TCR-mediated ThPOK induction promotes development of mature (CD24[−]) $\gamma\delta$ thymocytes

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T lymphocytes develop into two major lineages characterized by expression of the $\alpha\beta$ and $\gamma\delta$ T cell receptor (TCR) heterodimers. Within each major lineage, further specialization occurs, resulting in distinct subsets that differ in TCR specificity, phenotype and functional attributes. Thus, in the murine thymus, two distinct subsets of mature (CD24[−]) $\gamma\delta$ cells have been identified, that is NK1.1⁺ cells, which are enriched for V γ 1.1 usage and selectively produce IFN γ on stimulation, and CCR6⁺ cells, which are enriched for V γ 2 usage produce IL17. The upstream signals and transcriptional pathways that promote development of these distinct $\gamma\delta$ subsets remain relatively poorly understood. Here, we show that the Zn-finger transcription factor ThPOK has a critical function in the development of $\gamma\delta$ thymocytes. Thus, lack of functional ThPOK causes a marked reduction in the percentage and absolute number of mature $\gamma\delta$ thymocytes, and a particularly severe reduction of NK1.1⁺ cells. Conversely, constitutive ThPOK expression leads to a striking increase in mature NK1.1⁺ $\gamma\delta$ thymocytes. Further, we show that ThPOK induction in $\gamma\delta$ thymocytes is induced by strong TCR signals mediated by engagement with antibody or high-affinity endogenous ligands, and that an important ThPOK cis-acting element, the distal regulatory element (DRE), is sufficient for this TCR-dependent induction. These results show that ThPOK expression in $\gamma\delta$ thymocytes is regulated in part by the strength of TCR signalling, identify ThPOK as an important mediator of $\gamma\delta$ T cell development/maturation, and lend strong support to the view that development of a significant fraction of $\gamma\delta$ T cells depends on TCR engagement/signalling.

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Introduction

Most T lymphocytes undergo development in the thymus, in which they diverge into several lineages distinguished by the type and specificity of the T cell receptors (TCRs) they express, as well as their functional specializations. Developing T cells (thymocytes) progress through four stages defined according to their expression of the CD4 and CD8 surface markers, that is in order of maturity, CD4[−]CD8[−] (double negative or DN), CD4⁺CD8⁺ (double positive or DP) and CD4⁺CD8[−] or CD4[−]CD8⁺ (single positive or SP). Important lineage branch points occur at the DN and DP stages. At the DN stage, thymocytes diverge into alternate $\gamma\delta$ and $\alpha\beta$ lineages, depending on whether they express and transmit signals through the $\gamma\delta$ TCR or preTCR (an immature precursor of the $\alpha\beta$ TCR). Cells that adopt the $\gamma\delta$ lineage remain DN, whereas cells committed to the $\alpha\beta$ lineage progress to the DP stage. At the DP stage, $\alpha\beta$ thymocytes diverge again into alternate CD4 and CD8 lineages, depending on the ability of their $\alpha\beta$ TCR to recognize major histocompatibility complex (MHC) class II or class I ligands, respectively, which are expressed by antigen-presenting cells in the thymus.

Within the DN thymocyte compartment, development proceeds through additional stages defined according to the expression of the surface markers CD44 and CD25, that is in order of maturity, CD44⁺CD25[−] (DN1), CD44⁺CD25⁺ (DN2), CD44[−]CD25⁺ (DN3) and CD44[−]CD25[−] (DN4). $\gamma\delta$ TCR surface expression is initiated at the DN3>DN4 transition. Final maturation of $\gamma\delta$ DN4 thymocytes is marked by re-expression of CD44 and downmodulation of the maturation marker CD24, so that mature and immature $\gamma\delta$ thymocytes are defined by CD44⁺CD24[−] and CD44[−]CD24⁺ phenotypes, respectively. Consistent with functional maturity, CD44⁺CD24[−] $\gamma\delta$ thymocytes can proliferate and secrete cytokines in response to $\gamma\delta$ TCR engagement, whereas CD44[−]CD24⁺ $\gamma\delta$ thymocytes cannot (Haks *et al*, 2005; Haas *et al*, 2009). Mature $\gamma\delta$ thymocytes can be further subdivided into CCR6⁺ (NK1.1[−]) and NK1.1⁺ (CCR6[−]) subsets, which differ in their capacity to produce either IL17 or IFN γ cytokines, respectively (Haas *et al*, 2009; Do *et al*, 2010).

There is considerable accumulating evidence that TCR signalling has an important function in the development of $\gamma\delta$ thymocytes. First, almost all mature $\gamma\delta$ thymocytes express high levels of CD44 and many express NK1.1, both of which are activation markers that are inducible on $\gamma\delta$ thymocytes by antibody-mediated TCR crosslinking (Kreslavsky *et al*, 2009). Second, mature CD24[−] $\gamma\delta$ thymocytes display a skewed TCR V region repertoire, in particular a larger proportion of CD24[−] than CD24⁺ cells express V γ 1.1, suggesting that they have undergone selection based on their TCR specificity for intrathymic ligands (Lees *et al*, 2001). Third, thymocytes expressing a transgenic $\gamma\delta$ TCR of defined specificity, the KN6 TCR, undergo maturation to the CD24[−] stage only in the presence of their specific intrathymic

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ligand, whereas in its absence, they undergo alternative development to the $\alpha\beta$ lineage (Pereira *et al*, 1992; Haks *et al*, 2005). TCR signal strength seems to influence $\gamma\delta$ development in at least two respects: (1) as mentioned, strong TCR signals seem to promote commitment to the $\gamma\delta$ lineage at the expense of $\alpha\beta$ commitment (Hayes *et al*, 2003, 2005; Haks *et al*, 2005; Lauritsen *et al*, 2006) and (2) within the $\gamma\delta$ lineage, differentiation into specialized effector subsets seems to be controlled by differences in TCR signal strength. In particular, strong engagement by intrathymic ligands seems to promote development of IFN γ -producing $\gamma\delta$ cells (Jensen *et al*, 2008). Although TCR signalling thus has an important function in $\gamma\delta$ development, the downstream pathways and transcription factors that mediate alternate developmental signals remain to be defined.

Recently, we and others identified the Zn-finger transcription factor ThPOK as an important regulator of CD4/CD8-lineage choice by $\alpha\beta$ thymocytes (He *et al*, 2005; Sun *et al*, 2005). Thus, functional deficiency or constitutive expression of ThPOK perturbs normal lineage choice and causes $\alpha\beta$ thymocytes to develop exclusively to the CD8 or CD4 lineages, respectively (Keefe *et al*, 1999; He and Kappes, 2006). Given that both CD4 and $\gamma\delta$ development has been postulated to be controlled by quantitative differences in TCR signalling (Matechak *et al*, 1996; Haks *et al*, 2005), we reasoned that the downstream transcription pathways used in these processes might be partly overlapping. Therefore, we have explored whether ThPOK might also have a function in the development of $\gamma\delta$ thymocytes. In this study, we report that ThPOK is indeed expressed in a subset of developing $\gamma\delta$ thymocytes, and that its expression is promoted by TCR stimulation *in vitro* and correlates with TCR signal strength *in vivo*. Second, we show that ThPOK is important for development of mature (CD24 $^{-}$) $\gamma\delta$ thymocytes, particularly the NK1.1 $^{+}$ subset. These results provide strong support for the view that development of a substantial fraction of $\gamma\delta$ thymocytes depends on TCR engagement and signalling, and indicate that ThPOK is an important downstream effector of this process.

Results

ThPOK is expressed in the $\gamma\delta$ T cell lineage

We and others have earlier reported that ThPOK is selectively induced during development to the SP CD4 stage, and is not expressed by SP CD8 cells or by their common DP precursor (He *et al*, 2005; Sun *et al*, 2005). ThPOK expression in the earlier DN subset has not been specifically examined. We, therefore, used transgenic reporter lines in which GFP is expressed under the control of ThPOK regulatory elements to assess ThPOK expression in DN thymocytes at the single-cell level. DN thymocytes can be divided into three categories, according to their TCR surface expression, that is TCR^{low/-} cells, which either express no TCR or express the preTCR (which is hard to detect by surface staining), $\alpha\beta$ TCR $^{+}$ cells, which are mostly NKT cells, a sublineage of $\alpha\beta$ cells that are derived from DP precursors, and $\gamma\delta$ TCR $^{+}$ thymocytes, a distinct lineage, which diverges from the predominant $\alpha\beta$ lineage at the DN stage. There is mounting evidence that development to the $\gamma\delta$ lineage requires relatively strong TCR signals, compared with the development to the alternate $\alpha\beta$ lineage (Haks *et al*, 2005; Hayes *et al*, 2005;

Kreslavsky *et al*, 2008). Our initial analysis of ThPOK expression used the ThPOK-GFP transgene, in which reporter expression is controlled by a 12 kb ThPOK genomic fragment, including all earlier identified ThPOK control elements and promoters (Figure 1A) (He *et al*, 2008). FACS analysis of thymocytes from ThPOK-GFP reporter mice showed specific GFP expression in the CD4, but not CD8 lineages, as earlier reported (data not shown). More importantly, this analysis also revealed GFP expression in a significant fraction of DN thymocytes (Figure 1B). Most GFP $^{+}$ DN thymocytes exhibit an $\alpha\beta$ TCR $^{+}$ CD44 $^{+}$ phenotype, indicating that they likely belong to the $\alpha\beta$ NKT subset. Development and function of iNKT cells is severely perturbed in mice lacking functional ThPOK, indicating an important function for ThPOK in their differentiation (Engel *et al*, 2010). In addition, a significant fraction of $\gamma\delta$ TCR $^{+}$ thymocytes also expresses GFP (Figure 1B). Real-time RT-PCR analysis showed that endogenous ThPOK transcripts were expressed in GFP $^{+}$, but not GFP $^{-}$ $\gamma\delta$ subsets from ThPOK-GFP mice, showing that reporter expression provides an accurate reflection of ThPOK transcriptional activity in the $\gamma\delta$ lineage and is not a transgenic artefact (data not shown). Earlier, we and others have shown that a 500 bp distal regulatory element (DRE) located at the 5' end of the ThPOK locus is necessary and sufficient for CD4-lineage-specific expression of ThPOK (He *et al*, 2008; Setoguchi *et al*, 2008). To assess whether the DRE element is also sufficient for reporter expression in $\gamma\delta$ TCR $^{+}$ DN thymocytes, we used a transgenic DRE-GFP reporter line in which GFP expression is controlled by the DRE element in conjunction with a minimal hCD2 promoter (which by itself is insufficient for expression in thymocytes) (Figure 1A) (He *et al*, 2008). Significantly, we found that most mature (CD24 $^{-}$) $\gamma\delta$ TCR $^{+}$ DN thymocytes in these DRE-GFP reporter mice express the reporter, similar to ThPOK-GFP mice (Figure 1B). The fact that the DRE-GFP reporter mediates stage-specific ThPOK expression in mature $\gamma\delta$ thymocytes suggests that this small 500 bp DRE element is an important target of upstream factors that induce ThPOK in $\gamma\delta$ thymocytes. Real-time RT-PCR analysis of sorted DN thymocyte subsets from DRE-GFP mice showed that GFP $^{+}$ $\gamma\delta$ TCR $^{+}$ cells express endogenous ThPOK mRNA (in addition to the expected GFP transcripts), whereas GFP $^{-}$ $\gamma\delta$ TCR $^{+}$ thymocytes do not (Figure 1C). Hence, DRE-GFP similar to ThPOK-GFP reporter expression is regulated coordinately with endogenous ThPOK transcription. ThPOK levels in mature $\gamma\delta$ TCR $^{+}$ thymocytes are about four-fold less than for mature (CD69 $^{-}$) SP CD4 thymocytes (Supplementary Figure 1A). Of note, neither reporter construct includes ThPOK coding exons (the region detected by our ThPOK RT-PCR assay), so that ThPOK transcripts are unambiguously derived from the endogenous ThPOK locus rather than the reporter transgene. Finally, we examined whether DN cells lacking $\alpha\beta$ TCR or $\gamma\delta$ TCR expression, as well as markers of other haematopoietic lineages (Lin $^{-}$ cells) showed any reporter expression. Only some cells within the Lin $^{-}$ DN1 fraction showed GFP expression (see Supplementary Figure 1B). These GFP $^{+}$ DN1 cells are negative for ckit (CD117) expression, and so do not belong to the CD117 $^{+}$ DN1a/b subsets that are believed to be the major canonical T cell precursors (Porritt *et al*, 2004). Instead, they seem to belong to the DN1d/e subsets, which apparently do not contribute significantly to canonical T cell development, and whose lineage potential is, therefore,

Figure 1 ThPOK expression by DN thymocyte subsets. **(A)** Diagrams of ThPOK-GFP and DRE-GFP reporter constructs. White bar represents ThPOK gene, with exons and DRE indicated in solid black and horizontal stripes, respectively. **(B)** FACS analysis of GFP expression by indicated gated DN thymocyte subsets from ThPOK-GFP or DRE GFP transgenic mice (left-hand panels). Note distinct population of GFP+ CD24- cells in $\gamma\delta$ TCR+ thymocytes from reporter lines, which are absent in control mice. **(C)** Real-time RT-PCR analysis of GFP and endogenous ThPOK expression in sorted $\gamma\delta$ TCR+ thymocyte subsets from DRE-GFP transgenic mice (right panel). Note the correlation between GFP and endogenous mRNA expression. Data is derived from three DRE-GFP mice, and error bars represent s.d.

unclear and potentially heterogeneous (Porritt *et al*, 2004; Benz *et al*, 2008). Although it is formally possible that these Lin- GFP+ DN1d/e cells represent precursors of $\gamma\delta$ TCR+ GFP+ DN3/DN4 cells, this seems unlikely given that we detect no transitional GFP+ DN2 cells. Of note, because of the very low number of GFP+ DN1d/e cells, we were unable to perform RT-PCR to confirm that these cells also express endogenous ThPOK mRNA, so that it cannot be excluded that they represent a transgenic artefact.

ThPOK expression in $\gamma\delta$ thymocytes marks mostly mature/activated cells

The fact that GFP+ $\gamma\delta$ thymocytes are mostly CD24-, indicated that ThPOK expression by $\gamma\delta$ thymocytes was developmentally regulated. To explore this further, we carried out a detailed analysis of GFP expression by $\gamma\delta$ thymocyte subsets using additional cell surface markers. $\gamma\delta$ thymocytes can be divided into developmental stages based on differential surface expression of the CD24, CD44 and CD25 markers. The most immature DN thymocytes that express surface $\gamma\delta$ TCR belong to the CD24+CD44-CD25+ (DN3) stage, which then gives rise to the CD24+CD44-CD25- (DN4) stage, and finally to the CD24-CD25- (mature $\gamma\delta$) stage. The majority of mature CD24-CD25- $\gamma\delta$ thymocytes re-express CD44 and so resemble the earlier DN1 (CD44+CD25-) stage, but can be readily distinguished by expression of $\gamma\delta$ TCR and absence of CD24. Eight-colour FACS analysis of $\gamma\delta$ thymocytes from DRE-GFP reporter mice showed that the

highest level of GFP expression and the highest proportion of GFP+ cells occurred within the mature CD24-CD44+ fraction, although a significant fraction of immature CD24+CD44- cells also showed reporter expression, albeit mostly at lower levels (Figure 2A, bottom left panel). This suggests that immature GFP+ thymocytes may be precursors of mature GFP+ cells. Although most mature (CD24-) cells express the ThPOK reporter, they fall into distinct GFP^{int} and GFP^{hi} subsets (Figure 2A). Mature $\gamma\delta$ thymocytes have been separated into distinct CCR6+ and NK1.1+ lineages, which possess mutually exclusive capacities to produce IL17 and IFN γ , respectively (Haas *et al*, 2009). NK1.1 is induced on antibody-mediated TCR crosslinking of $\gamma\delta$ TCR+ DN3 cells, suggesting that NK1.1+ $\gamma\delta$ thymocytes have received a particularly strong TCR signal *in vivo* (Kreslavsky *et al*, 2008). Interestingly, CCR6+ (NK1.1-) and NK1.1+ (CCR6-) $\gamma\delta$ thymocyte subsets exhibit different GFP expression levels, that is they are predominantly GFP^{int} (intermediate) and GFP^{hi} (high), respectively (Figure 2A). RT-PCR analysis indicates that both GFP^{int} and GFP^{hi} mature $\gamma\delta$ subsets express endogenous ThPOK mRNA, but that mRNA expression is somewhat lower in GFP^{hi} cells (Figure 2B). This difference is most likely explained by the long half-life of GFP protein, which may persist/accumulate even if mRNA production levels are low or declining. We postulate that NK1.1+ cells or their immediate precursors transiently express high levels of ThPOK, which then decline, whereas CCR6+ cells express moderate levels, which remain stable.

Figure 2 ThPOK expression by $\gamma\delta$ thymocytes increases with maturation. (A) FACS analysis of $\gamma\delta$ TCR⁺ DN thymocytes from DRE-GFP mice stained with anti- $\gamma\delta$ TCR, -TCR β , -CD24, -CD44 and -NK1.1. GFP expression is shown for indicated $\gamma\delta$ TCR⁺ subsets (left-hand panels). Note that GFP expression is evident in a minority of immature (CD24⁺) $\gamma\delta$ TCR⁺ thymocytes, but a majority of mature (CD24⁺) $\gamma\delta$ TCR⁺ cells. (B) Real-time RT-PCR analysis of endogenous ThPOK expression for indicated sorted $\gamma\delta$ TCR⁺ thymocyte subsets from DRE-GFP transgenic mice (right panel). (C) FACS analysis of $\gamma\delta$ TCR⁺ DN thymocytes from DRE-GFP mice stained with anti-CD24, -CD44, -CD25 and -V γ 1.1. DN3, DN4 and mature (CD24⁺-CD44⁺) fractions were gated and reanalysed for GFP expression. V γ 1.1 expression is plotted for gated GFP⁺ and GFP⁻ subsets from each stage (right panels). Shaded histograms represent background fluorescence of equivalent gated thymocyte subsets from non-transgenic control mice.

To assess whether ThPOK expression also marks $\gamma\delta$ thymocytes expressing a distinct TCR repertoire, we compared V γ usage by GFP⁺ and GFP⁻ $\gamma\delta$ subsets. As NK1.1⁺ $\gamma\delta$ cells are reported to be enriched for V γ 1.1 usage (Azuara *et al*, 1997; Lees *et al*, 2001), we specifically examined

whether GFP⁺ $\gamma\delta$ thymocytes also showed such a preference. Indeed, among mature (CD24⁺) $\gamma\delta$ TCR⁺ thymocytes from adult mice, ~50% of GFP⁺ cells used V γ 1.1, compared with ~20% of equivalent mature GFP⁻ $\gamma\delta$ cells, indicating a significant divergence in TCR repertoire between the GFP⁺

and GFP[−] fractions (Figure 2C, bottom right and centre panels). Increased V γ 1.1 usage among ThPOK-expressing cells suggests the possibility that ThPOK transcription is triggered by $\gamma\delta$ TCR engagement, as V γ 1.1⁺ cells are thought to be enriched for self-reactive specificities (Lees *et al*, 2001; Lauritsen *et al*, 2009). Interestingly, enrichment for V γ 1.1 usage was evident in GFP⁺ cells even at the DN3 stage, the first stage at which $\gamma\delta$ TCR surface expression can be detected, consistent with a function for ThPOK early in $\gamma\delta$ development (Figure 2C, top right panel). The correlation between ThPOK expression and V γ 1.1 usage at the DN3 stage suggests that ThPOK transcription may be triggered by $\gamma\delta$ TCR engagement.

ThPOK is induced in $\gamma\delta$ thymocytes by strong TCR signals

As outlined above, the predominantly activated (CD44⁺) phenotype of ThPOK⁺ $\gamma\delta$ thymocytes as well as their increased V γ 1.1 usage both suggest that ThPOK transcription by these cells may be induced by strong TCR engagement. To directly test this hypothesis, we stimulated GFP[−] $\gamma\delta$ TCR⁺ DN3 and DN4 thymocytes from DRE-GFP mice with plate-bound anti- $\gamma\delta$ TCR antibody for 5 days. Significantly, stimulated but not untreated control cultures exhibited substantial GFP upregulation, strongly supporting a causal relationship between TCR engagement and ThPOK induction (Figure 3A). Nevertheless, it might be argued that TCR stimulation in these experiments leads to outgrowth of a minor contaminating population of GFP⁺ cells, rather than *de novo* generation of such cells from GFP[−] precursors. To exclude this possibility, we carried out similar antibody stimulation experiments using an immature DN thymic lymphoma cell line, Scid.adh (Carleton *et al*, 1999), transfected with the KN6 $\gamma\delta$ TCR (Bonneville *et al*, 1989). Antibody-mediated TCR stimulation of these KN6.Scid.adh cells leads to induction of the activation marker CD69 and downmodulation of CD25 consistent with partial differentiation, as earlier reported (Carleton *et al*, 1999). Importantly, these cells also exhibit dose-dependent upmodulation of ThPOK (Supplementary Figure 2). As KN6.Scid.adh cells are clonally derived and, therefore, unlikely to exhibit any pre-existing heterogeneity in ThPOK expression, this result provides compelling evidence for *de novo* induction of ThPOK in response to TCR stimulation. To test whether ThPOK expression *in vivo* also depends on TCR signalling, we used transgenic mice expressing the KN6 $\gamma\delta$ TCR, on either H-2^d or H-2^b backgrounds. The KN6 $\gamma\delta$ TCR represents one of the few examples of $\gamma\delta$ TCRs for which the ligand is known and in which ligand affinity can be modulated. In particular, the KN6 $\gamma\delta$ TCR recognizes the non-classical MHC class Ib products T10^d and T10^b with relatively low and high affinity, respectively (Adams *et al*, 2008). FACS and RT-PCR analysis of KN6⁺ thymocytes shows striking differences in ThPOK expression between the H-2^d and H-2^b backgrounds. In the presence of the weak T10^d ligand, the majority of KN6⁺ thymocytes, including mature (CD24[−]) thymocytes express low or undetectable levels of ThPOK, as assessed by DRE-GFP reporter expression or by RT-PCR analysis of endogenous ThPOK mRNA (Figure 3B and C). Only a minor NK1.1⁺ CD44⁺ CD24[−] population, comprising <0.5% of total $\gamma\delta$ cells, shows substantial ThPOK expression by RT-PCR (data not shown). In contrast, in the presence of the strong T10^b ligand, most KN6⁺ thymocytes exhibit GFP reporter expression, at both immature (DN3,

DN4) and mature stages, showing a clear correlation between high affinity of $\gamma\delta$ TCR for endogenous ligand and ThPOK induction (similar results were obtained with both DRE-GFP and ThPOK-GFP mice) (Figure 3B and data not shown). To conversely test the consequences of impaired TCR signalling on ThPOK induction in $\gamma\delta$ thymocytes, we used mice lacking Id3 (Pan *et al*, 1999). Id3 is an inhibitor of E proteins that is activated by TCR signalling and promotes thymocyte survival and proliferation (Bain *et al*, 2001; Xi *et al*, 2006). We have earlier shown that Id3 is important for mediating strong TCR signals that promote $\gamma\delta$ development (Haks *et al*, 2005; Lauritsen *et al*, 2009). RT-PCR analysis of sorted $\gamma\delta$ thymocyte subsets from Id3^{−/−} versus wt mice, showed that ThPOK mRNA levels were significantly reduced in mature $\gamma\delta$ thymocytes from Id3^{−/−} mice, supporting a function for Id3 in ThPOK induction in $\gamma\delta$ cells (Supplementary Figure 3A). As earlier shown, the V γ repertoire in Id3^{−/−} mice is skewed dramatically towards usage of V γ 1.1, which may reflect rescue of these putatively autoreactive cells from negative selection (Lauritsen *et al*, 2009; Ueda-Hayakawa *et al*, 2009) (Supplementary Figure 3B). Overall, the above results provide compelling evidence that ThPOK induction in DN thymocytes is mediated by TCR engagement, and that it requires relatively strong stimuli.

Function of ThPOK in $\gamma\delta$ development

To investigate whether ThPOK has any function in $\gamma\delta$ development, we next assessed $\gamma\delta$ thymocyte proportions and surface marker expression in mice lacking functional ThPOK (ThPOK^{HD/HD} or HD/HD mice) (Davé *et al*, 1998). Note that HD/HD mice are congenic for the HD mutation (backcrossed for 20 generations to the C57BL/6 background) and are maintained through HD/HD $\times$ HD/+ intercrosses to produce age-matched HD/HD and HD/+ littermates of a uniform genetic background. FACS analysis of adult HD/HD and HD/+ mice shows that proportions and absolute number of total $\gamma\delta$ thymocytes are similar, but that HD/HD mice exhibit a marked three-fold reduction of mature (CD24[−]) $\gamma\delta$ thymocytes (Figure 4A, top left panels). As mentioned, mature CD24[−] $\gamma\delta$ thymocytes have been subdivided into distinct CCR6⁺ and NK1.1⁺ lineages (Lees *et al*, 2001; Haas *et al*, 2009). Absolute numbers of both subsets are reduced in HD/HD mice, but the NK1.1⁺ subset is affected most severely, such that the ratio of CCR6⁺ versus NK1.1⁺ cells is reversed (Figure 4A and B). As noted above, both CCR6⁺ and NK1.1⁺ $\gamma\delta$ subsets express ThPOK, as measured by DRE-GFP reporter expression, but the NK1.1⁺ subset is enriched for cells expressing highest GFP levels. The GFP^{hi} subset was severely depleted in mature $\gamma\delta$ thymocytes from HD/HD mice, consistent with the preferential loss of NK1.1⁺ thymocytes (Supplementary Figure 4). Analysis of V γ region usage at the mature (CD24[−]) stage showed a significant decrease in V γ 1.1 usage in HD/HD mice, indicative of a shift in the TCR repertoire (20% V γ 1.1⁺ cells in HD/HD mice, compared with 45% in HD/+ littermates) (Figure 4A, right panels). By comparison, the relative usage of V γ 2 by mature $\gamma\delta$ thymocytes is slightly increased in HD/HD mice (45% of mature $\gamma\delta$ thymocytes in HD/HD mice, compared with 38% in HD/+ littermates), although the absolute number of mature V γ 2⁺ cells is also significantly decreased (Supplementary Figure 5). We further examined the effect of ThPOK deficiency on $\gamma\delta$ development

Figure 3 ThPOK is induced in $\gamma\delta$ thymocytes by TCR signalling. (A) $\gamma\delta$ TCR+ GFP- DN thymocytes were isolated from DRE-GFP mice, according to sort gate at left, cultured for 5 days on OP9-DL1 cells in the presence or absence of anti- $\gamma\delta$ TCR and reanalysed by FACS for expression of GFP, $\gamma\delta$ TCR and -NK1.1 (right-hand panels). GFP expression is shown for gated $\gamma\delta$ TCR+ subsets after culture. (B) Analysis of ThPOK expression by $\gamma\delta$ TCR+ DN thymocytes from H-2^{d/d} or ^{b/d} mice carrying the KN6 $\gamma\delta$ TCR transgene. Thymocytes from DRE-GFP+ mice were stained with anti- $\gamma\delta$ TCR, -TCR β , -CD24, -CD44 and -CD25, and GFP expression was assessed in the indicated gated subsets (left-hand panels). Red and green histograms represent equivalent gated populations from transgenic and non-transgenic mice, respectively. (C) Levels of endogenous ThPOK mRNA were assessed in indicated sorted $\gamma\delta$ TCR+ subsets from KN6+ mice H-2^{d/d} or ^{b/d} mice (not carrying the DRE-GFP transgene) by real-time RT-PCR analysis (right panel).

Figure 4 ThPOK deficiency impairs $\gamma\delta$ thymocyte maturation. **(A)** FACS analysis of $\gamma\delta$ TCR+ DN thymocytes from 1-month-old HD/HD and HD/+ littermates, showing expression of CD24, CD44, NK1.1, CCR6, V γ 1.1 and V γ 2 for indicated gated subsets. **(B)** Absolute numbers of indicated $\gamma\delta$ TCR+ thymocyte subsets from 6-week-old HD/HD ($n=4$), HD/+ ($n=5$) and ThPOK^{const} mice ($n=5$). Error bars indicate s.d.

early in ontogeny, that is in neonates. Consistent with adults, there was a severe reduction in the overall number of mature $\gamma\delta$ thymocytes. Most $\gamma\delta$ thymocytes in wt neonates express the V γ 2 segment, and ~40% of V γ 2+ thymocytes are mature. Similarly, in HD/HD neonates, most $\gamma\delta$ thymocytes express V γ 2, but only ~20% of these cells belong to the mature CD24- fraction (Supplementary Figure 6). Hence, the effect of ThPOK deficiency on $\gamma\delta$ development is already apparent early in ontogeny, when there has been little/no

opportunity for post-maturation outgrowth of particular $\gamma\delta$ subsets. Finally, we examined the effect of ThPOK deficiency on development of thymocytes expressing a single $\gamma\delta$ TCR specificity, that is the KN6 $\gamma\delta$ TCR on the H-2^b background (in which ThPOK is highly induced). A high proportion of KN6+ H-2^b HD/+ thymocytes develop to the mature (CD24-) stage, and a significant number accumulate in the periphery, consistent with earlier studies (Supplementary Figure 7, right panels) (Pereira *et al*, 1992). In the absence of functional

Figure 5 Constitutive ThPOK expression promotes $\gamma\delta$ thymocyte maturation. (A) FACS analysis of total DN or $\gamma\delta$ TCR + DN thymocytes from 1-month-old ThPOK^{const} and wt littermates stained with anti- $\gamma\delta$ TCR, -TCR β , -CD24, -CD44, -NK1.1 and -CCR6. (B) FACS analysis of $\gamma\delta$ TCR + DN thymocytes from 6-week-old ThPOK^{const} and wt littermates, stained with anti-V γ 1.1, -NK1.1 and -CD24. (C) FACS analysis of total lymph node or spleen cells from ThPOK^{const} and wt littermates stained with anti- $\gamma\delta$ TCR and -TCR β .

ThPOK, both the proportions of mature KN6 + thymocytes as well as peripheral KN6 + cells are greatly reduced, indicating a severe defect in development (Supplementary Figure 7, left panels). Overall, these results show that ThPOK has an important physiological function in promoting development of diverse $\gamma\delta$ subsets including endogenous V γ 1.1 + and V γ 2 + subsets, as well as KN6 transgenic thymocytes.

Given that ThPOK deficiency impairs normal $\gamma\delta$ development, we reasoned that ectopic ThPOK expression might conversely promote $\gamma\delta$ maturation. To test this, we used a transgenic line expressing ThPOK constitutively during T cell development under the control of the CD4 promoter/enhancer (ThPOK^{const} mice). Of note, although the CD4 enhancer/promoter construct is predicted to initiate transcription at the DN4/DP transition, expression of this ThPOK^{const} transgene is already detected at the DN2 stage (the reason for this unexpectedly early expression pattern is unknown, but presumably depends on the founder-specific transgene integration site) (Supplementary Figure 8A). Significantly, ThPOK^{const} mice exhibit a five to seven-fold increase in the proportion of mature (CD24–CD44 +) thymocytes most of which exhibit an NK1.1 + V γ 1.1 + phenotype, supporting an

important function for ThPOK in development, proliferation and/or survival of mature $\gamma\delta$ thymocytes, and of the NK1.1 + $\gamma\delta$ subset in particular (Figures 4B and 5A–C). The proportion of $\gamma\delta$ cells in the spleen and lymph nodes of ThPOK^{const} mice was also increased, although the absolute number of cells in the spleen was not (Figures 5C and 6C). Interestingly, some $\gamma\delta$ TCR + thymocytes and a substantial proportion of peripheral $\gamma\delta$ cells express CD4, suggesting that persistent/increased ThPOK expression can lead to adoption of some features of the CD4-lineage commitment programme by $\gamma\delta$ cells (Supplementary Figure 8C). In ThPOK^{const} mice carrying the KN6 $\gamma\delta$ TCR transgene in the presence of the low-affinity T10^d ligand, CD44 and NK1.1 expression was significantly upmodulated in both immature and mature $\gamma\delta$ subsets, suggesting a function in thymocyte activation (data not shown). In view of the significant effects of ThPOK deficiency and ThPOK overexpression on $\gamma\delta$ thymocyte proportions and V γ usage, we conclude that ThPOK is, in fact, an important regulator of development, survival and/or proliferation of these subsets.

To test whether ThPOK affects proliferation of $\gamma\delta$ thymocytes, we carried out *in vivo* BrdU labelling of wt, HD/HD and

Figure 6 Effect of ThPOK deficiency on peripheral $\gamma\delta$ T cell subsets. **(A)** FACS analysis of total or gated $\gamma\delta$ TCR+ splenocytes, as indicated, from HD/HD, HD/+ and ThPOK^{const} mice, showing expression of NK1.1, CCR6 and DRE-GFP reporter. **(B)** FACS analysis of $\gamma\delta$ TCR+ splenocytes, showing V γ 2 expression for indicated gated NK1.1/CCR6 subsets. **(C)** Absolute numbers of indicated $\gamma\delta$ TCR+ splenic subsets from 1-month-old HD/HD ($n=4$), HD/+ ($n=5$) and ThPOK^{const} mice ($n=5$). Error bars indicate s.d.

ThPOK^{const} thymocytes. After a short BrdU pulse, the amount of incorporation by $\gamma\delta$ thymocytes was found to correlate inversely with the level of maturity, that is $\gamma\delta$ TCR+ DN3 cells exhibited greatest incorporation, whereas $\gamma\delta$ TCR+ DN4 and mature (CD24-CD44+) $\gamma\delta$ TCR+ cells exhibited progressively less (Supplementary Figure 9A, left-hand panels). Only after a longer chase period did any label move into the mature fraction. Of note, there was no consistent difference in the proportion of BrdU-labelled $\gamma\delta$ TCR+ DN3 or DN4 cells between wt, HD/HD and ThPOK^{const} mice, suggesting that ThPOK has little effect on proliferation of these cells. The fact that mature $\gamma\delta$ TCR+ cells took up virtually no label in these short pulse experiments indicates that they proliferate very slowly. We tested whether extended

BrdU treatment would eventually lead to BrdU incorporation by mature $\gamma\delta$ TCR+ cells because of recruitment of cells labelled at earlier stages. Indeed, after 7 days of continuous BrdU treatment, 15–20% of mature $\gamma\delta$ TCR+ cells became labelled (Supplementary Figure 9A, right-hand panels). Again, there was no difference in the percentage of labelled mature cells in HD/HD or ThPOK^{const} mice with respect to wt controls, suggesting that turnover, that is the balance between recruitment and egress of cells from the mature $\gamma\delta$ TCR+ thymocyte compartment, is not significantly altered in these mice. We infer that ThPOK exerts its effect on an early developmental checkpoint that controls the rate at which $\gamma\delta$ cells progress to the mature stage, rather than at the level of mature $\gamma\delta$ cell proliferation.

ThPOK deficiency alters peripheral $\gamma\delta$ subset representation

Comparison of splenocytes from HD/+ and HD/HD mice revealed similar absolute numbers of $\gamma\delta$ cells, virtually all of which express low CD24 levels (Figure 6C; data not shown). However, the distribution of $\gamma\delta$ subsets, as defined by expression of NK1.1 and CCR6 markers, was markedly altered. In particular, the absolute number of CCR6+ $\gamma\delta$ cells was increased two to three-fold in HD/HD mice, whereas the number of CCR6– NK1.1– $\gamma\delta$ cells was reduced (Figure 6B and C). Splenic CCR6+ $\gamma\delta$ cells, such as CCR6+ $\gamma\delta$ thymocytes, exhibit almost exclusive usage of V γ 2 on both HD/+ and HD/HD backgrounds (Figure 6B). It, therefore, seems unlikely that the increase in CCR6+ $\gamma\delta$ splenocytes in HD/HD mice is due to diversion of cells from the NK1.1+ or NK1.1– CCR6– fractions, as the latter populations include much lower proportions of V γ 2+ cells. We also examined representation of CCR6+ and NK1.1+ $\gamma\delta$ subsets in the spleen of ThPOK^{const} mice. Interestingly, the number of NK1.1+ $\gamma\delta$ splenocytes was markedly reduced compared with wt mice, suggesting that constitutive expression of ThPOK may inhibit survival/expansion of these cells in the spleen (Figure 6C). Most NK1.1+ $\gamma\delta$ splenocytes from wt mice are GFP^{lo/-}, indicating that they normally downregulate ThPOK, consistent with the possibility that continued ThPOK expression is deleterious to persistence of NK1.1+ $\gamma\delta$ splenocytes (Figure 6A). Given that NK1.1+ and CCR6+ $\gamma\delta$ subsets have been shown to preferentially secrete IFN γ or IL17, respectively, on stimulation (Haas *et al*, 2009; Martin *et al*, 2009), we assessed whether this was also the case in HD/HD and ThPOK^{const} mice. In both kinds of mice, IFN γ and IL17 production remained largely restricted to NK1.1+ and CCR6+ $\gamma\delta$ subsets, respectively, indicating that acquisition of these effector functions is not regulated by ThPOK (Supplementary Figure 9B). In addition, the fraction of cells expressing each cytokine and the levels of expression were similar to wt mice.

CD122 and CD25 have also been used to distinguish different functional $\gamma\delta$ subsets in the periphery, including in the peritoneal cavity (Jensen *et al*, 2008; Shibata *et al*, 2008). CD122 and CD25 seem to identify similar, though not necessarily completely overlapping, $\gamma\delta$ subsets to those marked by NK1.1 and CCR6 expression, respectively. Thus, NK1.1+ and CCR6+ $\gamma\delta$ thymocytes have been shown to be largely CD122+ and CD122–, respectively (Haas *et al*, 2009), and peripheral CD122+ and CD122– (CD25+) subsets preferentially produce IFN γ and IL17A, respectively (Jensen *et al*, 2008; Shibata *et al*, 2008), similar to NK1.1+ and CCR6+ subsets. Analysis of peritoneal cavity $\gamma\delta$ cells in HD/HD mice revealed that the relative proportion and absolute number of CD122+ $\gamma\delta$ cells was selectively reduced, paralleling the reduction in NK1.1+ $\gamma\delta$ thymocytes in these mice (Supplementary Figure 10). These changes could reflect developmental or post-developmental effects occurring in the periphery.

Discussion

Although TCR engagement and signalling are widely acknowledged to have a critical function in $\alpha\beta$ T cell development, that is in thymic selection and CD4/CD8-lineage commitment, it remains controversial whether this is also

true for $\gamma\delta$ development. Direct evidence that TCR engagement and signalling can promote $\gamma\delta$ T cell development comes from studies showing that thymocytes expressing a transgenic $\gamma\delta$ TCR of defined specificity become functionally mature only in the presence of specific ligand (Haks *et al*, 2005). However, as endogenous ligands have not been identified for most $\gamma\delta$ thymocytes, it is unclear whether these findings are applicable to $\gamma\delta$ thymocytes in general. Furthermore, the transcriptional pathways that control $\gamma\delta$ development remain poorly characterized. Here, we provide evidence that the Zn-finger transcription factor ThPOK is induced by TCR signalling and promotes $\gamma\delta$ maturation in the thymus, and thus represents an important effector of TCR-mediated $\gamma\delta$ development.

We present two observations in support of a mechanistic link between TCR engagement on $\gamma\delta$ thymocytes and ThPOK induction. First, ThPOK is induced *in vitro* by strong antibody-mediated TCR stimuli both in primary $\gamma\delta$ thymocytes and in a $\gamma\delta$ TCR-expressing DN cell line. Second, ThPOK is expressed *in vivo* by almost all KN6 $\gamma\delta$ TCR transgenic thymocytes in the presence of the high-affinity T10^b ligand. The fact that KN6 thymocytes express ThPOK only in the presence of the high-affinity T10^b, but not the lower-affinity T10^d ligand, implies that ThPOK induction by $\gamma\delta$ thymocytes requires relatively strong TCR signals. This resembles control of ThPOK during $\alpha\beta$ development, in which ThPOK is induced preferentially in cells expressing class II-restricted TCRs, which are believed to transmit stronger TCR signals than class I-restricted TCRs (He *et al*, 2005). Our results indicate a direct causal relationship between TCR signalling and ThPOK induction in $\gamma\delta$ thymocytes, although it cannot be excluded that other non-TCR-mediated signals also contribute to ThPOK induction.

Our results further show that ThPOK has a critical function in controlling $\gamma\delta$ development, particularly of cells with high reactivity to thymic self-ligands. First, the proportion of mature (CD24–) $\gamma\delta$ thymocytes is severely reduced in HD/HD mice, and conversely greatly increased in ThPOK^{const} mice. Particularly affected in both kinds of mice are NK1.1+ cells, which are believed to be enriched for self-reactive specificities (Lees *et al*, 2001). Second, the V γ repertoire of mature $\gamma\delta$ thymocytes in both kinds of mice is significantly altered compared with wt littermates. In particular, the proportion of V γ 1.1+ cells within the mature $\gamma\delta$ thymocyte compartment is severely reduced in adult HD/HD mice and conversely increased in ThPOK^{const} mice. In DRE-GFP mice, the $\gamma\delta$ repertoire is already skewed towards V γ 1.1 usage in ThPOK-expressing DN3 cells, consistent with the idea that ThPOK affects $\gamma\delta$ selection from the earliest point at which surface $\gamma\delta$ TCR is expressed.

In the light of our evidence that ThPOK is induced in response to strong TCR signals, it may seem counter-intuitive that ThPOK is still expressed by $\gamma\delta$ thymocytes from Id3–/– mice, even though Id3 deficiency severely impairs development of KN6 thymocytes and DETC $\gamma\delta$ cells (Lauritsen *et al*, 2009). Significantly, mature $\gamma\delta$ thymocytes from Id3–/– mice almost all express the V γ 1.1 segment. The same phenomenon occurs in CD3 δ –/– mice in which TCR signalling is impaired by diminishing TCR surface expression (Park K and Kappes DJ, unpublished data). We suspect that preferential development of V γ 1.1+ cells in these situations

reflects a selective advantage for strongly self-reactive specificities, which seem to be disproportionately represented within the V γ 1.1+ fraction. High-affinity $\gamma\delta$ TCRs could compensate for downstream signalling deficiencies, for instance, by sustaining longer signals, and thus still promote ThPOK induction. Although no self-ligands for V γ 1.1+ cells have yet been identified, there is circumstantial evidence that cellular selection controls their development (Azuara *et al*, 1998). Furthermore, many V γ 1.1 thymocytes exhibit an activated CD44+ NK1.1+ phenotype, resembling $\alpha\beta$ NKT cells, which are well known to be selected by thymic self-antigens (Kronenberg and Engel, 2007). Hence, one might speculate that V γ 1.1+ cells developing on a signalling deficient background are particularly high-affinity cells, perhaps including cells that would normally undergo negative selection.

Although ThPOK+ (GFP+) $\gamma\delta$ thymocytes are enriched for V γ 1.1 usage (~50 versus ~30% among total $\gamma\delta$ cells), a large proportion of ThPOK+ cells express V γ 2 or other V γ segments, and in the absence of functional ThPOK, both mature V γ 1.1+ and V γ 2+ subsets are diminished. In contrast, another POK family factor found in $\gamma\delta$ thymocytes, Plzf, seems to be expressed almost exclusively by V γ 1.1+ thymocytes (Kreslavsky *et al*, 2009; Alonzo *et al*, 2010). Although the proportion of mature (CD24-) $\gamma\delta$ thymocytes is dramatically reduced in the absence of functional ThPOK, a significant number of mature $\gamma\delta$ thymocytes still develop, indicating that ThPOK is not absolutely required for $\gamma\delta$ cell development. Furthermore, KN6 thymocytes developing in the presence of the lower-affinity T10^d ligand do not express detectable ThPOK. Taken together, these observations suggest that a subset of $\gamma\delta$ thymocytes that recognizes ligand with lesser affinity does not require ThPOK for maturation. This raises the interesting question of why higher-affinity $\gamma\delta$ thymocytes would require ThPOK for their development. One possibility is that ThPOK expression protects these cells from negative selection.

Although, representation of mature $\gamma\delta$ thymocytes is drastically impaired by ThPOK deficiency, there is no corresponding decrease in total peripheral $\gamma\delta$ cell numbers in HD/HD mice. There are at least two possible, non-mutually exclusive, explanations for this observation: (1) although the rate of export of mature $\gamma\delta$ thymocytes is likely reduced in HD/HD mice, remaining emigrants may undergo homeostatic expansion in the periphery to fill available niches, and (2) immature $\gamma\delta$ thymocytes, which are not diminished in HD/HD mice, may be exported from the thymus and undergo post-thymic development in the periphery. Regarding the latter possibility, it remains unclear whether peripheral $\gamma\delta$ cells are descended mostly from mature or immature thymic precursors. *In vivo* labelling studies indicate that the majority of $\gamma\delta$ thymocytes that have recently entered the periphery (recent thymic emigrants or RTEs) exhibit a CD24+ phenotype, and undergo CD24 downmodulation only after arriving in the periphery (Kelly *et al*, 1993; Tough and Sprent, 1998), in contrast to $\alpha\beta$ RTEs, which are mostly CD24- (Kelly and Scollay, 1990). Further, it has been argued that the rate of production of CD24- $\gamma\delta$ thymocytes is too low to account for the appearance of new $\gamma\delta$ cells in the periphery (Zorbas and Scollay, 1993). Nevertheless, as $\gamma\delta$ RTEs are not uniformly CD24+ and, in fact, contain a substantial CD24-/lo fraction (Kelly *et al*, 1993; Zorbas

and Scollay, 1993), and as a high proportion of CD24+ RTEs seem to die off soon after reaching the periphery (Tough and Sprent, 1998), the relative contribution of CD24+ and CD24- RTEs to the long-lived peripheral $\gamma\delta$ pool remains difficult to estimate. Regardless, of the precise developmental route by which peripheral $\gamma\delta$ cells arise, they exhibit significant differences in subset distribution in ThPOK-deficient mice, consistent with an important function for ThPOK in development of these cells as well. In particular, they display a marked increase in the proportion of CCR6+ cells. Although CCR6+ cells from HD/HD mice are essentially all V γ 2+, as in wt mice, it will be interesting to assess in future whether they exhibit more subtle changes in TCR repertoire at the level of junctional diversity and/or V δ usage.

The present results considerably expand the known functions of ThPOK in controlling T lymphoid development and function. Three distinct functions have now been reported: (1) promoting CD4-lineage commitment at the expense of CD8 commitment (He *et al*, 2005; Sun *et al*, 2005), (2) promoting clonal expansion and functional responses of CD8 T cells during acute viral infection (Setoguchi *et al*, 2009), and (3) promoting maturation of a subset of $\gamma\delta$ thymocytes with high affinity for intrathymic ligands, as shown here. In all three situations, it seems that ThPOK induction occurs in response to a relatively strong TCR stimulus, as mimicked by antibody-mediated TCR crosslinking (He *et al*, 2008; Setoguchi *et al*, 2009), arguing that this is a general mechanism governing ThPOK expression in T lymphocytes. Strikingly, the DRE element is sufficient to mediate appropriate stage-specific ThPOK induction both during CD4 and $\gamma\delta$ development (He *et al*, 2008), arguing that ThPOK transcriptional control is regulated similarly in these two situations. Clearly, however, downstream outcomes in terms of lineage-specific gene expression programme are quite different for each subset. This could reflect different kinetics/levels of ThPOK induction, cell-intrinsic differences in transcriptional regulatory networks and/or differences in environmental cues and co-stimulatory signals.

During the submission process of this manuscript, a report appeared, which also showed expression of ThPOK in a significant fraction of $\gamma\delta$ thymocytes and splenocytes (Alonzo *et al*, 2010). This study focused primarily on development of a small subset of $\gamma\delta$ cells defined by expression of the V γ 1.1/V δ 6.3 heterodimer. Although many V γ 1.1+ V δ 6.3+ splenocytes showed high or intermediate expression levels of ThPOK, the frequency of such cells was apparently not significantly diminished by the absence of ThPOK. This is consistent with our analysis, which shows that ThPOK deficiency primarily affects maturation and subset distribution of $\gamma\delta$ thymocytes, but has more subtle effects on peripheral $\gamma\delta$ cells.

The precise function of ThPOK in $\gamma\delta$ development remains to be elucidated. The present data suggests that ThPOK acts early in the development of $\gamma\delta$ thymocytes to drive their maturation and/or effector differentiation, rather than late in the development to promote survival/expansion of mature $\gamma\delta$ thymocytes. Future experiments to compare the developmental potential of immature $\gamma\delta$ TCR+ thymocytes from ThPOK deficient and wt thymocytes at the single-cell level may clarify these questions.

Materials and methods

Mice

HD/HD (Davé *et al*, 1998), ThPOK-GFP (He *et al*, 2008) (referred to as F2F3), DRE-GFP (He *et al*, 2008) (referred to as F2c-pCD2), KN6 transgenic (Ishida *et al*, 1990) and Id3^{-/-} mice (Pan *et al*, 1999) have been earlier described. ThPOK^{const} mice were generated by inserting wild-type ThPOK cDNA into a pCD4 vector consisting of the CD4 promoter and proximal enhancer (Ellmeier *et al*, 1999). Animal care was in accordance with National Institutes of Health (NIH) guidelines.

TCR stimulation assays

For *in vitro* stimulation of thymocytes, $2-5 \times 10^5$ sorted thymocytes were cultured on OP9-DL1 monolayers in DMEM with 10% FCS on 96-well plates, as described (Haks *et al*, 2005). Plates were untreated or precoated with 0.02 $\mu\text{g}/\text{ml}$ anti- $\gamma\delta$ TCR (UC7-13D5; BD-Pharmingen), as indicated. For *in vitro* stimulation of KN6.Scid.adh cells, 1×10^6 cells were cultured overnight in 96-well plates that were untreated or precoated with anti-CD3e (2C11) or - $\gamma\delta$ TCR (UC7-13D5; BD Pharmingen) at the indicated concentration. The KN6.Scid.adh cell line was generated by retroviral transduction of Scid.adh cells with the KN6 TCR γ and TCR δ subunits connected by the 2A Tescovirus linker peptide, as described (Lauritsen *et al*, 2009).

Flow cytometry

Cells were prepared from thymus, and other lymphoid organs, and analysed by flow cytometry according to standard procedures. All antibodies were obtained commercially (from BioLegend, eBioscience, BD-PharMingen, or R&D Systems), except for anti-V γ 1.1 (kindly provided by R O'Brien, NJH, Denver).

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Intracellular cytokine assays

For measurement of intracellular cytokine levels, sorted CCR6+ and NK1.1+ $\gamma\delta$ subsets were cultured for 4 h in Iscove's MDM (with 10% FCS and 1000 IU/ml human rIL2) in the presence of 50 ng/ml PMA and 2 $\mu\text{g}/\text{ml}$ ionomycin, and for an additional 3 h in the presence of GolgiStop protein transport inhibitor (BD). Treated cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD), and stained with either anti-IFN γ or -IL17A antibodies.

Real-time RT-PCR

Real-time RT-PCR analysis for ThPOK and EGFP was carried out according to the probe-based method and analysed by the comparative Ct method (compared with β -actin). Primer and probe sequences are available on request.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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