LETTERS

Early events in the thymus affect the balance of effector and regulatory T cells

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In cellular immunology the critical balance between effector and regulatory mechanisms is highlighted by serious immunopathologies attributable to mutations in *Foxp3*, a transcription factor required for a major subset of regulatory T (T_R) cells[1–3]. Thus, many studies have focused on the developmental origin of T_R cells, with the prevailing view that they emerge in the thymus from late-stage T-cell progenitors whose T-cell receptors (TCRs) engage high affinity (agonist) ligands[4–6]. This study questions the completeness of that interpretation. Here we show that without any obvious effect on TCR-mediated selection, the normal differentiation of mouse γδ T cells into potent cytolytic and interferon-γ-secreting effector cells is switched towards an aggregate regulatory phenotype by limiting the capacity of $CD4^+CD8^+$ T-cell progenitors to influence *in trans* early γδ cell progenitors. Unexpectedly, we found that the propensity of early TCR-$\alpha\beta^+$ progenitors to differentiate into $Foxp3^+$ T_R cells is also regulated *in trans* by $CD4^+CD8^+$ T-cell progenitor cells, before agonist selection.

Thymocyte differentiation progresses from early $CD4^-CD8^-$ (double negative) progenitors either directly to TCR-$\gamma\delta^+$ cells, or to $CD4^+CD8^+$ (double positive) cells, which are selected by TCR engagement into mature 'single-positive' $CD4^+$ and $CD8^+$ TCR-$\alpha\beta^+$ cells. The double-negative to double-positive transition depends on a pre-TCR comprising TCR-β and pre-Tα chains, and mice lacking either harbour very few double-positive cells. Of note, double-positive cells function not only as progenitors of single-positive cells, but also 'condition' double-negative cell differentiation *in trans*[7]. Reflecting this, γδ cells in pTα-deficient (*Ptcra*$^{-/-}$) and TCR-β-deficient (*Tcrb*$^{-/-}$) mice differentiate with abnormally low expression of interferon-γ (IFN-γ) and cytolytic effector molecules. Nonetheless, other gene products (for example, XCL1) are expressed normally[7], suggesting that rather than being generally impaired, γδ cell progenitors lacking *trans*-conditioning might differentiate along an alternative pathway. Consistent with this, we found that genes expressed by activated splenic γδ cells in *Tcrb*$^{-/-}$ but not wild-type mice resembled those expressed by activated skin-resident dendritic epidermal T cells (DETCs) that naturally develop in the fetus before double-positive cells accumulate[8] (Fig. 1a). Resting splenic γδ cells showed less resemblance (Fig. 1b), which, together with a lack of DETC-associated Vγ5 expression (Supplementary Fig. 1), refutes the possibility that *Tcrb*$^{-/-}$ splenocytes are simply DETCs aberrantly localized to the spleen.

On several genetic backgrounds, DETC-deficient mice spontaneously develop cutaneous immunopathologies attributable to dysregulated αβ T cells[9]. Thus, although DETCs display cytolytic and pro-inflammatory potentials[10], their aggregate phenotype *in vivo* is regulatory. We therefore determined whether activated splenic γδ cells in *Tcrb*$^{-/-}$ mice also display aggregate regulatory activity. The membranes of TCR-$\alpha\beta^+CD25^{lo}CD4^+$ effector cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE), the dilution of which monitors cytokinesis. At 72 h or 86 h after activation only ~5–10% of cells remained undivided (Fig. 1c). Conversely, in 1:1 and 1:0.1 co-cultures with unlabelled $CD25^{hi}CD4^+$ T_R cells[11,12], ~50% and ~20%, respectively, of cells remained undivided. 1:1 co-cultures with wild-type γδ splenocytes conferred no such regulation, whereas in co-cultures with γδ cells from *Tcrb*$^{-/-}$ mice, ~25% of effector T cells remained undivided. This suppressive capacity did not require γδ cell subsetting, for example, by CD25 expression. Thus, γδ splenocytes developing independently of a normal double-positive pool resemble activated DETCs: both populations contain cytolytic and IFN-γ-producing effectors, but on aggregate are regulatory.

Immunoregulation is a critically important function provided by different mechanisms. Whereas natural αβ T_R cell activity relies on *Foxp3* transcription[1–3], other regulatory cells, including DETCs, do not[10]. Indeed, Foxp3 has not been reported in γδ cells, and we could not detect *Foxp3* RNA in TCR-$\gamma\delta^+$ splenocytes from C57.BL/6J or FVB/n mice, or from TCR-α-deficient (*Tcra*$^{-/-}$) mice that lack αβ T cells but harbour a large double-positive compartment (Fig. 2a). By contrast, *Foxp3* RNA was amplified from γδ cells of *Ptcra*$^{-/-}$ and *Tcrb*$^{-/-}$ mice, and Foxp3-protein-expressing γδ cells were reproducibly detected among splenocytes from *Tcrb*$^{-/-}$ but not wild-type mice (Fig. 2a, b). Similarly, TCR-$\gamma\delta^+$ double-negative thymocytes from *Tcrb*$^{-/-}$ and *Ptcra*$^{-/-}$ (but not wild type) mice also expressed Foxp3 (Fig. 2c, d). Thus, although it was only in a minority (1–2%) of TCR-$\gamma\delta^+$ cells (Supplementary Fig. 2), the expression of Foxp3 in *Tcrb*$^{-/-}$ mice emphasized the switch towards aggregate regulatory T-cell differentiation in the context of double-positive deficiency. Indeed, *Foxp3* RNA was also detected in γδ thymocytes from mice lacking lymphotoxin-β receptor (LTβR) (Fig. 2d) that contributes to *trans*-conditioning by double-positive cells[7].

Many experiments support the proposal that $CD4^+CD25^{hi}$TCR-$\alpha\beta^+$ T_R cells are selected by high-affinity TCR agonist engagement of double-positive cells[4–6]. Thus, $Foxp3^+$ γδ cells in *Tcrb*$^{-/-}$ mice might reflect an artefactual rescue by TCR-γδ of double-positive cells that then engage agonist and select as T_R cells. However, whereas TCR-γδ will rescue some double-positive differentiation in *Tcrb*$^{-/-}$ mice[13], such TCR-$\gamma\delta^+$ double-positive cells usually die without maturation[13–15]. Indeed, *Foxp3* RNA was not detectable in double-positive cells from *Tcrb*$^{-/-}$ mice (Fig. 2e). Moreover, the 'rescue hypothesis' does not obviously explain *Foxp3* RNA in LTβR-deficient (*Ltbr*$^{-/-}$)

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γδ thymocytes (Fig. 2d). Instead, we pursued the hypothesis that the aggregate regulatory phenotype of γδ cells in *Tcrb*$^{-/-}$ mice might reflect an uncharacterized route of regulatory T-cell differentiation.

Despite pTα deficiency, some double-negative to double-positive maturation accompanied by limited cell expansion occurs in *Ptcra*$^{-/-}$ mice, giving rise to a TCR-αβ^{+}CD4^{+} thymocyte pool ~130 times smaller than wild type[16] (Supplementary Table 1). CD25^{hi}Foxp3^{+} cell numbers are similarly reduced, confirming that the pre-TCR is necessary for T_R-cell differentiation, but this decline is only ~20-fold, and 25–30% of *Ptcra*$^{-/-}$ CD4^{+} thymocytes are CD25^{hi}Foxp3^{+} compared with 2.5–4.5% in wild-type mice (Fig. 3a). Hence, effector and regulatory CD4^{+} T cells do not share the same dependence on pTα. CD4^{+} cell numbers in the periphery of *Ptcra*$^{-/-}$ mice were less overtly reduced (possibly because of homeostatic expansion), but again effects on T_R cells were more mild, with the CD25^{hi}Foxp3^{+} phenotype shown by ~23–32% of CD4^{+} splenocytes compared with 6–11% in wild type (Fig. 3b; see also Supplementary Table 1). Moreover, regulatory function per cell was equal to or greater than wild type (Fig. 3c). This enrichment for T_R cells in *Ptcra*$^{-/-}$ mice is highly selective: for example, intestinal TCR-αβ^{+}CD8αα^{+} cells are markedly depleted (Supplementary Table 1). Because pTα function precedes double-positive differentiation, its mutation would not be predicted to affect agonist selection into mature CD4^{+} (SP4) thymocytes, supporting which the CD4^{+} thymocyte TCR repertoire is equivalent to wild type (Supplementary Table 2).

To verify independently the relationship between pTα, double-positive cells and T_R differentiation, we examined pTα transgenic

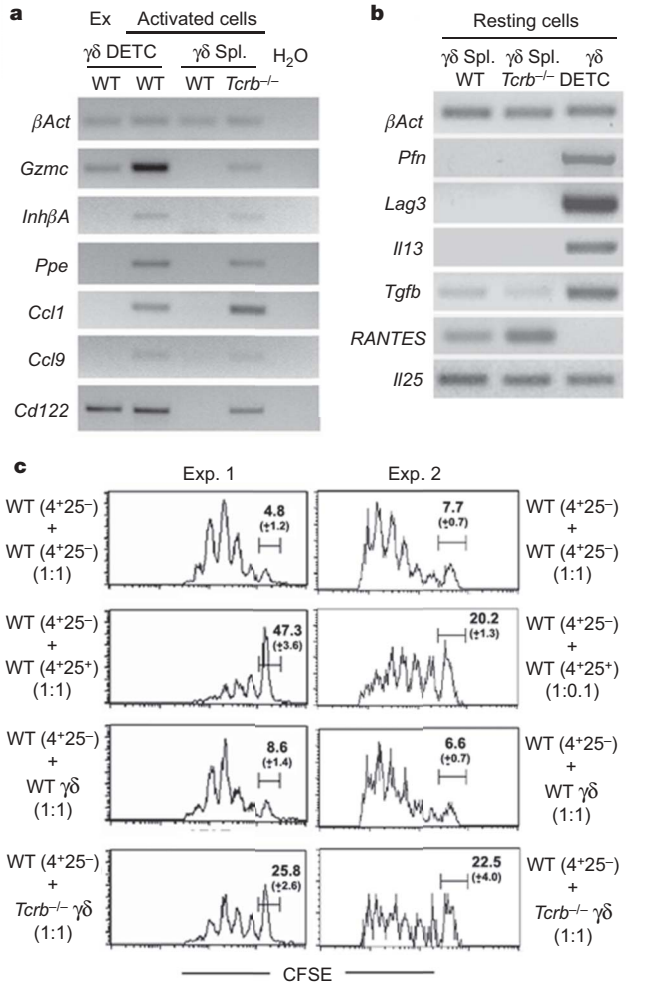

Figure 1 | Activated splenic γδ cells from *Tcrb*$^{-/-}$ mice express activated DETC-specific genes and display regulatory potential *in vitro*. **a**, Semi-quantitative RT–PCR on representative ($n > 3$) cDNA from wild-type TCR-γδ^{+} DETCs *ex vivo* (Ex, γδ DETC, WT), and anti-CD3ε monoclonal-antibody-activated wild-type TCR-γδ^{+} DETCs (activated cells, γδ DETC, WT), wild-type splenic TCR-γδ^{+} cells (activated cells, γδ Spl., WT), and *Tcrb*$^{-/-}$ splenic TCR-γδ^{+} cells (activated cells, γδ Spl., *Tcrb*$^{-/-}$). **b**, Semi-quantitative RT–PCR for genes as indicated, on representative ($n > 3$) cDNA from resting wild-type splenic TCR-γδ^{+} cells (γδ Spl., WT), resting *Tcrb*$^{-/-}$ splenic TCR-γδ^{+} cells (γδ Spl., *Tcrb*$^{-/-}$) or resting *ex vivo* wild-type TCR-γδ^{+} DETCs (γδ, DETC). **c**, CFSE-labelled wild-type CD4^{+}CD25^{-} splenic T cells (5×10^4) were cultured (in triplicate) with either wild-type splenic CD4^{+}CD25^{-} cells (5×10^4), wild-type splenic CD4^{+}CD25^{+} cells (5×10^4 (Exp. 1); 5×10^3 (Exp. 2)), and splenic γδ cells (5×10^4) from wild-type or *Tcrb*$^{-/-}$ mice, plus soluble anti-CD3ε monoclonal antibody (+antigen presenting cells) for 72 h (Exp. 1) or 86 h (Exp. 2). Average percentage of undivided cells (±s.d.) is indicated.

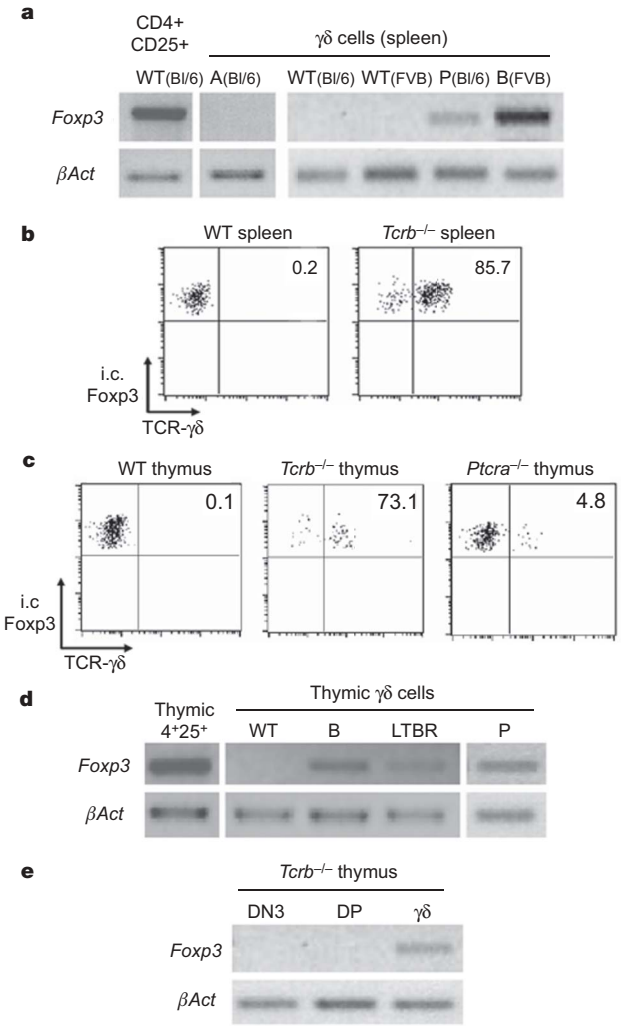

Figure 2 | γδ cells from *Tcrb*$^{-/-}$ and *Ptcra*$^{-/-}$ mice express Foxp3. **a**, Representative ($n > 3$) RT–PCR for *Foxp3* (compared to β-actin) in splenic γδ cells from C57Bl/6J or FVB/n strains of wild-type (WT), *Tcra*$^{-/-}$ (A), *Ptcra*$^{-/-}$ (P) or *Tcrb*$^{-/-}$ (B) mice. The Foxp3^{+} control is wild-type splenic TCR-αβ^{+}CD4^{+}CD25^{+} cells. **b**, **c**, Representative ($n > 6$) intracellular (i.c.) Foxp3 against TCR-γδ plots of (Foxp3^{+}) splenocytes from wild-type and *Tcrb*$^{-/-}$ mice, or thymocytes from wild-type, *Tcrb*$^{-/-}$ or *Ptcra*$^{-/-}$ mice. As these gatings represent minor populations (see text), representative un-gated plots are shown in Supplementary Fig. 2. Quadrant percentages are indicated. **d**, **e**, Representative ($n > 3$) RT–PCR for *Foxp3* in thymocytes from wild-type (WT), *Tcrb*$^{-/-}$ (B), *Ltbr*$^{-/-}$ (LTBR) or *Ptcra*$^{-/-}$ (P) mice as in **a**. DN3 is CD4^{-}CD8α^{-}TCR-αβ^{-}TCR-γδ^{-}B220^{-}NK1.1^{-}DX5^{-}CD25^{+}CD44^{-}; DP (double positive) is CD4^{+}CD8α^{+}; γδ is CD4^{-}CD8α^{-}TCR-γδ^{+}.

mice expressing different levels of pTα on wild-type and *Ptcra*$^{-/-}$ backgrounds[17]. Such mice confirmed an inverse correlation between double-positive cells and the representation of Foxp3^{+} cells among CD4^{+} cells: as *Ptcra*$^{-/-}$ mice were provided with transgenic pTα, more double-positive cells developed and the regulatory:effector balance declined to normal (2.5–4.5%) (Fig. 3d). This inverse relationship was almost superimposable upon that between double-positive cells and γδ cells, expressed as a percentage of total thymocytes[17] (Fig. 3e), but differed completely from the relationship of double-positive cells to unfractionated CD4^{+} thymocytes (Fig. 3f).

pTα expression might dictate T_R-cell representation via a cell-autonomous effect or an effect *in trans*, as exemplified by *trans*-conditioning[7]. To distinguish between these, *Ptcra*$^{-/-}$ thymocyte differentiation was examined in sub-lethally irradiated mice reconstituted with admixed *Ptcra*$^{-/-}$ and wild-type bone marrow, using congenic markers for each donor and the host. After 10–14 weeks, signatory *Ptcra*$^{-/-}$ thymocyte development was evident in a large double-negative compartment, a relatively small double-positive compartment, and some single-positive cells, but this was set against a backdrop of a large double-positive pool supplied by the progeny of wild-type donor and/or recovering host bone marrow (Fig. 4a). In this context, the regulatory:effector ratio among *Ptcra*$^{-/-}$ thymocytes was quantified as the percentage of CD4^{+} cells displaying the T_R phenotype. At minimum there was partial rescue, and three of seven mice displayed equivalent regulatory:effector ratios (for example, 3.9% and 4.5%, respectively) in cells derived from *Ptcra*$^{-/-}$ and wild-type progenitors. Thus, the establishment of a normal ratio can be rescued *in trans* without any obligatory cell-autonomous provision of pTα. Because normal double-positive differentiation is associated with increased thymus size, one might consider size as the primary influence on T_R-cell differentiation. Indeed, a thymus lacking a large double-positive pool does not properly support thymocyte proliferation[18]. However, reflecting variable susceptibility to irradiation, reconstituted thymi varied in size from ~200% to <10% of normal, yet the regulatory:effector cell ratio was similar wherever double-positive cells composed the major thymocyte subset (Fig. 4b). Likewise, γδ cells differentiating from *Ptcra*$^{-/-}$ progenitors in the context of large double-positive compartments regained the aggregate effector gene profile of wild-type γδ cells (data not shown).

Of the four early thymocyte double-negative (DN) stages, *trans*-conditioning targets DN1/DN2 cells (ref. 7 and data not shown) before TCR gene rearrangement. Reflecting this, full genome profiling revealed 360 RNAs ≥2-fold upregulated and 97 RNAs reciprocally downregulated in DN2 cells from wild-type compared with *Ptcra*$^{-/-}$ mice (data not shown). Upregulated RNAs included the *ICER* (also known as *Crem*) transcriptional repressor and many other genes (for example, *Nor1*, *Ccl4*, *Rgs1*, *Nurr1* and *Bcl2a1*) that originally identified *trans*-conditioning[7] (Supplementary Table 3). To further characterize *trans*-conditioned cells, we exploited a transgenic mouse in which the *ICER* promoter drives *lacZ* expression[7]. Numerous *trans*-conditioned genes were expressed more strongly by flow-sorted ICER^{hi} DN2 cells, which also appeared as large blasts compared with smaller ICER^{lo} cells (Supplementary Fig. 3). This suggested that size could separate conditioned (large) DN2-L thymocytes from unconditioned (small) DN2-S thymocytes using

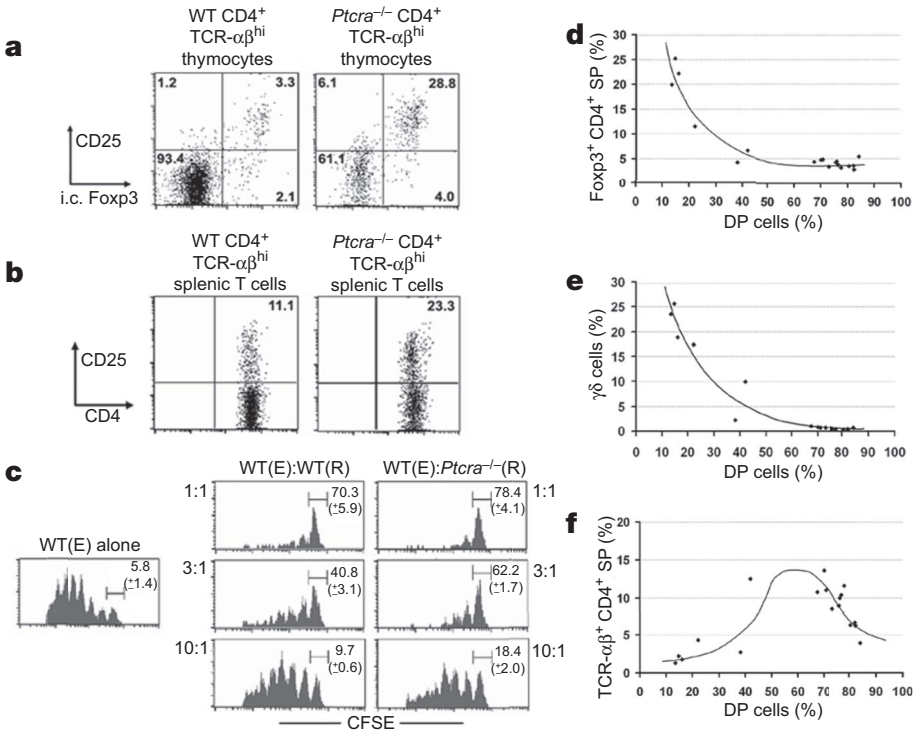

Figure 3 | *Ptcra*$^{-/-}$ CD4^{+}TCR-αβ^{hi} thymocytes and splenocytes are enriched for Foxp3^{+} regulatory cells. a, **b**, Representative ($n > 6$) CD25 or intracellular (i.c.) Foxp3 plots of wild-type or *Ptcra*$^{-/-}$ CD4^{+}TCR-αβ^{hi} single-positive thymocytes or splenocytes. Quadrant percentages are indicated. **c**, CFSE-labelled wild-type CD4^{+}CD25^{-} splenic effector T cells (5×10^4) (WT(E)) were cultured (in triplicate) with either wild-type (WT(R)) or *Ptcra*$^{-/-}$ (*Ptcra*$^{-/-}$(R)) splenic CD4^{+}CD25^{+} cells in the presence of soluble anti-CD3ε monoclonal antibody (+antigen presenting cells) for 72 h. Average percentage of undivided cells (±s.d.) is indicated. **d**–**f**, Adult mice thymus ($n = 18$) from *Ptcra*$^{+/-}$.*Lck-Ptcra-transgene*$^{+/-}$ × *Ptcra*$^{-/-}$ crosses were analysed for percentage CD4^{+}CD8αβ^{+} cells (DP cells (%)), percentage TCR-γδ^{+} cells (γδ cells (%)) and percentage CD4^{+}CD8αβ^{-}TCR-αβ^{+} single-positive cells (TCR-αβ^{+}CD4^{+} SP (%)). The percentage of TCR-αβ^{+}CD4^{+} single-positive cells that was also Foxp3^{+} was also assessed (Foxp3^{+}CD4^{+} SP (%)). Lines in the scatter graphs are suggestive of possible correlation.

unmanipulated wild-type mice. Although size-separation is inevitably imprecise (for example, small cells will include conditioned cells that recently underwent cytokinesis), profiling showed that the aggregate transcriptomes of DN2-L and DN2-S cells were distinct, and by hierarchical clustering analysis DN2-S cells resembled *Ptcra*$^{-/-}$ DN2 cells more than DN2-L cells (Supplementary Fig. 4), consistent with reduced *trans*-conditioning.

To monitor the developmental potentials of the purified DN2 subsets, paired fetal thymic organ cultures (FTOCs) were used. DN2-S cells differentiated slowly, and by day 8 the double-positive pool was much smaller than in the faster differentiating DN2-L cultures (Fig. 4c). However, SP4 cells arose, and using three independent paired FTOCs harvested on days 13–21, we compared the expression of *Cd25*, *Foxp3* and seven genes (*Gitr*, *Lag3*, *Ppe*, *Ctla4*, *Cd27*, *Cd28* and *Cd122*) reportedly enriched in wild-type CD25^{hi} versus CD25^{lo} SP4 cells[19–22]. All were overexpressed in CD25^{hi} SP4 cells from wild-type and *Ptcra*$^{-/-}$ mice, and in SP4 cells derived from DN2-S relative to DN2-L cultures, even without prior CD25 subsetting. Differential expression of *Foxp3* was highly significant at $P < 0.01$, as measured by two-tailed *t*-test. Conversely, cyclin D3 was enriched in wild-type CD25^{lo} SP4 cells and in DN2-L-derived SP4 cells (Fig. 4d, e). In sum, from wild-type DN2 thymocytes (that temporally precede pre-TCR selection into the double-positive pool and subsequent TCR-mediated selection) one can purify DN2-S cells that show reduced evidence of *trans*-conditioning and that differentiate into cells enriched in hallmarks of the T_R-cell phenotype. Unlike other models, these data readily explain the bias towards regulatory differentiation in the αβ and γδ T-cell compartments of mutant mice lacking normal double-positive compartments. A revised model that may be useful as a basis for further experimentation (Fig. 4f; see also Supplementary Fig. 5) proposes that *trans*-conditioning of DN2 progenitors promotes replication and blasting (DN2-L cells) before further pre-TCR-dependent proliferation at the DN4 to double-positive stage. Their progeny that engage partial agonists positively select as CD4$^+$ effectors, whereas those engaging agonists on epithelial or bone-marrow-derived cells die by negative selection. Conversely, some DN2 cells will ordinarily receive less *trans*-conditioning, perhaps stochastically, or because of anatomical (niches) and/or cellular heterogeneity. Such cells differentiate as DN2-S cells without overt expansion before pre-TCR-dependent proliferation. The distinct gene expression of DN2-S-derived double-positive cells then dictates that those engaging agonists, for example on epithelial cells, select as T_R cells, rather than deleting. Thus, whereas effector cell differentiation in *Ptcra*$^{-/-}$ mice is impaired by reduced cell-autonomous expansion and *trans*-conditioning, only the former impairs regulatory cell differentiation. Mathematical models are consistent with unconditioned cells being a major source of T_R cells for which agonist selection[4–6] would be a final, essential manifesting step. It has also been noted that the promotion of T_R-cell differentiation by SHP-1 deficiency is consistent with T_R cells arising from a distinct progenitor

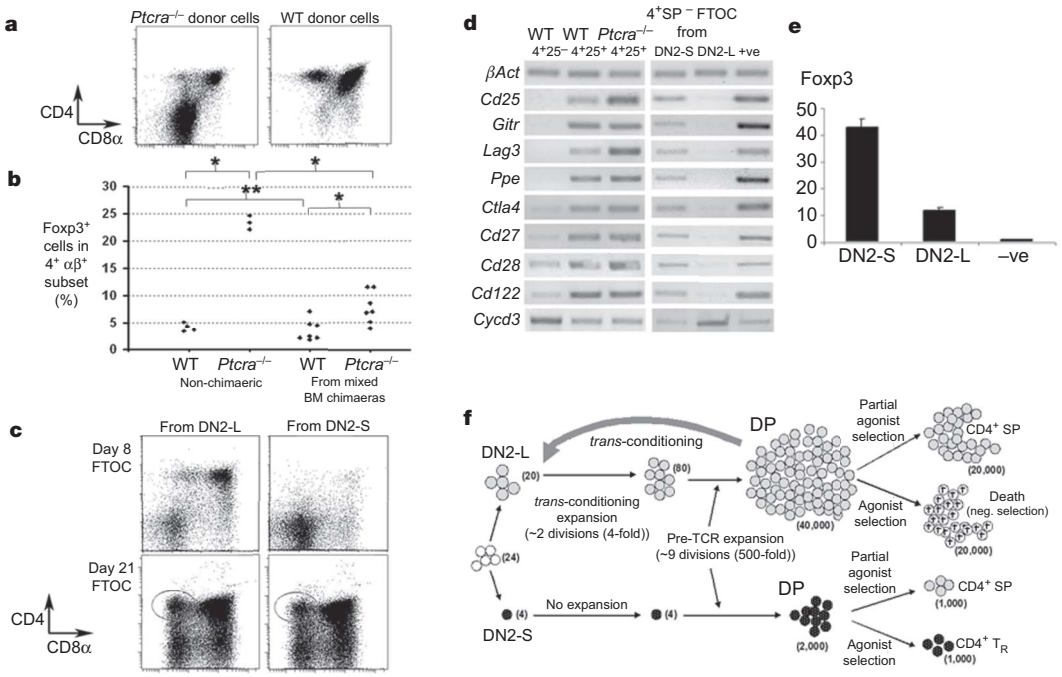

Figure 4 | *Trans*-conditioning regulates the differential capacity of early thymic progenitors to develop into T cells with regulatory characteristics. **a**, Representative ($n = 7$) CD4/CD8α plots of wild-type (Ly5.1) and *Ptcra*$^{-/-}$ (Ly5.2) donor thymocytes from a 12-week bone-marrow chimaera. **b**, Graph displaying percentage of CD4$^+$TCR-αβ$^+$ thymocytes that are Foxp3$^+$ from wild-type and *Ptcra*$^{-/-}$ non-chimaeric mice, and from mixed wild-type/*Ptcra*$^{-/-}$ bone-marrow chimaeras described in **a**. *P*-values are shown; asterisk, $P < 0.05$; double asterisk, not significant at $P = 0.05$. **c**, CD4/CD8α plots of wild-type DN2 thymocytes (CD4$^-$CD8α$^-$TCR-αβ$^-$TCR-γδ$^-$B220$^-$NK1.1$^-$DX5$^-$CD25$^+$CD44$^+$) size-sorted into the largest 20%, DN2-L, and the smallest 20%, DN2-S, and cultured as 8- or 21-day FTOC (1×10^4 cells per lobe). CD4$^+$ single-positive (SP) gates for analyses in panels **d** and **e** are shown (ovals). **d**, Representative ($n = 3$) RT–PCR for regulatory T-cell-associated genes in: wild-type CD4$^+$CD8α$^-$CD25$^-$ cells (WT 4$^+$25$^-$); wild-type CD4$^+$CD8α$^-$CD25$^+$ cells (WT 4$^+$25$^+$); *Ptcra*$^{-/-}$ CD4$^+$CD8α$^-$CD25$^+$ cells (*Ptcra*$^{-/-}$ 4$^+$25$^+$); and CD4$^+$CD8α$^-$ cells from 13-day FTOC (as described for **c**; DN2-S and DN2-L). **e**, Real-time PCR for Foxp3 in CD4$^+$CD8α$^-$ cells from 21-day FTOC (as described for **c**). Units for the *y* axis are Foxp3/HPRT ($\times 10^4$). cDNA from *Rag1*$^{-/-}$ thymus is a negative control (−ve). Results are significantly different by both two-tailed Student's *t*-test (27.1, $P < 0.01$; SPSS v.13.0 software) and non-parametric Mann–Whitney *U*-test ($P < 0.05$). Data show mean +s.d. **f**, Revised working model for thymic T_R-cell development. DN2-L cells upregulate gene expression and undergo expansion by *trans*-conditioning (mediated by WT DP cells), whereas DN2-S cells do neither. DN2-L cells develop into conventional double-positive cells that either positively select on partial agonist or negatively select on full agonist. In contrast, DN2-S cells produce a minor, yet distinct double-positive subset that differentiates to CD4$^+$ T_R cells when encountering full agonist on epithelial cells. This scheme predicts that pTα-deficiency will reduce total thymocyte output and alter the effector:regulatory T-cell balance towards T_R cells. Numbers indicate relative thymocyte number (see Supplementary Fig. 5).

pool[23], which might additionally explain the seeming ceiling on T_R-cell numbers even in TCR-transgenic mice where there is increased opportunity for agonist selection[24,25].

That the regulatory:effector balance increases whenever thymic double-positive pools are reduced may partially explain T_R-cell enhancement in pregnancy, because double-positive cells are depleted, purportedly by steroid hormones[26]. Chronic infections may also deplete thymic double-positive cells, and it will be intriguing if there are parallel increases in regulatory potential, possibly to limit immunopathology. Although lymphotoxin determines several aspects of *trans*-conditioning of γδ cells[7], its contribution to the regulatory:effector balance is less clear. Although its deficiency may reduce conditioning, thus promoting T_R-cell differentiation, its impairment of AIRE expression may limit T_R-cell selection. Thus, future studies must aim to define the molecules that act in early thymocyte differentiation to determine the regulatory:effector balance in the αβ and γδ T-cell compartments.

Note added in proof: Since the preparation of this paper, the over-representation of T_R cells in *Ptcra*$^{-/-}$ mice has also been reported, with additional explanations[30].

METHODS

Mice. Mice have been described previously[16,17,27–29]. For generation of mixed bone-marrow chimaeras, 5×10^6 bone-marrow cells from wild-type (C57/Bl6 Ly5.1$^+$Thy1.2$^+$) or *Ptcra*$^{-/-}$ (Ly5.2$^+$Thy1.2$^+$) mice were mixed at wild-type:*Ptcra*$^{-/-}$ ratios of 1:1, 1:5 or 1:10 and injected intravenously into irradiated (900 rad) wild-type recipient mice (C57Bl/6 Ly5.2$^+$Thy1.1$^+$). Analysis was at 12 weeks.

Flow cytometry. Antibodies were from eBioscience or BD Pharmingen. Pre-enriched double-negative thymocytes were obtained by complement lysis (low-tox-M rabbit complement, Cedarlane) of CD4$^+$ and CD8$^+$ cells using hybridomas; RL172; 31M (gift of R. Ceredig). Intracellular staining of Foxp3 used the eBioscience protocol (number 72-5775-40). Visualization of β-galactosidase expression has been described[7].

RT–PCR. DNase-treated (RQ1, Promega) total RNA became oligodT-primed cDNA using the PowerScript cDNA synthesis kit (Clontech). Semi-quantitative PCR was performed on cDNA equivalent to $1–2 \times 10^3$ cells (Advantage2, Clontech) on a Tetrad DNA engine (MJ Research). Real-time PCR was performed on an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems) using SYBR GreenER qPCR SuperMix (Applied Biosystems). For primers see Supplementary Table 4.

***In vitro* regulatory T-cell assay.** Sorted, CFSE-labelled (Molecular Probes), wild-type CD4$^+$CD25$^-$ T cells (5×10^4 per well) from lymph node/spleen were targets. Sorted, DDAO-SE-labelled (Molecular Probes), wild-type or *Ptcra*$^{-/-}$ lymph node/splenic CD4$^+$CD25$^+$ or γδ cells were potential regulators. Red-blood-cell-depleted mitomycin-C-treated (Sigma) splenocytes were used as antigen presenting cells (5×10^4 per well). Cells were mixed in 96-well round-bottom plates, activated with soluble anti-CD3ε monoclonal antibody (2C-11, Pharmingen) for 72–84 h, and analysed by flow cytometry.

Fetal thymic organ cultures. C57Bl/6 day-15 fetal thymic lobes were treated for 5 days with 2-deoxyguanosine (1.35 mM) in FTOC media (RPMI 10% FCS, 50 μM β-mercaptoethanol, 10 mM HEPES, L-Glut/Pen/Strep). Hanging-drop cultures in Terasaki plates with 1×10^4 thymocytes for 24 h were followed by 8–21-day culture on nucleopore filters in FTOC media, before FACS analysis.

Transcription profiling. Total RNA was amplified and labelled by MessageAmp II aRNA amplification kit and Illumina TotalPrep RNA amplification kit (Ambion). cRNA was hybridized to Sentrix Mouse-6 Expression Beadchips (Illumina). Analysis used Genespring GX7.3 (Agilent Technologies).

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