Published in final edited form as:
*Eur J Immunol*. 2010 September ; 40(9): 2385–2390. doi:10.1002/eji.201040534.

# The sequential activation of Gata3 and Thpok is required for the differentiation of CD1d-restricted CD4+ NKT cells

**Lie Wang**[1], **Tiffany Carr**[2], **Yumei Xiong**[1], **Kathryn F. Wildt**[1], **Jinfang Zhu**[3], **Lionel Feigenbaum**[4], **Albert Bendelac**[2], and **Rémy Bosselut**[1]

[1]Laboratory of Immune Cell Biology, Center for Cancer Research (CCR), NCI, NIH, Bethesda, Maryland, USA

[2]Department of Pathology, Howard Hughes Medical Institute, Committee on Immunology, University of Chicago, Chicago, Illinois, USA

[3]Laboratory of Immunology, NIAID, NIH

[4]NCI-SAIC, Frederick, Maryland, USA

## Summary

While most CD4+ T cells are MHC class II-restricted, a small subset, including CD1d-restricted 'invariant' iNKT cells, are selected on non-classical MHC-I or MHC-I-like molecules. We previously showed that the sequential activation of two zinc finger transcription factors, Gata3 and Thpok, promotes the differentiation of conventional, MHC II-restricted thymocytes into CD4+ T cells. In the current study, we show that a Gata3-Thpok cascade is required for the differentiation of CD4+ iNKT cells. Gata3 is required for iNKT cells to express *Thpok*, whereas Thpok is needed for their proper differentiation, and notably for them to maintain CD4 and terminate CD8 expression. These findings identify the sequential activation of Gata3 and Thpok as a hallmark of CD4+ T-cell differentiation, regardless of MHC restriction.

## Introduction

While the vast majority of CD4+ T cells are MHC II-restricted, small but functionally important contingents of CD4+ cells are restricted by other MHC or MHC-like molecules. The most abundant of these cells recognize lipid-bound CD1d molecules, express markers typical of NK cells, including the surface antigen NK 1.1, and can be identified in vitro through their binding to tetramers of α-galactosylceramide (Gal-ceramide)-bound CD1d [1]. NKT cells express an invariant TCRα chain and a limited repertoire of TCRβ chains (hence their name, invariant NKT cells, iNKT), and NKT cell development is largely dependent on the transcription factor PLZF [2, 3]. Although they are selected by MHC I-like CD1d molecules, iNKT cells typically do not express CD8, and appear as $CD4^+CD8^-$ (CD4 single positive (SP)) or $CD4^-CD8^-$ (double negative (DN)). This is unlike other subsets of MHC I-restricted cells that acquire effector function in the thymus and adopt a CD8-SP phenotype [4, 5]. Currently, it is unclear why, unlike conventional MHC I-restricted thymocytes, iNKT precursors differentiate into CD4 rather than CD8 cells.

Address for correspondence: Rémy Bosselut, Laboratory of Immune Cell Biology, NCI, NIH, Building 37, Room 3015, 37 Convent Drive, Bethesda, MD 20892-4259, USA, phone 301 402 48 49, fax 301 402 48 44, remy@helix.nih.gov.

**Conflict of interest**
The authors declare no financial or commercial conflict of interest.

The transcription factors Gata3 and Thpok are both required for the generation of CD4+ T cells from $CD4^+CD8^+$ (double positive, DP) thymocytes [6]. Gata3 is expressed throughout T cell differentiation and is required at multiple branch points during T cell development or effector differentiation [7]. Gata3 gene expression is transiently up-regulated during the differentiation of CD4-lineage thymocytes [8]; it is required at an early step of CD4+ T cell development, and has been proposed to promote the expression of genes specifically expressed in CD4+ T cells, or required for their differentiation [9]. Thpok is not expressed in DP thymocytes and its up-regulation during CD4-lineage differentiation requires Gata3 [9–11]. Thpok is required for the commitment of MHC II-restricted thymocytes to the CD4-lineage, notably by repressing the expression of the transcription factor Runx3 and of cytotoxic genes, and by promoting CD8 silencing [12–15].

In the current study, we found that Thpok is expressed at high level in CD1d-restricted NKT cells in the thymus and peripheral lymphoid organs. Thpok is required for the differentiation of CD4+ iNKT cells and for their repression of CD8, a function reminiscent of the one it serves in MHC II-restricted T cells. We further document that *Thpok* expression in iNKT cells is highly dependent on Gata3. We conclude from these findings that a similar cascade, sequentially involving Gata3 and Thpok, promotes the differentiation of MHC II- and CD1d-restricted thymocytes into CD4+ T cells.

## Results and discussion

We previously described a bacterial artificial chromosome (BAC) reporter transgene in which the first *Thpok* coding exon (exon 2) has been replaced by a GFP cDNA [9, 15] ( Supporting Information Fig. 1, thereafter referred to as $Thpok^{GFP}$ transgene). In two independently derived mouse lines, this transgene expressed GFP in essentially all CD4+ T cells and mature CD4-SP thymocytes but not in CD8-SP thymocytes, or in naïve or memory spleen or LN CD8+ T cells [9]. However, we found low-level GFP expression in activated $Thpok^{GFP}$ CD8+ cells (Fig. 1A), in agreement with a recent report [16]. Immunoblot analyses demonstrated expression of endogenous Thpok protein (Fig. 1A). although at levels well below those in CD4+ cells.

This suggested that *Thpok* expression in T cells was not strictly limited to MHC II-restricted cells. To further explore this possibility, we introduced the $Thpok^{GFP}$ reporter into MHC II-deficient mice. In such mice, a small subset of thymocytes expressed GFP, of which most were $TCR^{hi}$ $CD24^{lo}$ (Fig. 1B). These cells shared two additional characteristics: they did not express CD8 and were $CD44^{hi}$ (Fig. 1B, C). Invariant NKT (iNKT) cells, which recognize CD1d-bound lipids [17], form the main subset of CD4+ T cells in MHC-II-deficient mice and are $TCR^{hi}$ $CD24^{lo}$ $CD44^{hi}$, prompting us to assess whether they express *Thpok*. Supporting this idea, most $GFP^+$ thymocytes in MHC-II-deficient $Thpok^{GFP}$ mice expressed the NKT cell marker NK1.1 (Fig. 1C). Indeed, most iNKT thymocytes, identified using α-Gal-ceramide CD1d tetramers, expressed the *Thpok* reporter (Fig. 1D and Supporting Information Fig. 2, and so did peripheral iNKT cells (Fig. 1E).

iNKT cells derive from DP thymocytes [18], cease CD8 expression early during their differentiation and therefore become CD4-SP cells. Subsequently, a subset of them terminates CD4 expression and becomes $CD4^-CD8^-$ DN thymocytes. These DN iNKT cells also expressed GFP, although slightly less than their CD4-expressing counterparts (Fig. 1D). This differs from MHC II-restricted cells which all express both Thpok and CD4. Future studies will determine the mechanistic basis for this difference. Unlike CD4-differentiating thymocytes and naïve CD4+ T cells [13, 15], iNKT cells co-express *Runx3* and *Thpok*, with similar *Runx3* levels in CD4+ and DN subsets (Y. X. and R. B., unpublished results); whether or not Runx3 affects CD4 expression in iNKT cells remains to determine, although

we and others previously found that Thpok antagonizes the *Cd4*-silencing function of Runx3 [14, 15, 19].

To examine whether Thpok is required for iNKT cell development, we evaluated iNKT populations in *Thpok*-deficient mice [9]. While there was no reproducible difference in the frequency of CD1d-specific cells between *Thpok*-deficient and *Thpok*-sufficient thymi, *Thpok*-deficient iNKT cells failed to express CD4, and a subset of them expressed low levels of CD8 (Fig. 2A). iNKT cells were also found in normal frequency in the spleen and liver of *Thpok*-deficient mice, where they also displayed a DN or CD8-expressing phenotype (Fig. 2B and data not shown). Thus, unlike the related zinc finger transcription factor PLZF [2, 3], Thpok is not needed for the development of iNKT cells, but it is required for their continued CD4 expression. Of note, *Thpok*-deficient iNKT cells expressed less CD8 than conventional CD8+ cells, and a subset of them reexpressed CD8α but not CD8β (Fig. 2B, right and Supporting Information Fig. 3A). This suggested that so far unidentified transcription factors, expressed in conventional CD8+ cells but not in iNKT cells, promote CD8 expression. In addition, *Thpok*-deficient iNKT cells had impaired expression of markers of effector function, including NK1.1 and Granzyme B (Fig. 2C, 2D), although not of CD44 (Supporting Information Fig. 3B), indicating that Thpok controls multiple aspects of iNKT cell differentiation. The reduced Granzyme B expression by *Thpok*-deficient iNKT cells (Fig. 2D) was especially noticeable since Thpok represses Granzyme B expression in conventional T cells [12, 15]. Thus, *Thpok* affects multiple aspects of iNKT cells development in addition to their coreceptor expression. A recent report similarly found that *Thpok* promotes IFNγ expression in iNKT cells [20], whereas it has the opposite effect in conventional CD4+ cells [15].

Similar to their *Thpok*-deficient counterparts, *Gata3*-deficient iNKT cells have reduced CD4 expression [21]. We had previously proposed a sequential model for Gata3 and Thpok function during the development of MHC II-restricted CD4+ T cells, whereby Gata3 is required for *Thpok* expression and Thpok promotes CD4-commitment and CD8 silencing [9]. Consequently, we wondered whether Gata3 was required for *Thpok* expression in iNKT cells. To assess this, we evaluated the expression of *Thpok* mRNA by RT-PCR in iNKT cells purified from *Gata3*-sufficient mice and from mice in which floxed *Gata3* alleles were deleted in DP cells by a *Cd4*-Cre transgene (referred to as $Gata3^{\Delta DP}$ mice)[22]. These analyses showed a 90% reduction in *Thpok* expression in *Gata3*-deficient relative to wild-type iNKT cells (Fig. 3A). To determine whether this resulted from an homogenous reduction of *Thpok* expression, we introduced the $Thpok^{GFP}$ transgene into $Gata3^{\Delta DP}$ mice. Consistent with our previous finding that Gata3 is required for *Thpok* expression, there was little or no GFP fluorescence in conventional thymocytes ($CD44^{lo}$ $NK1.1^{-}$) in $Thpok^{GFP}$ $Gata3^{\Delta DP}$ mice (Supporting Information Fig. 4A). In iNKT thymocytes, *Gata3* disruption made GFP expression barely detectable, compared to their *Gata3*-sufficient counterparts (Figs. 3B, columns 2 and 4, and Supporting Information Fig. 4B). We conclude from these experiments that Gata3 promotes *Thpok* expression in iNKT cells.

As previously reported [21], Gata3 disruption resulted in the disappearance of the CD4+ iNKT subset. We observed a minor re-expression of CD8 on $Gata3^{\Delta DP}$ iNKT thymocytes (data not shown and Fig. 3B), with substantial biological variability among mice. This re-expression was not nearly as pronounced as on *Thpok*-deficient iNKT cells, and it is possible that this is due to the small residual *Thpok* expression in *Gata3*-deficient iNKT thymocytes (Supporting Information Fig. 4B). Of note, *Gata3* disruption results in drastically reduced peripheral iNKT cell numbers [21], whereas *Thpok* disruption had no such effect, indicating that Gata3 serves other functions in addition to promoting *Thpok* expression. This is supported by the altered development of *Gata3*-deficient iNKT thymocytes, illustrated by

the premature expression of NK1.1 by *Gata3*- but not *Thpok*-deficient iNKT thymocytes (Ref. [21] and Supporting Information Fig. 3B).

The expression of *Thpok* by CD1d-selected iNKT cells indicates that *Thpok* can be expressed in thymocytes in the absence of MHC-II co-engagement of TCR and CD4. The selection of CD1d-restricted iNKT cells differs from that of conventional T cells in two critical aspects [1, 23]. First, selecting CD1d molecules are expressed by DP thymocytes but not by the thymic epithelium [24]. Second, the selection of iNKT cells requires homotypic interactions between SLAM family receptors and signaling through the adaptor SAP, none of which is involved in selection of conventional T cells [25]. Future work will determine whether either factor promotes *Thpok* expression by iNKT cells. Because CD1d does not bind CD8, the expression of *Thpok* by iNKT cells is consistent with the 'kinetic signaling' model of lineage choice, which proposes that CD8-independent TCR signaling in thymocytes promotes CD4 differentiation, and therefore results in *Thpok* expression [26].

The expression of *Thpok* in iNKT cells depends on Gata3, similar to what we previously reported in MHC II-restricted thymocytes [9]. However, while *Gata3*-deficient MHC II-restricted thymocytes fail to differentiate into mature T cells, iNKT cells develop despite *Gata3* disruption, allowing us to dissect effects of Gata3 on cell development from those on *Thpok* expression. As a result, we could identify a *Gata3* requirement for *Thpok* expression in iNKT cells, distinct from that for selection. Such a demonstration had not been possible in MHC II-restricted thymocytes, which in absence of *Gata3* are arrested before the stage at which they would normally express *Thpok* [9, 27]. We had previously shown that Gata3 was recruited to the *Thpok* locus in MHC II-restricted thymocytes [9]. Recent 'Chipseq' large scale analyses have found that Gata3 binds the same site, close to the 3′ extremity of *Thpok* intron 1 in iNKT cells [within DNase I hypersensitivity site A in Ref. 9] (J.Z., William E. Paul and Keji Zhao, unpublished data). In MHC II-restricted thymocytes, this activity of Gata3 is part of a broader network of positive and negative transcriptional regulators that determine *Thpok* expression [6, 28], and it is likely that this is the case in iNKT cells as well.

Previous studies have led to the idea that *Thpok* is up-regulated as a result of MHC II signaling in thymocytes [10, 11, 13, 14]. We now add an important amendment to this paradigm, namely that *Thpok* is also expressed in iNKT cells. As in conventional MHC II-restricted thymocytes T cells, Thpok is required for proper repression of CD8 expression by CD1d-restricted iNKT thymocytes and for their development into CD4+ cells, and its expression is Gata3-dependent. We propose that the sequential activation of Gata3 and Thpok is a characteristic of CD4+ T cells, regardless of their antigenic specificity.

## Materials and Methods

### Mice

*Thpok*$^{-/-}$, *Gata3*$^{\Delta DP}$ and the *Thpok*$^{GFP}$ line were previously described [9, 22]. MHC II-deficient mice were from Jax [29]. Mice were housed in Specific Pathogen Free facilities and analyzed between 4 and 12 weeks of age. Animal procedures were approved by relevant NIH Animal Care and Use Committees.

### Antibodies

The following mAb were obtained from BD Pharmingen and used for staining: TCRβ (H57-597), CD4 (RM4.4 or GK1.5), CD8 (53-6.7), CD24 (M1/69), CD44 (IM7), CD69, and NK1.1 (PK136). PBS-57-loaded and control (unloaded) mouse CD1d tetramer were from the NIH tetramer core facility.

### Cell preparation and staining

Cells were prepared and analyzed by flow cytometry according to previously described procedures, using either an LSR II flow cytometer (BD Biosciences) or a modified (Cytek) FacsCalibur cytometer (BD Biosciences) [9]. Cell sorting was performed as described using Facs Vantage or Facs Aria instruments (BD Biosciences) [9]. CD4 or CD8 peripheral cells were purified using Dynal beads (Invitrogen), activated with antibodies against CD3 and CD28 and cultured under 'Th2' condition as described [15].

### Gene and protein expression analyses

Gene expression was analyzed by RT-PCR using Taqman reagents, probes (*Zbtb7b* Mm00784709_s1) and an ABI PRISM 7500HT sequence detection system, all from Applied Biosystems, following published procedures [12, 15]. Gene expression values were normalized to *Actb* in the same sample. For protein analyses, cells were lyzed in 1% Triton-containing buffer; anti-Thpok immunoprecipitation and immunoblotting was carried out as previously described [9].

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank Ehydel Castro for expert mouse technical assistance, Barbara Taylor for help with flow cytometry, Takeshi Egawa for insight on the control of *Cd4* expression, Renaud Lesourne, Paul Love and Bill Paul for helpful discussions and reagents, and B.J. Fowlkes, Paul Love and Al Singer for reading the manuscript. This work was supported in part by the Intramural Research Programs of the National Cancer Institute, Center for Cancer Research, and of the National Institute of Allergy and Infectious Diseases, NIH.

## References

1. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. Annu Rev Immunol. 2007; 25:297–336. [PubMed: 17150027]
2. Savage AK, Constantinides MG, Han J, Picard D, Martin E, Li B, Lantz O, Bendelac A. The transcription factor PLZF directs the effector program of the NKT cell lineage. Immunity. 2008; 29:391–403. [PubMed: 18703361]
3. Kovalovsky D, Uche OU, Eladad S, Hobbs RM, Yi W, Alonzo E, Chua K, et al. The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. Nat Immunol. 2008; 9:1055–1064. [PubMed: 18660811]
4. Schwartzberg PL, Mueller KL, Qi H, Cannons JL. SLAM receptors and SAP influence lymphocyte interactions, development and function. Nat Rev Immunol. 2009; 9:39–46. [PubMed: 19079134]
5. Prince AL, Yin CC, Enos ME, Felices M, Berg LJ. The Tec kinases Itk and Rlk regulate conventional versus innate T-cell development. Immunol Rev. 2009; 228:115–131. [PubMed: 19290924]
6. Wang L, Bosselut R. CD4-CD8 lineage differentiation: Thpok-ing into the nucleus. J Immunol. 2009; 183:2903–2910. [PubMed: 19696430]
7. Ho IC, Tai TS, Pai SY. GATA3 and the T-cell lineage: essential functions before and after T-helper-2-cell differentiation. Nat Rev Immunol. 2009; 9:125–135. [PubMed: 19151747]
8. Hernandez-Hoyos G, Anderson MK, Wang C, Rothenberg EV, Alberola-Ila J. GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation. Immunity. 2003; 19:83–94. [PubMed: 12871641]
9. Wang L, Wildt KF, Zhu J, Zhang X, Feigenbaum L, Tessarollo L, Paul WE, et al. Distinct functions for the transcription factors GATA-3 and ThPOK during intrathymic differentiation of CD4(+) T cells. Nat Immunol. 2008; 9:1122–1130. [PubMed: 18776904]

10. Sun G, Liu X, Mercado P, Jenkinson SR, Kypriotou M, Feigenbaum L, Galera P, Bosselut R. The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. Nat Immunol. 2005; 6:373–381. [PubMed: 15750595]
11. He X, He X, Dave VP, Zhang Y, Hua X, Nicolas E, Xu W, et al. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. Nature. 2005; 433:826–833. [PubMed: 15729333]
12. Jenkinson SR, Intlekofer AM, Sun G, Feigenbaum L, Reiner SL, Bosselut R. Expression of the transcription factor cKrox in peripheral CD8 T cells reveals substantial postthymic plasticity in CD4-CD8 lineage differentiation. J Exp Med. 2007; 204:267–272. [PubMed: 17296789]
13. Egawa T, Littman DR. ThPOK acts late in specification of the helper T cell lineage and suppresses Runx-mediated commitment to the cytotoxic T cell lineage. Nat Immunol. 2008; 9:1131–1139. [PubMed: 18776905]
14. Muroi S, Naoe Y, Miyamoto C, Akiyama K, Ikawa T, Masuda K, Kawamoto H, Taniuchi I. Cascading suppression of transcriptional silencers by ThPOK seals helper T cell fate. Nat Immunol. 2008; 9:1113–1121. [PubMed: 18776907]
15. Wang L, Wildt KF, Castro E, Xiong Y, Feigenbaum L, Tessarollo L, Bosselut R. The zinc finger transcription factor Zbtb7b represses CD8-lineage gene expression in peripheral CD4+ T cells. Immunity. 2008; 29:876–887. [PubMed: 19062319]
16. Setoguchi R, Taniuchi I, Bevan MJ. ThPOK derepression is required for robust CD8 T cell responses to viral infection. J Immunol. 2009; 183:4467–4474. [PubMed: 19734230]
17. Benlagha K, Weiss A, Beavis A, Teyton L, Bendelac A. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. J Exp Med. 2000; 191:1895–1903. [PubMed: 10839805]
18. Egawa T, Eberl G, Taniuchi I, Benlagha K, Geissmann F, Hennighausen L, Bendelac A, Littman DR. Genetic evidence supporting selection of the Valpha14i NKT cell lineage from double-positive thymocyte precursors. Immunity. 2005; 22:705–716. [PubMed: 15963785]
19. Wildt KF, Sun G, Grueter B, Fischer M, Zamisch M, Ehlers M, Bosselut R. The transcription factor zbtb7b promotes CD4 expression by antagonizing runx-mediated activation of the CD4 silencer. J Immunol. 2007; 179:4405–4414. [PubMed: 17878336]
20. Engel I, Hammond K, Sullivan BA, He X, Taniuchi I, Kappes D, Kronenberg M. Co-receptor choice by V{alpha}14i NKT cells is driven by Th-POK expression rather than avoidance of CD8-mediated negative selection. J Exp Med. 2010
21. Kim PJ, Pai SY, Brigl M, Besra GS, Gumperz J, Ho IC. GATA-3 regulates the development and function of invariant NKT cells. J Immunol. 2006; 177:6650–6659. [PubMed: 17082577]
22. Zhu J, Min B, Hu-Li J, Watson CJ, Grinberg A, Wang Q, Killeen N, et al. Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. Nat Immunol. 2004; 5:1157–1165. [PubMed: 15475959]
23. Borowski C, Bendelac A. Signaling for NKT cell development: the SAP-FynT connection. J Exp Med. 2005; 201:833–836. [PubMed: 15781574]
24. Coles MC, Raulet DH. NK1. 1+ T cells in the liver arise in the thymus and are selected by interactions with class I molecules on CD4+CD8+ cells. J Immunol. 2000; 164:2412–2418. [PubMed: 10679077]
25. Griewank K, Borowski C, Rietdijk S, Wang N, Julien A, Wei DG, Mamchak AA, et al. Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. Immunity. 2007; 27:751–762. [PubMed: 18031695]
26. Singer A, Adoro S, Park JH. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. Nat Rev Immunol. 2008; 8:788–801. [PubMed: 18802443]
27. Pai SY, Truitt ML, Ting CN, Leiden JM, Glimcher LH, Ho IC. Critical roles for transcription factor GATA-3 in thymocyte development. Immunity. 2003; 19:863–875. [PubMed: 14670303]
28. Collins A, Littman DR, Taniuchi I. RUNX proteins in transcription factor networks that regulate T-cell lineage choice. Nat Rev Immunol. 2009; 9:106–115. [PubMed: 19165227]
29. Madsen L, Labrecque N, Engberg J, Dierich A, Svejgaard A, Benoist C, Mathis D, Fugger L. Mice lacking all conventional MHC class II genes. Proc Natl Acad Sci U S A. 1999; 96:10338–10343. [PubMed: 10468609]

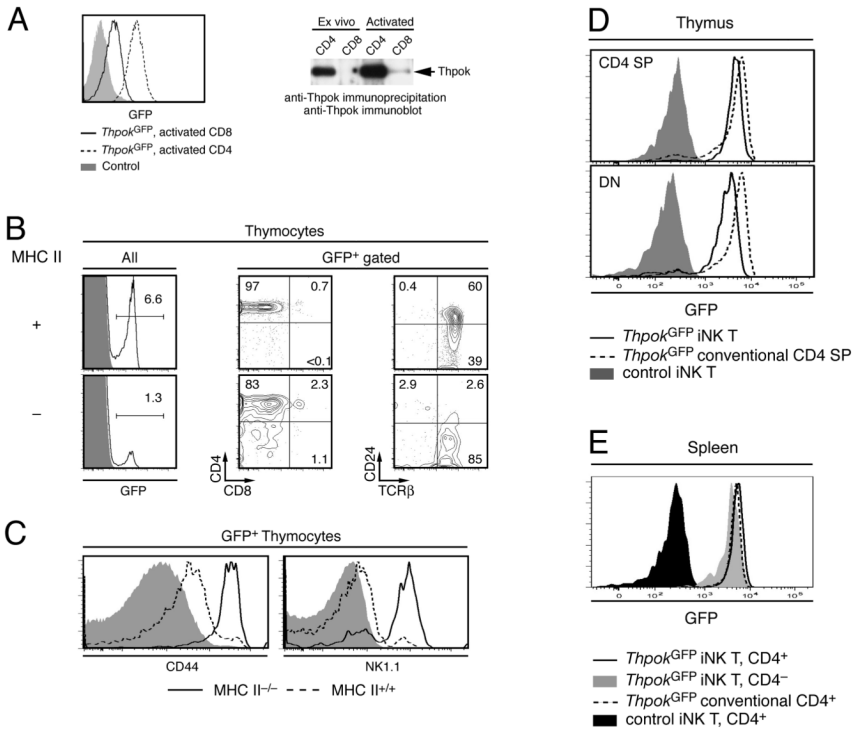


**Figure 1. Expression of the *Thpok*$^{GFP}$ reporter in iNKT cells**

(A) GFP expression on *Thpok*$^{GFP}$ CD8+ (plain line) or CD4+ (dashed line) T cells 7 days after activation, overlaid on background fluorescence from similarly activated control cells (grey-shaded) (left). Expression of Thpok protein was analyzed by immunoprecipitation and immunoblot on freshly isolated (left) and *in vitro* activated (day 7) CD4+ and CD8+ T cells (right). (B, C) Thymocytes from MHC II-deficient or -sufficient mice carrying the *Thpok*$^{GFP}$ reporter were analyzed by multicolor flow cytometry. (B) GFP fluorescence (plain lines) is overlaid over background fluorescence from control non-transgenic mice (grey shaded). Two parameter contour plots gated on GFP-expressing cells show CD4 vs. CD8 (middle) and TCRβ vs. CD24 expression. (C) Overlaid histograms show expression of CD44 (left) and NK1.1 (right) on GFP$^{+}$ cells from MHC II-deficient (plain lines) and -sufficient (dashed lines). Grey-shaded histograms show staining with an isotype-control antibody. (D) Overlaid histograms show GFP fluorescence (plain lines) on CD4-SP (top) and DN (bottom) iNKT thymocytes from *Thpok*$^{GFP}$ mice, and background fluorescence (grey-shaded) on the same populations from control mice. Subsets were defined by expression of CD4, CD8 and CD24, and reactivity with α-Gal-ceramide-loaded CD1d tetramer, as shown in Supporting Information Fig. 2A. The dashed line shows GFP fluorescence on conventional CD4-SP cells from *Thpok*$^{GFP}$ mice. (E) GFP fluorescence is shown on CD4$^{+}$ (plain line) and CD4$^{–}$ (grey-shaded) iNKT cells, and on conventional CD4+ T cells (dashed lines), all from the spleen of *Thpok*$^{GFP}$ mice, as gated in Supporting Information Fig. 2B. The black-filled histograms depicts background fluorescence on CD4$^{+}$ iNKT cells from control mice. Data (A-E) are representative of at least two experiments.

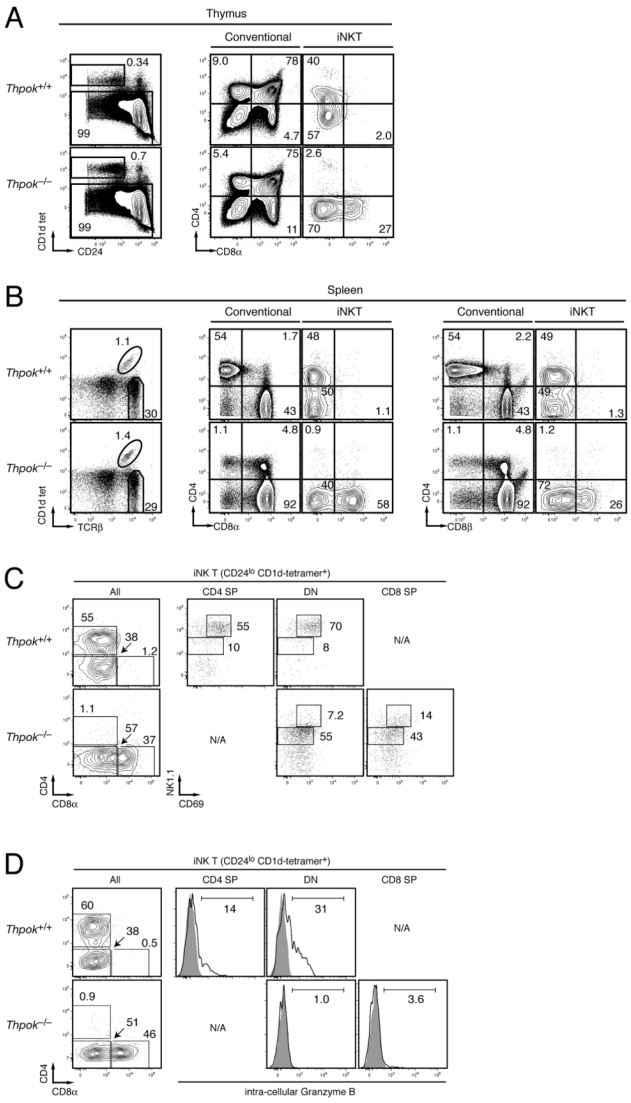


**Figure 2. *Thpok* is required for the differentiation of CD4$^+$ iNKT cells**

(A) Conventional and iNKT thymocytes, defined on the basis of α-Gal-ceramide-loaded CD1d binding and expression of CD24 (left) are analyzed for CD4 and CD8α expression (right). (B) Contour plots (right) display expression of CD8α vs. CD4 or CD8β vs. CD4 on conventional T cells and iNKT cells in the spleen, gated as shown in the top left plots. Data (A, B) are representative of 7 mice of each genotype analyzed in four separate experiments. (C, D) Expression of surface CD69 and NK1.1 (C), or of intra-cellular Granzyme B (D) is analyzed on iNKT thymocytes from wild-type and *Thpok*-deficient defined on the basis of CD24 expression and CD1d-tetramer binding as in (A) and gated for CD4 and CD8α expression as shown in the leftmost plots. Data are representative of three mice of each genotype anlyzed in two separate experiments. Grey-shaded histograms in (D) show staining with an isotype control.

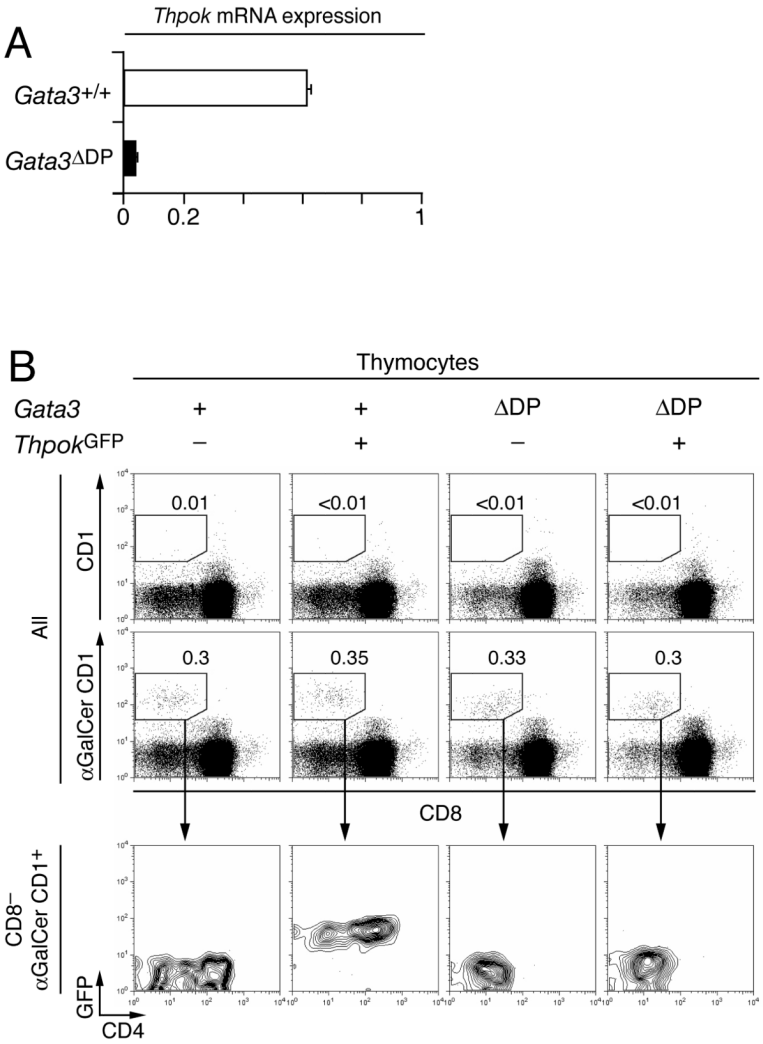


**Figure 3. Gata3 promotes *Thpok* expression in both iNKT and conventional CD4+ T cells**

(A) Expression of *Thpok* mRNA was measured by real-time RT-PCR on sorted iNKT thymocytes ($CD24^{lo}$ CD1d-$tetramer^{+}$) from $Gata3^{+/+}$ and $Gata3^{\Delta DP}$ mice. Values were normalized to β-actin expression in the same samples and are displayed relative to *Thpok* mRNA expression in conventional CD4-SP thymocytes, set to 1. Data are representative of two distinct determinations from two separate sorts. (B) iNKT cell populations are defined on two parameters plots of CD8 expression and α-Gal-ceramide-CD1d binding; the top row show absence of binding of unloaded CD1d tetramers as a specificity control (top panels). Two parameter plots show CD4 vs. GFP expression on gated iNKT cells. Note the reduced CD4 levels on $Gata3^{\Delta DP}$ iNKT thymocytes and their detectable residual GFP expression (rightmost plot). Representative of three experiments (the other two identifying iNKT thymocytes by their expression of NK.1.1).