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Prinz, I., Sansoni, A., Kissenpfennig, A., Ardouin, L., Malissen, M., & Malissen, B. (2006). Visualization of the earliest steps of gammadelta T cell development in the adult thymus. *Nature Immunology*, 7(9)(9), 995-1003. <https://doi.org/10.1038/ni1371>

Published in:
Nature Immunology

Document Version:
Early version, also known as pre-print

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Visualization of the earliest steps of $\gamma\delta$ T cell development in the adult thymus

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The checkpoint in $\gamma\delta$ cell development that controls successful T cell receptor (TCR) gene rearrangements remains poorly characterized. Using mice expressing a reporter gene 'knocked into' the *Tcrd* constant region gene, we have characterized many of the events that mark the life of early $\gamma\delta$ cells in the adult thymus. We identify the developmental stage during which the *Tcrd* locus 'opens' in early T cell progenitors and show that a single checkpoint controls $\gamma\delta$ cell development during the penultimate CD4⁺CD8⁺ stage. Passage through this checkpoint required the assembly of $\gamma\delta$ TCR heterodimers on the cell surface and signaling via the Lat adaptor protein. In addition, we show that $\gamma\delta$ selection triggered a phase of sustained proliferation similar to that induced by the pre-TCR.

In mice, most T cells express T cell receptors (TCRs) consisting of α - and β -chains, whereas a minor population expresses an alternative form made of γ - and δ -chains. Based on expression of CD25 and CD44, the least mature double-negative (DN) CD4⁺CD8⁺ thymocytes can be organized according to the following maturation sequence: CD44⁺CD25⁺ (DN1) → CD44⁺CD25⁺ (DN2) → CD44^{neg-lo}CD25⁺ (DN3) → CD44⁺CD25⁺ (DN4). DN4 cells give rise to double-positive (DP) CD4⁺CD8⁺ thymocytes, a few of which develop further into single-positive CD4⁺CD8⁺ or CD4⁺CD8⁺ cells that migrate to the periphery.

Genetic studies have defined two sequential checkpoints at which developing $\alpha\beta$ cells undergo programmed cell death if they fail to fulfill specific requirements¹. Transition through the earliest checkpoint requires assembly of the pre-TCR. This 'molecular sensor' ensures that only those DN3 cells containing productive *Tcrb* rearrangements develop into DN4 and DP thymocytes. Because *Tcrd* rearrangements occur after transition to the DP stage, the pre-TCR lacks a TCR α chain. It is composed of a pre-TCR α -TCR β heterodimer and of CD3 signaling subunits. The phenotypic transition induced by the pre-TCR is referred to as 'TCR β selection' and is associated with an intense phase of cell proliferation. The earliest β -selected thymocytes correspond to a minor subset of DN3 cells called 'DN3L cells' and are larger than their precursors, which are called 'DN3E cells'. Preselection DN3E cells are noncycling and contain random *Tcrb* rearrangements, whereas β -selected DN3L cells are cycling and enriched in productive *Tcrb* rearrangements². DP cells assemble a second molecular sensor consisting of a TCR α -TCR β heterodimer and of CD3 subunits. Successful differentiation into single-positive cells requires low-affinity interactions between TCR $\alpha\beta$ heterodimers and

self peptides bound to major histocompatibility complex molecules. This last developmental transition is known as positive selection.

Knowledge of $\gamma\delta$ cell development remains limited³. *Tcrb*, *Tcrd* and *Tcrd* rearrangements are postulated to occur simultaneously during DN2 and DN3 stages, and most $\gamma\delta$ cells are DN and probably diverge from $\alpha\beta$ T cell progenitors before the upregulation of CD4 and CD8 expression. Mice lacking *Tcrb* (*Tcrb*^{-/-} mice), which are incapable of β -selection, have fewer DN3L cells. However, mice lacking both *Tcrb* and *Tcrd* have even fewer DN3L cells, suggesting that a small fraction of DN3L cells can be generated by expression of TCR δ chains, and such δ -selected cells probably give rise to mature TCR $\gamma\delta$ ⁺ DN4 cells⁴. Consistent with that view, DN4 cells in *Tcrb*^{-/-} mice are enriched in productive *Tcrd* rearrangements, whereas these rearrangements are random in DN3 cells^{4,5}.

Evidence suggests that in contrast to β -selection, δ -selection does not result in extensive proliferation of the selected cells⁴. Also at variance with the idea that $\gamma\delta$ cells encounter a developmental checkpoint at the DN3 stage is the observation that only 7–10% of DN4 cells contain intracellular TCR $\gamma\delta$ heterodimers⁶. Provided that such cells constitute the immediate precursors of mature DN4 $\gamma\delta$ cells, those results, together with results published before⁷, suggest that $\gamma\delta$ -selection takes place at the DN4 stage and not at the DN3 stage. Therefore, the available data leave open the issue of whether the checkpoint thought to control $\gamma\delta$ cell development operates at the DN3 or DN4 stage.

Because there is no phenotypic marker other than TCR $\gamma\delta$ itself for tracking thymocytes committed to the $\gamma\delta$ lineage, delineation of $\gamma\delta$ cell development has relied mainly on monitoring of the expression of TCR $\gamma\delta$ heterodimers using antibodies that recognize epitopes

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Received 30 May; accepted 6 July; published online 30 July 2006; doi:10.1038/ni1371

Figure 1 Characterization of *Tcrd-H2BEGFP* mice. (a) *Tcrd-H2BEGFP* construct, containing an IRES-driven reporter cassette (I) encoding an H2B (H)–EGFP (G) fusion protein in the 3' untranslated region of *Tcrd*. To prevent any potential interference with rearrangement and expression of the targeted *Tcrd* allele, the 'knock-in' approach is limited to insertion of the IRES-driven cassette and of a residual *loxP* site (triangle) in exon C4 of *Tcrd*. Filled boxes, exons C1–C3. (b) Fluorescence microscopy of a small intestinal villus section stained with anti-B220 (red) and DAPI (4',6-diamidino-2-phenylindole; blue). Original magnification, $\times 100$. (c) Flow cytometry of IELs prepared from wild-type (gray filled histogram) and *Tcrd-H2BEGFP* (black line) small intestines and stained for CD3 ϵ , TCR $\alpha\beta$, TCR $\gamma\delta$ and NK1.1. EGFP fluorescence is analyzed in gated TCR $\gamma\delta^+$ CD3 ϵ^+ T cells, which represent 24.1% of TCR $\alpha\beta$ –NK1.1 $^-$ IELs. (d) Flow cytometry of marker expression on gated EGFP $^{\text{hi}}$ IELs. FSC, forward scatter. Data are representative of at least five independent experiments.

generated by the association of TCR γ and TCR δ chains^{8,9}. It has therefore been impossible to identify $\gamma\delta$ T cell precursors before the stage at which they express TCR $\gamma\delta$ heterodimers⁶. Here we have developed mice expressing a reporter gene 'knocked into' the *Tcrd* constant region gene, thereby circumventing that technical limitation. Using these mice, we have characterized $\gamma\delta$ T cell development from the point of opening of the *Tcrd* locus in a subset of early T cell progenitors to the point of emergence of mature TCR $\gamma\delta^+$ DN4 thymocytes.

RESULTS

Generation of knock-in mice

We introduced an expression cassette encoding an internal ribosomal entry site (IRES) and a fusion of human histone H2B and enhanced green fluorescence protein (EGFP)¹⁰ in the 3' untranslated region of the *Tcrd* constant (C) gene (Fig. 1a and Supplementary Figs. 1 and 2 online). 'Knock-in' mice with the intended '*Tcrd-H2BEGFP*' mutation were derived from Bruce-4 C57BL/6 embryonic stem cells¹¹. *Tcrd-H2BEGFP* mice were born at the expected frequencies, and homozygous mice were healthy and had normal T cell development (Supplementary Fig. 3 online).

The thymus and periphery of *Tcrd-H2BEGFP* mice contained $\alpha\beta$ T cells, natural killer (NK) cells and NKT cells in numbers identical to those of wild-type C57BL/6 mice (Supplementary Fig. 4 online). The $\gamma\delta$ T cells from *Tcrd-H2BEGFP* and wild-type mice expressed identical amounts of TCR $\gamma\delta$ heterodimers on the cell surface, indicating that insertion of the IRES-driven cassette in the 3' untranslated region did not affect *Tcrd* expression (data not shown). Compared with age- and sex-matched wild-type mice, *Tcrd-H2BEGFP* mice had slight lower absolute numbers of thymic but not of peripheral $\gamma\delta$ T cells (Supplementary Fig. 4). We injected equal numbers of lineage-negative (CD5 $^-$ CD45R $^-$ CD11b $^-$ Gr-1 $^-$ 7-4 $^-$ Ter119 $^-$) bone marrow cells purified from wild-type and *Tcrd-H2BEGFP* mice into mice lacking recombination-activating gene 2 and the gene encoding common cytokine receptor γ -chain. Wild-type and *Tcrd-H2BEGFP* bone marrow cells contributed equally to the resulting peripheral $\gamma\delta$ T cell pool (Supplementary Fig. 5 online). Control mice reconstituted with either wild-type or *Tcrd-H2BEGFP* bone marrow had $\gamma\delta$ T cells with either no (negative) EGFP expression (EGFP $^{\text{neg}}$) or high EGFP expression (EGFP $^{\text{hi}}$), respectively (data not shown). These data suggest that our 'knock-in' approach did not notably impair $\alpha\beta$ or $\gamma\delta$ T cell development and therefore caused no adverse effect that may have confounded interpretation of experimental data.

H2BEGFP expression in T cells and thymocytes

Expression of the H2BEGFP reporter readily identified intraepithelial lymphocytes (IELs) in the small intestine (Fig. 1b). All CD3 ϵ^+ TCR $\gamma\delta^+$ IELs from *Tcrd-H2BEGFP* mice were EGFP $^{\text{hi}}$ (Fig. 1c), whereas wild-type CD3 ϵ^+ TCR $\gamma\delta^+$ IELs were EGFP $^{\text{neg}}$. Reciprocally, most EGFP $^{\text{hi}}$ IELs expressed TCR $\gamma\delta$ and CD3 ϵ at their surface (Fig. 1d). We noted a few EGFP $^{\text{hi}}$ CD3 ϵ^- TCR $\gamma\delta^-$ IELs, which probably corresponded to activated IELs that had internalized their TCR $\gamma\delta$ –CD3 complexes. The distribution of amounts of CD3 ϵ and TCR $\gamma\delta$ on the surface of TCR $\gamma\delta^+$ IELs was rather broad, and CD3 ϵ and TCR $\gamma\delta$ surface amounts varied in direct proportion to each other (Fig. 1c,d). This observation probably reflects the fact that each combination of γ - and δ -chains has a distinct pairing efficiency and rate of export to the cell surface, which influences the amount of TCR $\gamma\delta$ expressed on the surface of each clone¹². In contrast, the distribution of EGFP fluorescence in $\gamma\delta$ cells produced a sharp peak (Fig. 1d). We obtained similar results by analysis of $\gamma\delta$ T cells in the spleen and lymph nodes (data not shown). Therefore, EGFP fluorescence genuinely defines peripheral $\gamma\delta$ T cells independently of the amount of TCR $\gamma\delta$ expressed on the cell surface.

The frequencies and absolute numbers of DN1, DN2, DN3 and DN4 cells were not substantially different in wild-type and *Tcrd-H2BEGFP* mice (Fig. 2a and Supplementary Fig. 3). Analysis of the expression of EGFP versus TCR $\gamma\delta$ on gated DN1, DN2, DN3 and DN4 thymocytes was very informative. Wild-type DN cells were all EGFP $^{\text{neg}}$ (Fig. 2b), whereas in *Tcrd-H2BEGFP* mice, DN cells could be categorized into EGFP $^{\text{hi}}$, EGFP intermediate (EGFP $^{\text{int}}$) and EGFP $^{\text{neg}}$ populations. Most EGFP $^{\text{neg}}$ cells were DN1 cells (Fig. 2b), which represent cells not committed to the T cell lineage as well as early T cell progenitors that have not yet initiated *Tcrd* rearrangement (discussed below). EGFP $^{\text{hi}}$ DN cells represented approximately 5% of total thymocytes and all expressed TCR $\gamma\delta$ heterodimers on the cell surface. EGFP $^{\text{hi}}$ DN cells were either DN4 (23.9% $\pm$ 3.3% of DN4 cells) or DN1 (10.1% $\pm$ 3.2% of DN1 cells) cells. In contrast, DN2 and DN3 subsets contained few EGFP $^{\text{hi}}$ TCR $\gamma\delta^+$ cells (0.8% $\pm$ 0.3% of DN2 and 1.0% $\pm$ 0.3% of DN3). EGFP $^{\text{int}}$ cells were present in all four DN subsets, had no detectable expression of TCR $\gamma\delta$ heterodimers on the cell surface and constituted most of the DN2 (96.3% $\pm$ 1.4%), DN3 (97.6% $\pm$ 1.4%) and DN4 (64.9% $\pm$ 8.1%) subsets. A unique subpopulation of EGFP $^{\text{int}}$ DN3 cells with low to high expression of surface TCR $\gamma\delta$ heterodimers is discussed below.

During somatic rearrangement of antigen receptor gene loci, accessibility to variable-(diversity)-joining (V(D)J) recombinase, a key event known as 'locus opening', is reflected by the presence of germline

transcripts¹³. To assess whether the intermediate amounts of EGFP found in most DN cells resulted from germline transcription of the *Tcrd* locus, we crossed *Tcrd*-H2BEGFP mice with recombination-activating gene 1-deficient (*Rag1*^{-/-}) mice. Germline *Tcrd* transcripts can be detected specifically in DN thymocytes of *Rag1*^{-/-} mice¹⁴. Analysis of *Tcrd*-H2BEGFP *Rag1*^{-/-} thymi showed the presence of only EGFP^{neg} and EGFP^{int} cells (Fig. 2c). As in *Tcrd*-H2BEGFP *Rag1*^{+/+} thymi, the DN2 and DN3 subsets consisted mostly of EGFP^{int} cells, whereas the DN1 subset consisted of both EGFP^{neg} and EGFP^{int} cells. Three conclusions can be drawn from the data reported above. First, the lack of EGFP^{hi} cells in *Tcrd*-H2BEGFP *Rag1*^{-/-} mice indicates that their development depends on *Tcrd* rearrangements. Second, germline transcription of the *Tcrd* locus is sufficient to produce the intermediate amounts of EGFP fluorescence found in most DN cells. Third, most DN2 and DN3 cells in *Tcrd*-H2BEGFP mice and *Tcrd*-H2BEGFP *Rag1*^{-/-} mice were EGFP^{int} and thus 'evenly' transcribed *Tcrd* genes.

Mapping the opening of the *Tcrd* locus

DN1 cells constitute a heterogeneous mixture of cells¹⁵. Through analysis of the surface expression of CD117 (c-Kit), CD24, CD44 and CD25, true DN1 cells can be more accurately identified and the DN1-to-DN2 transition can be analyzed precisely^{16,17}. Accordingly, a precursor-progeny pair referred to as DN1a (CD24⁺CD117⁺) and DN1b (CD24^{lo}CD117⁺) has been defined among DN1 cells and shown to give rise to DN2 cells (CD117⁺CD127⁺) and DN3 cells (CD117^{lo}CD127^{lo})^{16,17}. We used the same combination of markers to characterize EGFP^{int} DN1 cells in *Tcrd*-H2BEGFP mice (Fig. 3). In contrast to DN2 cells, which were all EGFP^{int}, DN1a cells were homogeneously EGFP^{neg}. Notably, cells at the DN1b stage had heterogeneous amounts of EGFP expression, with a continuum of cells extending from the position of EGFP^{neg} cells to that of EGFP^{int} cells. This suggests that the *Tcrd* locus becomes transcriptionally active at the DN1b stage. Notably, the DN1 subset from *Tcrd*-H2BEGFP *Rag1*^{-/-} thymi also contained EGFP^{int} cells, indicating that the onset of *Tcrd* germline transcription occurred independently of RAG expression (Fig. 2c).

The most efficient T cell progenitors in the thymus are called early T cell progenitors (ETPs) and are equivalent to DN1a plus DN1b thymocytes^{18,19}. The cytokine receptor Flt3 (also called CD135) is expressed on 5–20% of the ETP population and Flt3⁺ ETPs are less mature than their Flt3^{lo} counterparts^{18,19}. Analysis of *Tcrd*-H2BEGFP mice showed that the Flt3⁺ DN1 subset contained EGFP^{neg} cells (Fig. 3b). Therefore, analysis of *Tcrd*-H2BEGFP mice allowed us to map the opening of the *Tcrd* locus to the DN1b stage of T cell development.

A single checkpoint controls $\gamma\delta$ cell development

We next analyzed the unique population of DN3 cells that was present in *Tcrd*-H2BEGFP mice and had low to high expression of surface

Figure 2 H2BEGFP expression in DN thymocytes. (a) Flow cytometry of CD44 and CD25 marker expression on the surface of gated DN thymocytes (mouse genotypes, above plots). WT, wild-type. Numbers in quadrants correspond to the percentage of cells in each among DN cells. (b,c) Flow cytometry of EGFP and surface TCR $\gamma\delta$ expression on gated DN subsets of thymocytes (mouse genotypes, left margins). Data are representative of at least five (c) or ten (b) independent experiments. Vertical dashed lines define EGFP^{neg}, EGFP^{int} and EGFP^{hi} cells.

TCR $\gamma\delta$ heterodimers (Fig. 2b). On a dot plot comparing TCR $\gamma\delta$ and EGFP expression, these cells formed a continuum extending from EGFP^{int}TCR $\gamma\delta$ ^{lo} to EGFP^{hi}TCR $\gamma\delta$ ^{hi} (Fig. 4a). These cells constituted approximately 1% of the DN3 compartment and may represent selected $\gamma\delta$ T cells in transit to the DN4 stage. The pre-TCR delivers signals that permit $\alpha\beta$ T cell development^{1,20}, and the Lat adaptor protein is essential for pre-TCR signaling, as *Lat*^{-/-} mice have a complete developmental block at the DN3 stage²¹. Likewise, mature $\gamma\delta$ T cells are absent from *Lat*^{-/-} mice^{21,22}, suggesting that Lat also relays signals important for $\gamma\delta$ T cell development. To analyze whether TCR $\gamma\delta$ ⁺ DN3 cells need Lat to transit to the DN4 stage, we crossed *Tcrd*-H2BEGFP mice with *Lat*^{-/-} mice. The absence of Lat did not prevent surface expression of TCR $\gamma\delta$ heterodimers on DN3 cells (Fig. 4a). However, these cells were unable to mature beyond the EGFP^{int}TCR $\gamma\delta$ ^{lo} stage. Similar percentages of EGFP^{int}TCR $\gamma\delta$ ^{lo} DN3 cells were present in *Tcrd*-H2BEGFP thymi and *Tcrd*-H2BEGFP *Lat*^{-/-} thymi (Fig. 4a).

We also generated *Tcrd*-H2BEGFP mice lacking the CD3 ϵ signaling subunit (*Tcrd*-H2BEGFP *Cd3e*^{-/-} mice). As expected based on published studies^{23,24}, *Tcrd*-H2BEGFP *Cd3e*^{-/-} thymocytes did not progress beyond the DN3 stage (data not shown). Moreover, the absence of a CD3 ϵ subunit prevented expression of TCR $\gamma\delta$ heterodimers

Figure 3 Mapping the *Tcrd* locus opening. **(a)** Flow cytometry of CD24 and CD117 expression on the surface of wild-type and *Tcrd-H2BEGFP* DN1 cells. Boxed cells with the most surface CD117 correspond to the DN1a plus DN1b subsets (DN1a+b) and, along with DN2 cells, underwent additional staining (right). CD24⁺ DN1b and DN2 cells have been boxed for comparison. Numbers in plots (right) indicate EGFP mean fluorescence intensity of boxed cells. **(b)** EGFP fluorescence in Flt3⁺ DN1 cells from wild-type mice (gray filled histogram) and *Tcrd-H2BEGFP* mice (black line). Data are representative of three independent experiments.

Pre-TCR signals upregulate the transcription of productively rearranged *Tcrb* genes during the DN to DP transition^{24,25}. Likewise, expression of signaling-proficient TCR $\gamma\delta$ heterodimers on the surface of EGFP^{int}TCR $\gamma\delta$ ^{lo}

DN3 cells from *Tcrd-H2BEGFP* mice upregulated the transcription of *Tcrd-H2BEGFP* 'units', as documented by an increase in both EGFP and TCR $\gamma\delta$ expression (**Fig. 4a**). This observation allowed definition of an EGFP^{hi}TCR $\gamma\delta$ ^{hi} post-selection DN3 stage. The resulting TCR $\gamma\delta$ ⁺ DN4 cells had homogeneously high expression of EGFP and surface TCR $\gamma\delta$ heterodimers. No DN3 cells in *Lat*^{-/-} mice had high expression of EGFP and TCR $\gamma\delta$ heterodimers, indicating that TCR $\gamma\delta$ heterodimers were unable to deliver such an inductive signal. Therefore, by using *Tcrd-H2BEGFP Lat*^{-/-} mice we were able to visualize $\gamma\delta$ T cell precursors that succeeded in assembling and expressing small amounts of TCR $\gamma\delta$ on their surface but failed to receive the signals needed for differentiation into EGFP^{hi}TCR $\gamma\delta$ ^{hi} DN3 intermediates and mature EGFP^{hi}TCR $\gamma\delta$ ^{hi} DN4 $\gamma\delta$ T cells. Our data further showed that TCR $\gamma\delta$ heterodimers can be readily detected at the surface of preselection

Figure 4 A single checkpoint controls $\gamma\delta$ cell development. **(a)** EGFP and surface TCR $\gamma\delta$ expression on DN subsets (above plots) from various mice (genotypes, left margin). Boxed areas indicate windows corresponding to EGFP^{int}TCR $\gamma\delta$ ^{lo} DN3 cells; percentage for each window corresponds to the percentage among DN3 cells. **(b)** Flow cytometry of DN3 cells (genotype, left margin) stained with antibodies specific for TCR $\gamma\delta$ and EGFP (left); boxed areas indicate windows corresponding to TCR $\gamma\delta$ ^{lo} (blue 'low' window) and TCR $\gamma\delta$ ^{hi} (red 'high' window) DN3 cells. Histograms (right) show marker expression on TCR $\gamma\delta$ ^{lo} (blue) and TCR $\gamma\delta$ ^{hi} (red) DN3 cells and on EGFP^{hi}TCR $\gamma\delta$ ^{hi} (green) DN4 cells. Data are representative of at least five independent experiments. Many events were recorded in **b** to visualize TCR $\gamma\delta$ ⁺ DN3 cells, leading to 'overloading' of the bottom part of the dot plot. As a result, *Tcrd-H2BEGFP* thymi and *Tcrd-H2BEGFP Lat*^{-/-} thymi seem to contain many EGFP^{neg} DN3 cells. However, consistent with the TCR $\gamma\delta$ -versus-EGFP dot plots in **a** and in **Figure 2b**, EGFP^{neg} cells constitute less than 2% of DN3 cells.

$\gamma\delta$ T cells and are thus not subjected to constitutive internalization as pre-TCR complexes²⁰.

Rearrangement and transcription of *Tcr* genes require signals from the interleukin 7 receptor and Janus kinase 3 (refs. 26–28). There were EGFP^{int} cells in the DN compartment in *Tcrd-H2BEGFP* Janus kinase 3-deficient mice, and EGFP^{hi} cells were absent from both the DN3 and DN4 compartments in *Tcrd-H2BEGFP* Janus kinase 3-deficient mice (Supplementary Fig. 6 online). These data indicate that expression of TCR γ chains is needed to induce early signs of $\gamma\delta$ -selection and that Janus kinase 3 is dispensable for *Tcrd* locus transcription. Given that expression of TCR δ chains is also required for $\gamma\delta$ cells to mature to the DN4 stage⁴, the assembly of complete TCR $\gamma\delta$ heterodimers is thus mandatory for $\gamma\delta$ cell development. Therefore, our data show that the single molecular sensor that controls $\gamma\delta$ cell development at the DN3 stage requires successful expression of $\gamma\delta$ TCR heterodimers and uses CD3 ϵ and Lat to deliver inductive signals.

Phenotypic changes induced by $\gamma\delta$ -selection

Analysis of *Tcrd-H2BEGFP* mice provided a unique 'snapshot' of TCR $\gamma\delta^+$ DN3 cells in transit to the DN4 compartment. After staining cells with antibodies specific for the interleukin 7 receptor α -chain (CD127), CD24 and CD5, we subdivided TCR $\gamma\delta^+$ DN3 cells from wild-type mice, *Tcrd-H2BEGFP* mice and *Tcrd-H2BEGFP* *Lat*^{-/-} mice into EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ and EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN3 subsets and compared

those with EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN4 cells (Fig. 4b). Wild-type TCR $\gamma\delta^+$ DN3 cells and EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ DN3 cells from *Tcrd-H2BEGFP* *Lat*^{-/-} mice lacked CD5 molecules at their surface (Fig. 4b and data not shown). In contrast, EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ DN3 cells from *Tcrd-H2BEGFP* thymi showed a bimodal CD5 distribution. A minority lacked CD5 and most expressed amounts of CD5 similar to their immediate EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN3 and EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN4 progeny (Fig. 4b). Therefore, signs of $\gamma\delta$ -selection were already apparent at the EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ DN3 stage. The transition from EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ to EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ was also associated with a notable decrease in CD127 expression (Fig. 4b). EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN3 cells had a CD127 distribution intermediate between that of CD127⁺EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ DN3 cells and that of CD127^{neg-lo}EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN4 cells (Fig. 4b). EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ DN3 cells from *Tcrd-H2BEGFP* mice and *Tcrd-H2BEGFP* *Lat*^{-/-} mice had similar amounts of CD127 expression (Fig. 4b). Therefore, in contrast to the CD5 upregulation that had already occurred at the EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ DN3 stage, analysis of CD127 expression showed that some phenotypic changes triggered by $\gamma\delta$ -selection can be delayed until the EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN3 stage. Although CD24 downregulation has been associated with intrathymic maturation of $\gamma\delta$ T cells, the generality of that finding has been disputed²⁹. We noted similar amounts of CD24 on the surfaces of EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN4 cells and EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ and EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN3 cells (Fig. 4b). A small

percentage of DN3 cells (1.5%) in wild-type thymi had low to high expression of surface TCR $\gamma\delta$ heterodimers and had a transitional phenotype similar to that of TCR $\gamma\delta^+$ DN3 cells isolated from *Tcrd-H2BEGFP* mice (Fig. 4b). Therefore, *Tcrd-H2BEGFP* mice genuinely reproduce the phenotypic changes associated with $\gamma\delta$ -selection in wild-type mice. Moreover, these data support the idea of the existence of an EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ DN3 $\rightarrow$ EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN3 $\rightarrow$ EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN4 developmental series. Like β -selection, $\gamma\delta$ -selection straddles the DN3-DN4 stages and is thus associated with rapid downregulation of CD25. As noted in developing $\alpha\beta$ T cells³⁰, CD5 expression was also developmentally regulated by TCR $\gamma\delta$ signals.

It is generally assumed that β -selection is associated with clonal expansion, whereas progenitors committed to the $\gamma\delta$ T cell lineage undergo little or no proliferation^{1,3,20}. *Tcrd-H2BEGFP* mice allowed us to revisit that issue using 7-amino-actinomycin D staining coupled to bromodeoxyuridine (BrdU) incorporation. Although forward scatter signals and immunofluorescence intensity were slightly modified by the fixation procedures, all populations of DN cells could be identified, with the notable exception of the EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ DN3 subset, which tended to merge with the bulk of EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ DN3 cells. That prevented us from drawing any conclusion regarding the mitotic activity of the EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ DN3 cells in *Tcrd-H2BEGFP* *Lat*^{-/-} thymi expressing TCR $\gamma\delta$ heterodimers unable to induce $\gamma\delta$ -selection. Notably, because of its

Figure 5 Cell proliferation is induced by $\gamma\delta$ -selection. (a) Forward-scatter analysis of the size of EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ DN3, EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN3 and EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN4 cells from wild-type and *Tcrd-H2BEGFP* mice; cells are defined as described in Figure 4. (b) DNA content analysis of DN thymocytes isolated after exposure of *Tcrd-H2BEGFP* mice to BrdU for 2 d. Cells were stained for CD25, CD44, TCR $\gamma\delta$ and BrdU and were incubated with 7-amino-actinomycin D. Outlined areas define windows corresponding to EGFP^{hi}TCR $\gamma\delta^+$ ($\gamma\delta$) and EGFP^{int}TCR $\gamma\delta^-$ (Bulk) DN3 and DN4 cells. DNA content was assessed in cells in each of these windows; numbers in histograms indicate the percentage of EGFP^{hi}TCR $\gamma\delta^+$ DN3 and DN4 cells with more than 2N DNA content. (c) BrdU incorporation and DNA content of the cell populations defined in b; numbers in quadrants indicate the percentage of cells in each window of the BrdU-versus-7-amino-actinomycin D (7-AAD) dot plot. Data are representative of at least five independent experiments.

association with histone H2B, EGFP localized exclusively to nuclei, and the chromatin-bound H2BEGFP fusion protein withstood the cell-permeabilization procedure required for BrdU detection (data not shown). Comparison of the size of EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ and EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN3 cells and of EGFP^{hi} TCR $\gamma\delta^+$ DN4 cells showed that DN3 cells were larger than their DN4 progeny (Fig. 5a). The large cell size noted for TCR $\gamma\delta^{\text{lo-hi}}$ DN3 cells correlated with cell proliferation, as 38.8% $\pm$ 2.6% of the EGFP^{int-hi} TCR $\gamma\delta^+$ DN3 cells had a greater-than-diploid content of DNA (more than 2N DNA), compared with 14.0% $\pm$ 2.0% in EGFP^{hi}TCR $\gamma\delta^+$ DN4 cells (Fig. 5b). Before selection, TCR $\gamma\delta^-$ DN3 cells and β -selected TCR $\gamma\delta^-$ DN4 subsets contained on average 14.8% $\pm$ 3.0% and 29.0% $\pm$ 7.1% of cells with more than 2N DNA, respectively (Fig. 5b,c). After continuous exposure of *Tcrd-H2BEGFP* mice to BrdU for 4 d, substantial proportions of both EGFP^{int-hi}TCR $\gamma\delta^+$ DN3 cells and their EGFP^{hi}TCR $\gamma\delta^+$ DN4 progeny incorporated BrdU (Fig. 5c and Supplementary Fig. 7 online). The number of labeled EGFP^{hi} DN3 and EGFP^{hi} DN4 cells increased rapidly over the first 3 d before reaching a plateau corresponding to approximately 90% of each population. Therefore, our results show that $\gamma\delta$ -selected DN3 cells undergo a quick maturation and efficiently cycle, with the percentage of cells with more than 2N DNA being similar to that found in β -selected DN4 cells.

DN cell subsets expressing TCR $\gamma\delta$ heterodimers

By using *Tcrd-H2BEGFP* mice and deliberately not gating out DN cells expressing surface CD3 ϵ , we have demonstrated an EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ DN3 $\rightarrow$ EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN3 $\rightarrow$ EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN4 developmental sequence and have shown that progression beyond the

EGFP^{int}TCR $\gamma\delta^{\text{lo}}$ DN3 stage is controlled by $\gamma\delta$ -selection. In previous studies attempting to study $\gamma\delta$ cell development, thymocytes expressing CD3 ϵ at their surface have been gated out. That approach eliminates both mature $\gamma\delta$ DN4 cells and a few mature peripheral $\gamma\delta$ cells that reenter the thymus, but coincidentally prevents the detection of $\gamma\delta$ progenitors with low expression of TCR $\gamma\delta$ -CD3 complexes at their surface. Because our approach preserved the thymic EGFP^{hi}TCR $\gamma\delta^{\text{hi}}$ DN cells, we next analyzed them by flow cytometry. Two well separated clusters of EGFP^{hi} cells were distinguished on a dot plot of CD24 versus CD44 staining (Fig. 6a). One cluster, called 'cluster A' here, had a CD24⁺CD44^{neg-int} phenotype and represented 81.6% $\pm$ 4.2% of the EGFP^{hi} DN cells. A second cluster, called 'cluster B' here, had a CD24⁺CD44^{hi} phenotype and represented 13.8% $\pm$ 3.3% of the EGFP^{hi} DN cells. We further analyzed these two clusters for expression of NK1.1, CD122, CD49b (DX5), CD127, CD62L, CD5, CD43, CD69, CD90.2 (Thy1.2), TCR $\gamma\delta$, TCR V γ 6.3 and TCR V γ 5 (Fig. 6b). Based on surface phenotype, cluster A was found to correspond to DN4 $\gamma\delta$ cells and thus constituted the end product of the developmental pathway that we characterized and that initiates at the DN3 stage. The $\gamma\delta$ T cells comprising the minor cluster B were readily distinguished from those in cluster A in that they showed characteristics of DN1 thymocytes and had high expression of CD127 (Fig. 6b). Based on expression of CD44 and CD62L, cluster B cells also seemed to be more activated than cluster A cells. Notably, cluster B cells showed a bimodal distribution when analyzed for NK1.1 expression (Fig. 6b): 71.9% $\pm$ 5.4% of cluster B cells lacked NK1.1, whereas 27.8% $\pm$ 5.7% of cluster B cells expressed NK1.1. In contrast, all cluster A cells lacked NK1.1. The NK1.1⁺ cells in cluster B did not

express CD62L but did express CD122 and comprised all of the DX5⁺ cells (Fig. 6b and data not shown). The NK1.1⁻ cells in cluster B had on average higher expression of $\gamma\delta$ TCR than did the NK1.1⁺ cells (Fig. 6c). The V δ 6.3 gene segment was used by 3–4% of cluster A cells and by approximately 50% of cluster B cells. In cluster B, NK1.1⁺ cells showed an even stronger bias toward V δ 6.3 usage (Fig. 6d). Therefore, the phenotype of the NK1.1⁺EGFP^{hi} $\gamma\delta$ T cell subset found in cluster B unequivocally identified it as the thymic NKT $\gamma\delta$ T cell subset^{31–33}. This unusual subset shares with NKT cells the expression of cell surface markers associated with activated or memory cells and the simultaneous production of large amounts of interleukin 4 and interferon- γ after activation^{31–33}. Despite its notable similarity with NKT $\alpha\beta$ T cells, very little is known about this minor $\gamma\delta$ T cell subset, and it remains to be determined whether its development follows a sequence distinct from that proposed for the main subset of adult thymic $\gamma\delta$ T cells (Supplementary Fig. 8 online).

DISCUSSION

The possibility to simultaneously analyze *Tcrd* gene transcription (using the H2BEGFP reporter) and TCR $\gamma\delta$ surface expression allowed us to decipher the earliest stages of $\gamma\delta$ cell development in adult thymus. Our findings included the 'visualization' of

Figure 6 Phenotype of EGFP^{hi} DN thymocytes. (a) Flow cytometry of EGFP^{hi} DN thymocytes gated and stained with antibodies specific for CD44 and CD24 surface molecules. SSC, side scatter. (b) Expression of markers (below histograms) on cluster A cells (grey filled histograms) and cluster B cells (black lines). (c,d) Flow cytometry of marker expression on cluster A and cluster B cells. Numbers in quadrants indicate the percentage of cells in each window. Data are representative of at least three independent experiments.

a principal $\text{EGFP}^{\text{int}}\text{TCR}\gamma\delta^{\text{lo}}$ DN3 $\rightarrow$ $\text{EGFP}^{\text{hi}}\text{TCR}\gamma\delta^{\text{hi}}$ DN3 $\rightarrow$ $\text{EGFP}^{\text{hi}}\text{TCR}\gamma\delta^{\text{hi}}$ DN4 developmental pathway and the demonstration that progression beyond the $\text{EGFP}^{\text{int}}\text{TCR}\gamma\delta^{\text{lo}}$ DN3 stage is controlled by $\gamma\delta$ -selection. Hints about the $\gamma\delta$ T cell developmental pathway demonstrated using adult *Tcrd-H2BEGFP* mice can be found in reports based on the use of mice lacking Lat, TCR β or pre-TCR α . For example, loss-of-function mutations in *Lat* have provided the opportunity to demonstrate the presence of a DN3 TCR $\gamma\delta^{\text{lo}}$ population²². In the absence of Lat, this population is not capable of maturing further and has a phenotype similar to that of the $\text{EGFP}^{\text{int}}\text{TCR}\gamma\delta^{\text{lo}}$ DN3 population found in *Tcrd-H2BEGFP Lat*^{-/-} mice²². Experiments using mice lacking pre-TCR α have demonstrated a pathway of $\gamma\delta$ T cell differentiation that originates at the DN3 stage and gives rise to mature DN4 $\gamma\delta$ T cells¹⁷. Using an indirect strategy combining expression of the CD27 marker and *in vivo* assays, one study concluded that $\gamma\delta$ selection occurs at the DN3 stage and inferred that those results obtained in *Tcrb*^{-/-} mice extend to wild-type mice³⁴. However, given that DP cells regulate the differentiation of early thymocyte progenitors and of developing $\gamma\delta$ T cells^{35,36}, the lack of DP cells in *Lat*^{-/-}, *Tcrb*^{-/-} and pre-TCR α -deficient mice might have influenced those results. In contrast, *Tcrd-H2BEGFP* mice provide a minimally manipulated, DP cell-replete environment that allows characterization of the developmental cascade beginning with the opening of the *Tcrd* locus and ending with the emergence of mature $\gamma\delta$ DN4 cells.

Using *Tcrd-H2BEGFP* mice, we have identified the DN1b stage as the precise time point corresponding to the opening of the *Tcrd* locus. Because of their lack of Flt3 expression, these DN1b cells probably correspond to late ETPs. Given that Notch drives the transition from early to late ETPs^{18,19}, it is plausible that Notch is involved in the induction of *Tcrd* transcripts³⁷. Interleukin 7 is important in the modulation of chromatin accessibility at *Tcr* loci³⁸. Therefore, the lack of interleukin 7 receptor on DN1a and DN1b cells¹⁶ suggests that *Tcr* loci become accessible to V(D)J recombinase at a later time point than *Tcrd* loci. Moreover, the lack of RAG transcripts in DN1b cells¹⁶ indicates that the *Tcrd* loci are not the object of rearrangement at that stage. In adult thymus, the first *Tcr* and *Tcrd* rearrangements are detectable at the DN2 stage³⁹. Because CD3 signaling subunits are also expressed in DN2 cells^{16,17}, it is possible that some DN2 cells successfully assemble surface TCR $\gamma\delta$ heterodimers and undergo $\gamma\delta$ -selection. In support of that view, *Tcrd-H2BEGFP* mice had a few $\text{EGFP}^{\text{hi}}\text{TCR}\gamma\delta^{\text{int-hi}}$ DN2 cells. However, their scarcity prevented us from establishing whether they can readily give rise to TCR $\gamma\delta^+$ DN4 cells.

After an early seminal study on $\gamma\delta$ cell development⁴, it has been accepted that $\gamma\delta$ cell development occurs in the absence of clonal expansion and it has been inferred that the pre-TCR has a unique capacity to induce proliferation of β -selected DN3 cells^{20,40}. It should be emphasized, however, that in that earlier work⁴, even the 'δ-selected' DN3 cells showed a notable cycling activity (38% and 64% of δ-selected and β-selected DN3 cells, respectively, contained more than 2N DNA). Using *Tcrd-H2BEGFP* mice, we have shown that the formation of TCR $\gamma\delta$ heterodimers in DN3 cells induced their maturation into DN4 cells through a process involving a phase of proliferation. Based on estimate of the proportions of cells in different phases of the cell cycle, the magnitude of this proliferation seemed to be similar to that induced by the pre-TCR. Continuous BrdU labeling showed that both EGFP^{hi} DN3 and EGFP^{hi} DN4 cells were generated at similar rates. Based on the numbers of preselection TCR $\gamma\delta^+$ DN3 and mature TCR $\gamma\delta^+$ DN4 cells, and given that EGFP^{hi} DN3 cell precursors and their EGFP^{hi} DN4 cell progeny have an identical rate of

turnover, it is possible to calculate that 'selected' $\gamma\delta$ T cells expand their populations approximately 20- to 30-fold. An expansion of such magnitude corresponds to four to five divisions, compared with the six to seven divisions thought to occur in β-selected cells^{41,42}. Provided that $\gamma\delta$ -selection requires not only cell surface TCR $\gamma\delta$ expression but also some form of ligand-mediated positive selection (discussed below), only a fraction of the 0.9×10^4 preselection TCR $\gamma\delta^+$ DN3 cells might fulfill those last requirements and embark into proliferation. Therefore, the four to five divisions postulated to result from $\gamma\delta$ -selection constitutes a minimal estimate. Consistent with the idea that both β- and $\gamma\delta$ -selected cells undergo extensive proliferation before becoming DP cells or mature TCR $\gamma\delta^+$ DN4 cells, respectively, CD25 showed the same rapid kinetics of extinction during β- and $\gamma\delta$ -selection^{43,44}.

An adult thymus contains approximately 300-fold more DP cells than $\gamma\delta$ cells. That difference can no longer be accounted for by the fact that $\gamma\delta$ -selection does not induce a phase of sustained proliferation. The assembly of TCR $\gamma\delta$ heterodimers in DN3 cells requires two successful productive V(D)J rearrangement events and is thus more demanding than the assembly of pre-TCR α -TCR β heterodimers, which requires only one. Consistent with that view, in *Tcrd-H2BEGFP Lat*^{-/-} mice the pool of preselection $\text{EGFP}^{\text{int}}\text{TCR}\gamma\delta^{\text{lo}}$ DN3 cells approximated 0.5% of DN3 cells, whereas in *Cd3e*^{-/-} mice the pool of preselection cells expressing TCR β chains was approximately 16% of DN3 cells²⁵. Provided that *Tcrb*, *Tcr* and *Tcrd* rearrangements occur concurrently and with the same frequency and that the fate of bipotent DN3 precursors is determined by the TCR isotype that assembles first, it is likely that the large differences in the numbers of $\gamma\delta$ and DP cells generated by $\gamma\delta$ - and β-selection, respectively, reflect mainly the distinct sizes of the corresponding preselection pools.

Analysis of *Tcrd-H2BEGFP* mice demonstrated that the outcome of *Tcrd* and *Tcr* rearrangements is sensed in a single step at the $\text{EGFP}^{\text{int}}\text{TCR}\gamma\delta^{\text{lo}}$ DN3 stage. That view is consistent with the fact that none of the CD3 components are required for the onset of either *Tcrd* or *Tcr* rearrangements⁴⁵. When DP cells undergo positive selection, most (97%) die either by default (lack of selection) or by negative selection¹. It has been argued that the two-checkpoint model followed by developing αβ T cells allows them to meet the stringent specificity requirements associated with TCRαβ selection more efficiently than if TCRαβ specificity were sensed in a single step⁴⁶. It is possible that developing $\gamma\delta$ T cells undergo some form of ligand-mediated positive selection. So far, no firm evidence has been obtained for the requirement of conventional major histocompatibility complex molecules during $\gamma\delta$ repertoire selection, and $\gamma\delta$ T cells can be found in normal numbers in mice deficient in major histocompatibility complex classes Ia and II (ref. 47). Developmentally 'programmed' use of certain V γ and V δ gene segments containing short homology repeats near their coding ends and the absence of terminal deoxynucleotidyl transferase activity allow some $\gamma\delta$ cell subsets to express TCRs with a single (or fairly limited number of) canonical V(D)J junction(s). The expression of these semi-invariant TCRs does not preclude the existence of a subsequent checkpoint governed by TCR $\gamma\delta$ clonotypic selection. For example, for dendritic epithelial T cells, cellular selection probably acts 'on top of' genetically 'programmed' rearrangement events^{48,49}. Whether the single checkpoint used by adult $\gamma\delta$ cells that express a larger TCR diversity reflects less-stringent specificity requirements than for TCRαβ⁺ DP thymocytes⁵⁰ or development regardless of TCR specificity remains to be determined.

In conclusion, *Tcrd-H2BEGFP* mice have allowed us to identify the subset of early thymocyte progenitors in which *Tcrd* locus opening

occurs. These mice have also permitted us to show that expression of TCR $\gamma\delta$ on the surface of a small subset of DN3 cells induces their maturation into $\gamma\delta$ DN4 cells through a process that involves a phase of intense proliferation. By making these minute subsets directly accessible, experiments using *Tcrd-H2BEGFP* mice should lay the groundwork needed to elucidate the gene networks that control cardinal events of $\gamma\delta$ T cell development.

METHODS

Mice. Mice were housed in specific pathogen-free conditions and were handled in accordance with French and European directives. All mouse protocols were approved by the Institut National de la Santé et de la Recherche Médicale Committee on Animal Welfare. The generation of *Tcrd-H2BEGFP* mice is detailed in **Supplementary Methods** online. Unless specified otherwise, analyses used 5- to 6-week-old mice. *Rag1*^{-/-} mice⁵¹, *Cd3e*^{-/-} mice²³ and *Lat*^{-/-} mice²² have been described.

Cell purification. DN thymocytes were isolated from wild-type and *Tcrd-H2BEGFP* mice as described¹⁷. Thymocyte suspensions prepared at a density of 20×10^6 cells/ml in RPMI 1640 medium containing 1% FCS and 10 mM HEPES and were incubated for 10 min at 37 °C with a mixture of rat immunoglobulin M directed against mouse CD8 (clone 3.168.8.1) and CD4 (clone RL 172.4), followed by the addition of a 1:10 dilution of Low-Tox rabbit complement (Cedarlane Laboratories). After being incubated for 30 min at 37 °C with frequent gentle mixing, viable DN cells were recovered after centrifugation at 20 °C over Ficoll. IELs were isolated as described⁵².

Antibodies. Phycoerythrin-conjugated anti-TCR $\gamma\delta$ (GL3), anti-TCR δ V6.3 (8F4H7B7), anti-CD24 (M1/69), anti-CD27 (LG.3A10), anti-CD43 (S7), anti-CD44 (IM7), anti-CD49b (pan-NK) (DX5), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD122 (TM- β 1) and anti-Flt3 (A2F10.1); biotin-conjugated anti-TCR $\gamma\delta$ (GL3), anti-CD24 (M1/69), anti-CD44 (IM7) and anti-CD62L (MEL-14); peridinin chlorophyll protein complex-indodicarbocyanine (Cy5.5)-conjugated anti-CD3e (145-2C11) and anti-CD4 (RM4-5); allophycocyanin-conjugated anti-CD3 (145-2C11), anti-CD8 α (53-6-7), anti-CD117 (2B8) and anti-NK1.1 (PK136); phycoerythrin-indodicarbocyanine-conjugated anti-CD4 (RM4-5), anti-CD44 (IM7) and anti-NK1.1 (PK136); peridinin chlorophyll protein complex-conjugated anti-CD5 (53-7-3); phycoerythrin-indodicarbocyanine (Cy5)-conjugated anti-CD5 (53-7-3); and allophycocyanin-indodicarbocyanine-conjugated anti-CD8 α (53-6-7) and anti-CD25 (PC61) were all purchased from BD Pharmingen. Phycoerythrin-indodicarbocyanine (Cy5.5)-conjugated anti-TCR β (H57-597); phycoerythrin-conjugated anti-CD127 (A7R34); and phycoerythrin-indodicarbocyanine (Cy5)-conjugated anti-CD127 (A7R34) and anti-CD90.2 (53-2-1) were obtained from eBioscience. Indodicarbocyanine (Cy5)-conjugated anti-CD44 (IM7) and phycoerythrin-conjugated anti-TCR δ V5 were gifts from S.H.E. Kaufmann (Max-Planck Institute for Infection Biology, Berlin, Germany) and P. Pereira (Pasteur Institute, Paris, France), respectively.

Flow cytometry. Before being stained, cells were incubated for 10 min on ice with rat serum and purified monoclonal antibody to the Fc receptor (2.4G2) to block Fc receptors. The FACSCalibur, LSR and FACSCanto systems (BD Biosciences) were used for multiparameter flow cytometry. Data were analyzed with CellQuest software (BD Biosciences) and FlowJo software (Treestar).

BrdU incorporation and cell cycle analysis. Mice were injected intraperitoneally with 1.5 mg BrdU (Sigma-Aldrich) in PBS and their drinking water was supplemented with 0.8 mg/ml of BrdU and 2% glucose. After 2 d, DN cells were prepared by complement lysis and were stained with antibodies specific for CD44, CD25 and TCR $\gamma\delta$. BrdU staining was done with the BrdU Flow kit (BD Biosciences) according to the manufacturer's recommendations. Cells were sequentially fixed and made permeable with BD Cytofix/Cytoperm Plus and Cytoperm buffers. After being washed in Permwash buffer, cells were treated with 30 μ g DNase for 30 min at 37 °C to expose BrdU epitopes. Cells were then washed and were stained for 20 min at 25 °C with allophycocyanin-conjugated anti-BrdU diluted in Permwash buffer. Cells were washed once more before being resuspended in PBS containing 1% FCS for flow cytometry. For DNA

labeling, the fluorescent dye 7-amino-actinomycin D (BD Biosciences) was added 5–10 min before flow cytometry acquisition.

Statistics. The number of cells in each specified subset was calculated as mean $\pm$ s.d. for at least three separate experiments.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank C.A. Stewart, D.J. Pennington (University of London, London, UK), W. Held (Ludwig Institute for Cancer Research, Lausanne, Switzerland) and R. Ceredig (Center for Biomedicine, Basel, Switzerland) for discussions; and P. Grenot, M. Barad, F. Danjan, M. Richelme, P. Perrin, C. Grégoire, M. Fallet, S. Sarazin and A. Gillet for advice. Supported by Centre National de la Recherche Scientifique (B.M.), Institut National de la Santé et de la Recherche Médicale (B.M.), Association pour la Recherche contre le Cancer (B.M.), Fondation pour la Recherche Médicale (B.M.), Ministère de l'Éducation Nationale et de la Recherche (Plate-forme RIO-MNG; B.M.), Agence Nationale de Recherches (B.M.), the European Community (MUGEN Network of Excellence; B.M.) and a Marie Curie Intra-European Fellowship within the 6th European Community Framework Program (I.P.).

AUTHOR CONTRIBUTIONS

All authors contributed to discussions of experimental design and data analysis; I.P. did all experimental studies unless otherwise indicated; A.S. provided technical assistance; A.K. helped design the 'knock-in' strategy; L.A. and M.M. generated the Janus kinase 3-deficient mice; and I.P. and B.M. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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