T cells. *P < 0.05, **P < 0.01, ***P < 0.001, n.s., not significant.

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