The EMBO Journal Vol.17 No.18 pp.5349–5359, 1998

Evidence implicating Gfi-1 and Pim-1 in pre-T-cell differentiation steps associated with β-selection

Thorsten Schmidt, Holger Karsunky, Bernd Rödel, Branko Zevnik, Hans-Peter Elsässer[1] and Tarik Möröy[2]

Institut für Zellbiologie (Tumorforschung), IFZ, Universitätsklinikum Essen, Virchowstrasse 173, D-45122 Essen and [1]Institut für Zytobiologie und Zytopathologie, Philipps Universität Marburg, Robert-Koch-Strasse 5, D-35033 Marburg, Germany

[2]Corresponding author
e-mail: moeroey@uni-essen.de

After rearrangement of the T-cell receptor (TCR) β-locus, early $CD4^-/CD8^-$ double negative (DN) thymic T-cells undergo a process termed 'β-selection' that allows the preferential expansion of cells with a functional TCR β-chain. This process leads to the formation of a rapidly cycling subset of DN cells that subsequently develop into $CD4^+/CD8^+$ double positive (DP) cells. Using transgenic mice that constitutively express the zinc finger protein Gfi-1 and the serine/threonine kinase Pim-1, we found that the levels of both proteins are important for the correct development of DP cells from DN precursors at the stage where 'β-selection' occurs. Analysis of the $CD25^+/CD44^{-,lo}$ DN subpopulation from these animals revealed that Gfi-1 inhibits and Pim-1 promotes the development of larger β-selected cycling cells ('L subset') from smaller resting cells ('E subset') within this subpopulation. We conclude from our data that both proteins, Pim-1 and Gfi-1, participate in the regulation of β-selection-associated pre-T-cell differentiation in opposite directions and that the ratio of both proteins is important for pre-T-cells to pass the 'E' to 'L' transition correctly during β-selection.

Keywords: β-selection/Gfi-1/Pim-1/pre-T-cell development/zinc finger protein

Introduction

T-cells develop from poorly defined precursor cells that infiltrate the thymus from the bone marrow and undergo a series of successive phenotypic transitions that are accompanied by several selection steps, the appearance of specific surface markers and DNA rearrangements of the antigen receptor loci (for a review see Fehling and von Boehmer, 1997). The most mature TCR α/β^+CD4^+ and TCR α/β^+CD8^+ cells (single positive cells, SP) emerge by a process called 'positive selection' from a pool of $CD4^+/CD8^+$ double positive (DP) cells that constitute the major part of the thymus (reviewed in Benoist and Mathis, 1997). DP cells develop from the $CD4^-/CD8^-$ double negative (DN) population that consists of bone marrow-derived precursor cells. These precursors undergo several differentiation and proliferative expansion steps regulated by members of the haematopoietin family of cytokines, particularly interleukin-7 (IL-7) (Murray *et al.*, 1989; Peschon *et al.*, 1994; von Freeden-Jeffrey *et al.*, 1995, 1997; Maraskovsky *et al.*, 1997) and the signalling through the pre-T-cell receptor (TCR) complex (Groettrup and von Boehmer, 1993; Fehling *et al.*, 1995) to give rise to $CD4^+/CD8^+$ DP cells. According to their differential expression of the surface molecules CD25 (IL-2 receptor α chain) and CD44 (Pgp-1), immature DN cells can be subdivided into four subpopulations (Pearse *et al.*, 1989; Godfrey and Zlotnik. 1993; Godfrey *et al.*, 1993). The two major DN subpopulations consist of $CD25^+/CD44^{-,lo}$ cells that give rise to $CD25^-/CD44^{-,lo}$ cells which then quickly upregulate CD4 and CD8 and constitute the DP population. The rearrangement of the TCR β-chain genes takes place in cells of the $CD25^+/CD44^{-,lo}$ DN population. DP cells and $CD25^-/CD44^{-,lo}$ DN cells emerging from this pool express functional TCR β-chains and are termed 'β-selected' (Mallick *et al.*, 1993; Dudley *et al.*, 1994). The process of β-selection is restricted to the $CD25^+/CD44^{-,lo}$ DN cells and appears to be governed by the assembly of a TCR β-chain, a pTα molecule and CD3 (Fehling and von Boehmer, 1997). During β-selection, $CD25^+/CD44^-$ DN cells that productively rearrange their TCR β locus and are able to form a pre-TCR complex start to proliferate and enter the next developmental stage; they downregulate CD25 expression and start to upregulate CD4 and CD8. Cells from the $CD25^+/CD44^{-,lo}$ DN population that fail to rearrange both TCR β alleles productively remain quiescent and die unless they have the potential to become γ/δ cells. A closer analysis of the $CD25^+/CD44^{-,lo}$ DN cells revealed that this population can be divided further into two subsets: one that contains larger, cycling cells (~15%, termed 'L' cells) with a high proportion of in-frame β-rearrangements and a second subset that represents the major pre-selected population. These cells are termed 'E' cells for 'expected size' and are smaller than the 'L' cells. They are resting cells without an enrichment of in-frame β-rearrangements and represent ~85% of the whole $CD25^+/CD44^{-,lo}$ DN population (Hoffmann *et al.*, 1996).

The Gfi-1 zinc finger protein is expressed at readily detectable levels almost exclusively in thymocytes, but its role in pre-T-cell development has not been investigated so far. The *gfi-1* gene was first discovered as an integration site for Moloney murine leukaemia virus (MoMuLV) in virally infected cells that were selected for IL-2 independence (Gilks *et al.*, 1993). Other studies involving MoMuLV-infected transgenic mice already carrying the *trans*-oncogenes *pim-1* and L-*myc* in their germline showed that the *gfi-1* gene is a frequent target for the integration of proviral DNA and is most likely involved in the accelerated progression of lymphoid malignancies in MoMuLV-infected lymphoid cells. These findings provided evidence that Gfi-1 can act synergistically at least

in the process of lymphomagenesis with Myc, a helix–loop–helix zinc finger (HLH-LZ) transcription factor, and Pim-1, a cytoplasmic serine/threonine kinase (Schmidt *et al.*, 1996; Zörnig *et al.*, 1996; Scheijen *et al.*, 1997). The *pim-1* gene was itself first identified as a MoMuLV proviral insertion site, and studies in *pim-1* transgenic mice demonstrated a low oncogenic potential for Pim-1 (Selten *et al.*, 1985; van Lohuizen *et al.*, 1989). Biochemical studies suggested that Gfi-1 functions as a transcriptional repressor that mediates its activity by sequence-specific DNA binding in a position-independent manner (Grimes *et al.*, 1996a; Zweidler-McKay *et al.*, 1996). Although the biological role of Gfi-1 remains to be clarified, several *in vitro* studies indicated that a constitutive expression can relieve peripheral mature T-cells from a requirement for IL-2 to overcome a G_1 arrest (Grimes *et al.*, 1996a) or, in general, could help to sustain cell proliferation of IL-2 dependent cells in the absence of the cytokine (Zörnig *et al.*, 1996). Surprisingly, Gfi-1 expression is highest in thymocytes (Gilks *et al.*, 1993) where potential signalling via the IL-2 receptor is restricted to the more mature cells that are programmed to leave the thymus to constitute the peripheral immune response.

Experimental results that could shed some light on the role of Gfi-1 in pre-T-cell development in the thymus do not exist at present. Similarly, Pim-1 is expressed in thymic pre-T-cells and its function in pre-T-cell development is not clear either, but MoMuLV infection experiments provided strong evidence that Gfi-1 and Pim-1 cooperate efficiently in T-cell tumorigenesis (see above). This suggests that both proteins are acting in complementary signal transduction pathways that have not yet been identified. Therefore, both proteins are likely to be of considerable interest with regard to a potential role in T-cell differentiation. To be able to gain first insight into the functions of Gfi-1 and Pim-1 in T-cell development and into the nature of their synergistic effects, we analysed gain-of-function mouse mutants that overexpress either Pim-1 or Gfi-1 specifically in T-cells. Analysis of these mouse models showed that high levels of Gfi-1 can inhibit the development of DP thymic T-cells from DN precursors. More precise inspection of the DN subpopulations revealed that the β-selection-associated development of larger cycling L cells from smaller resting E cells within the $CD25^+/CD44^{-,lo}$ subset was disturbed by Gfi-1, suggesting that Gfi-1 interferes directly with β-selection-associated processes. In contrast, we find that high levels of Pim-1 can promote pre-T-cell development through β-selection and that Pim-1 can relieve the Gfi-1-imposed block in Gfi-1/Pim-1 double transgenic mice. We propose a model in which Gfi-1 and Pim-1 can regulate the process of β-selection and in particular the E to L cell transition within the $CD25^+/CD44^{-,lo}$ DN pre-T-cell subset in opposite directions. Our findings infer that both proteins are likely candidates for factors that participate in the regulation of β-selection at a critical point in pre-T-cell differentiation.

Results

Thymic cellularity is reduced in mice overexpressing an lck-driven gfi-1 transgene

In order to investigate a possible function of Gfi-1 in T-cell development, we generated transgenic mice that constitutively express the *gfi-1* gene at a higher than endogenous level in the thymus. The murine *gfi-1* cDNA (Zörnig *et al.*, 1996) was placed between the proximal *lck* promoter and genomic sequences derived from the human growth hormone (hGH) gene (Figure 1A). This construct had been used successfully in earlier experiments (Zörnig *et al.*, 1996). The portion of the construct consisting of the *lck* promoter, the *gfi-1* cDNA and the hGH 3′-untranslated region (Figure 1A) was microinjected into fertilized oocytes according to standard procedures (see Materials and methods) and several founder animals were obtained. Two founders were chosen to establish transgenic lines by continuous backcrossing with C57/Bl6 animals (lines *lck-gfi-1* 124 and *lck-gfi-1* 223). Compared with wild-type animals, mice of both *lck-gfi-1* transgenic lines expressed the *gfi-1* transgene at elevated levels in the thymus; line 223 at slightly higher protein levels than line 124 (Figure 1A). The size and cellularity of the thymus were found to be reduced drastically in animals of both *lck-gfi-1* transgenic lines. Cell numbers dropped to ~5–20% of normal control littermates (Figure 1B). Accordingly, the size of transgenic thymi was significantly smaller but thymic architecture *per se* was not significantly altered (Figure 1C and D). The boundaries between thymic cortex and medulla, clearly visible in the normal control, are less pronounced in some areas but still present in thymi from transgenic mice (Figure 1C and D). Forced co-expression of the cytoplasmic protein kinase Pim-1 by crossing *E*μ *pim-1* transgenic mice that express the Pim-1 serine/threonine kinase in both T- and B-lymphoid compartments (van Lohuizen *et al.*, 1989) with *lck-gfi-1* animals could partially restore thymic cellularity in *lck-gfi-1* transgenic mice (Figure 1B). These findings suggested that overexpression of Gfi-1 impairs normal thymopoiesis and that Pim-1 can to some extent antagonize this activity of Gfi-1.

Differentiation from DN to DP cells is inhibited by high levels of Gfi-1 but can be rescued by co-expression of Pim-1

To identify the differentiation status and thereby the mechanism responsible for the observed loss in thymic cellularity in *lck-gfi-1* transgenics, we analysed the frequencies of CD4 and CD8 subsets by flow cytometry. Although all CD4/CD8 thymocyte subsets are present, both transgenic lines displayed drastic alterations in relative percentages and in absolute cell numbers of the different CD4/CD8 subpopulations (Figure 2A, D and E). In particular, a significant reduction in absolute cell numbers of the SP and the DP subsets was observed (Figure 2E), whereas the absolute cell numbers of the DN population remained largely unchanged compared with non-transgenic littermates (Figure 2E). In all cases, the transgenic line with the higher level of transgene expression (*lck-gfi-1* 223) consistently had a more profound phenotype (Figure 2A).

One possible reason for the apparent loss of $CD4^+/CD8^+$ DP cells could be that high level Gfi-1 expression renders these cells more susceptible to programmed cell death. However, thymocytes explanted from *lck-gfi-1* mice were no more prone to cell death than their counterparts from normal littermates when left untreated in medium or when apoptosis was induced with DNA-damaging agents or steroids (data not shown). It has been reported that Gfi-1 directly represses the expression of the Bax and Bak

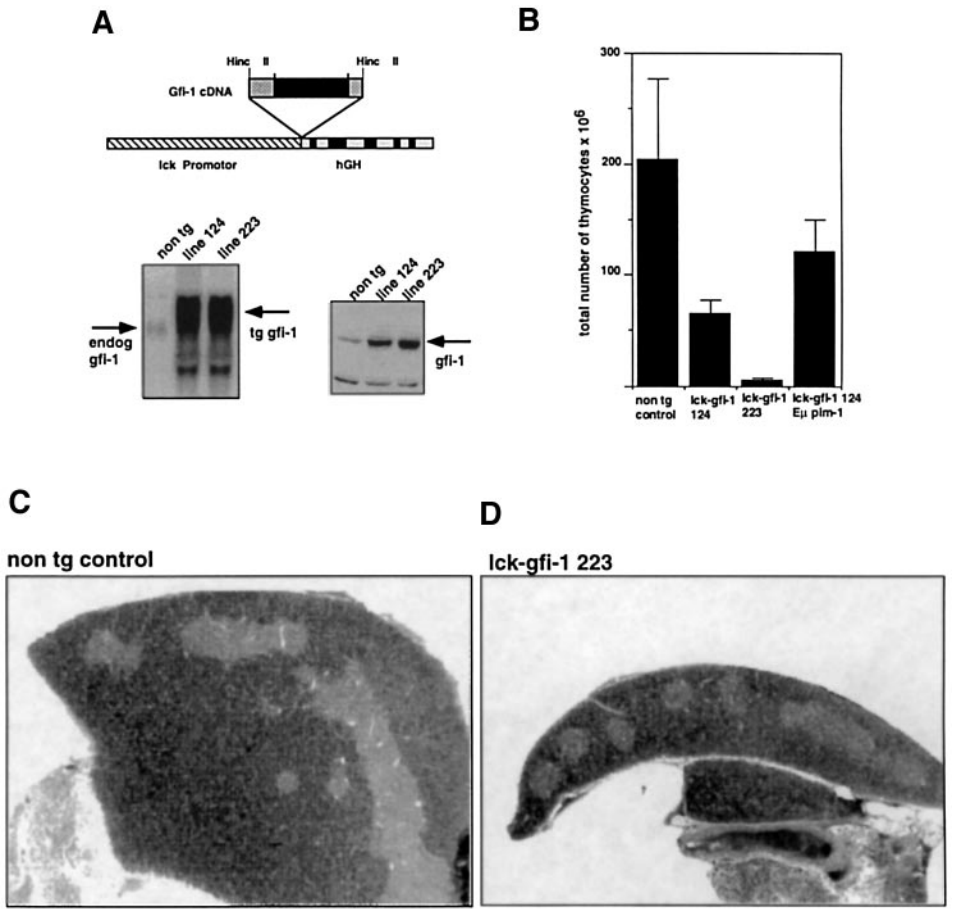

Fig. 1. Transgenic *gfi-1* construct, transgene expression and histological analysis of the thymus of *lck-gfi-1* transgenic mice. (**A**) Schematic representation of the construct used to generate *lck-gfi-1* transgenic mice and detection of endogenous and transgenic Gfi-1-specific mRNA (left panel) and Gfi-1 protein of two *lck-gfi-1* transgenic lines 124 and 223 in comparison with control thymus from non-transgenic littermates. Protein expression from the *gfi-1* transgene is slightly higher in line 223 than in line 124. (**B**) Total number of cells per thymus for non-transgenic control animals ($n = 10$) and for transgenic animals from the lines *lck-gfi-1* 124 ($n = 6$) and *lck-gfi-1* 223 ($n = 7$), and from doubly transgenic mice ($n = 4$) resulting from cross-breeding of the lines *Eμ pim-1* (van Lohuizen *et al.*, 1989; Zörnig *et al.*, 1996) and *lck-gfi-1* 124. All animals were 4–8 weeks old. (**C** and **D**) H/E staining of the histological sections demonstrates the size reduction of the transgenic thymus compared with a normal thymus. Shown are photomicrographs of stained histological sections through thymi from a normal control animal (C) and a *lck-gfi-1* transgenic mouse (D) representative of several individual animals of both transgenic lines (enlargement was 320-fold).

proteins (Grimes *et al.*, 1996b). However, no significant changes in the RNA expression levels of Bcl-2 family members, Bax, Bak, Bcl-x or Bcl-2, could be detected in thymi from *lck-gfi-1* transgenic mice (data not shown), indicating that the observed loss of DP cells in *lck-gfi-1* animals is not due to a higher susceptibility of these cells to undergo cell death. To rule out conclusively that apoptosis is responsible for the loss of DP cells, we crossed the *lck-gfi-1* transgenic mice of both lines (124 and 223) to animals that express the *Eμ bcl-2* transgene that has been shown previously to be active and expressed in B- and T-lymphoid cells and in particular to be able to rescue DP cells from apoptosis induced by a variety of stimuli (Sentman *et al.*, 1991). However, double *lck-gfi-1*/*Eμ bcl-2* transgenics do not show an altered phenotype compared with single *lck-gfi-1* transgenics, i.e. a similar degree of DP cell loss was observed in *gfi-1/bcl-2* double transgenics (Figure 2B). This inability of *bcl-2* to rescue the phenotype seen in *lck-gfi-1* mice clearly confirmed that the loss of cellularity and in particular the loss of DP cells seen in *lck-gfi-1* mice is not due to programmed cell death.

In a subsequent experiment, we analysed the double transgenic mice that resulted from a cross between *lck-gfi-1* transgenics from the line 124 and *Eμ pim-1* animals. In contrast to the *gfi-1/bcl-2* mice, we observed that the loss of DP and SP thymocyte subpopulations was indeed restored to almost normal numbers in double *pim-1/gfi-1* transgenics (Figure 2C), suggesting that Pim-1 is able to counteract the effect of Gfi-1 overexpression on thymic pre-T-cell subsets. One consequence of this combination of Pim-1 and Gfi-1 expression was the restoration of normal numbers of CD4/CD8 DP cells (see Figure 2) but also the rapid development of thymic lymphomas in double transgenic mice (Schmidt *et al.*, 1998). When the *lck-gfi-1* line 223 was used for crossings into the Pim-1 transgenics, the synergistic effect of the combination of Pim-1 and Gfi-1 was so strong that no tumour-free animals were obtained for analysis at 4–6 weeks of age (not shown).

V(D)J recombination or lineage decisions do not appear to be affected by high levels of Gfi-1

Examination of the expression of TCR α/β, TCR γ/δ, CD3 and CD25 by flow cytometry showed that both types

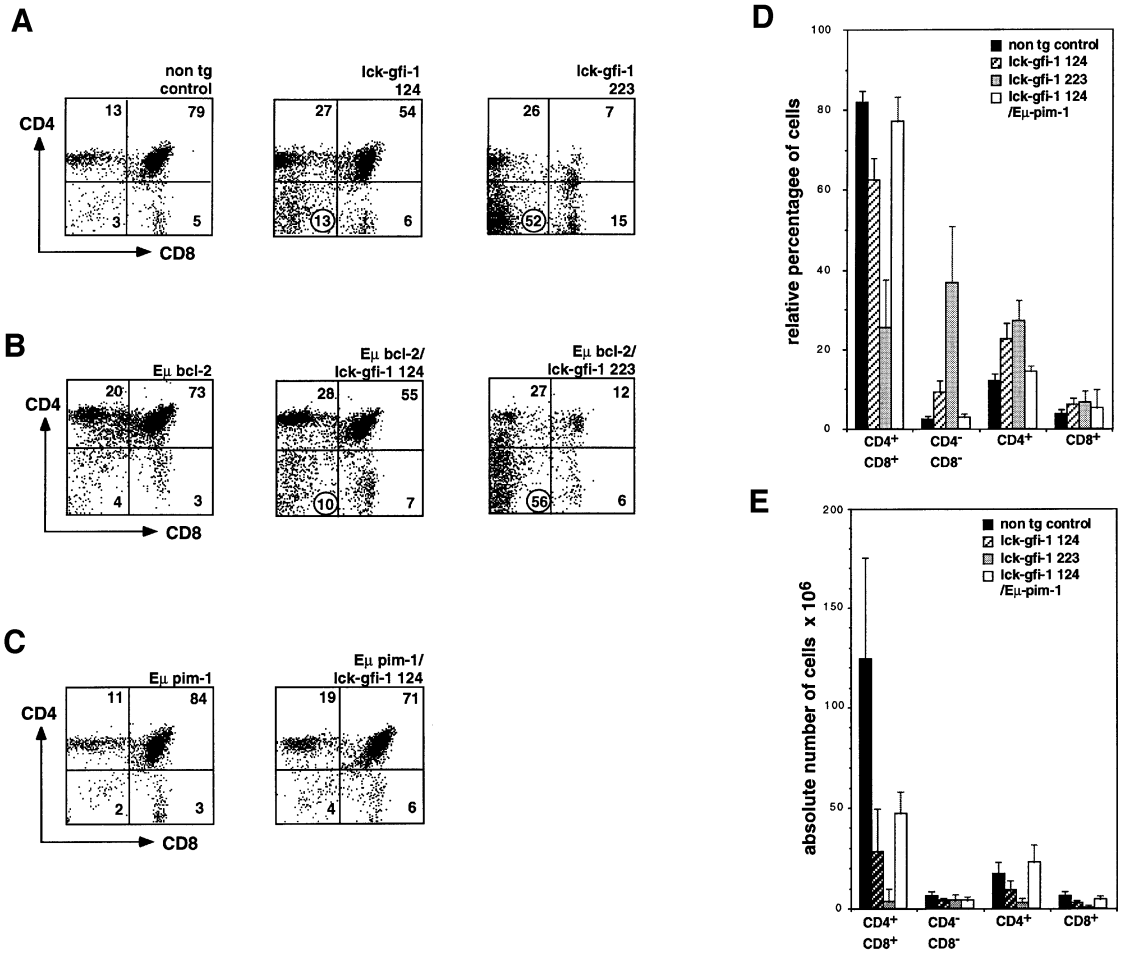

Fig. 2. Quantification of the major CD4- and CD8-bearing subpopulations in thymus from transgenic and normal mice. (**A**–**C**) Thymocytes from 4- to 8-week-old *lck-gfi-1* transgenic mice of both lines 124 and 223, Eμ *pim-1* animals, Eμ *bcl-2* animals, normal control mice and double transgenic mice from a cross between *lck-gfi-1* lines and Eμ *pim-1* or Eμ *bcl-2* animals were isolated, stained with fluorescein isothiocyanate (FITC)-labelled anti-CD4 and phycoerythrin (PE)-labelled anti-CD8 antibodies and analysed by flow cytometry. Documented are dot plots representative of several animals, with the relative percentage of cells bearing CD4 or CD8 falling into the respective quadrants [*lck-gfi-1* 124 (n = 7), *lck-gfi-1* 223 (n = 10), normal control littermates (n = 9), Eμ *bcl-2* (n = 5), Eμ *bcl-2/lck-gfi-1* 124 (n = 3), Eμ *bcl-2*/*lck-gfi-1* 223 (n = 2), Eμ *pim-1* (n = 3), Eμ *pim-1/lck-gfi-1* 124 (n = 6)]. (**D**) Relative percentages of cells that fall into the four CD4/CD8 subpopulations are shown for both *lck-gfi-1* lines, for double transgenic Eμ-*pim/lck gfi-1* 124 animals as well as for age-matched littermate controls. Shown are average values with standard deviations for several mice representing the two different transgenic lines *lck-gfi-1* 124 (n = 7) and *lck-gfi-1* 223 (n = 10), normal control littermates (n = 9) or Eμ *pim-1*/*lck-gfi-1* 124 double transgenics (n = 6). (**E**) Absolute numbers of cells of each CD4/CD8 subpopulation were counted from several 4- to 8-week-old mice of the *lck-gfi-1* line 124 (n = 4), the *lck-gfi-1* line 223 (n = 6), double Eμ *pim-1/lck-gfi-1* 124 mice and normal control littermates (n = 8) and are shown as average values with standard deviation.

of TCRs and CD3 molecules as well as the IL-2 receptor α-chain (CD25) are expressed on thymocytes of *lck-gfi-1* transgenics, including the major subtypes of TCR β- and α-chains (Vα8 subtype) (Figure 3). However, the relative numbers of TCR γ/δ-bearing cells as well as the relative numbers of $CD25^+$ and $CD3^{hi}$ cells are altered in thymocytes from both *lck-gfi-1* transgenics lines compared with normal control cells (Figure 3A and B), which is very probably due to the higher relative proportion of $CD4^-$/$CD8^-$ DN cells in transgenic thymi (see above; Figure 2). Absolute numbers of TCR α/β cells were drastically reduced in the transgenic mice down to 10% of control levels (Figure 3C), as was expected considering the loss of DP cells in the *lck-gfi-1* transgenics. Also, the absolute number of TCR γ/δ-bearing cells was reduced in *lck-gfi-1* animals albeit only to ~50% of the levels in non-transgenic controls (Figure 3D). The findings further confirm the notion that Gfi-1 overexpression blocks the development of DP from DN precursors, but demonstrate that this is very unlikely to be due to an altered V(D)J recombination programme, TCR assembly or CD3 expression. Our results rather suggest that high Gfi-1 levels mainly block the replenishment of the thymus with TCR α/β DP and SP cells by inhibiting differentiation of DP cells from the DN precursor pool.

Gfi-1 and Pim-1 overexpression can influence the frequencies and proliferation of $CD4^-/CD8^-$ (DN) subpopulations in the thymus

Next, we tested the effect of Gfi-1 and Pim-1 overexpression on the different subsets within the DN subpopulation. The analysis of the expression of CD25 had already shown that the relative number of cells bearing this surface marker was increased in the *lck-gfi-1* transgenic mice (Figure 3A). The DN thymocyte subpopulation can be subdivided further into several cellular subsets according

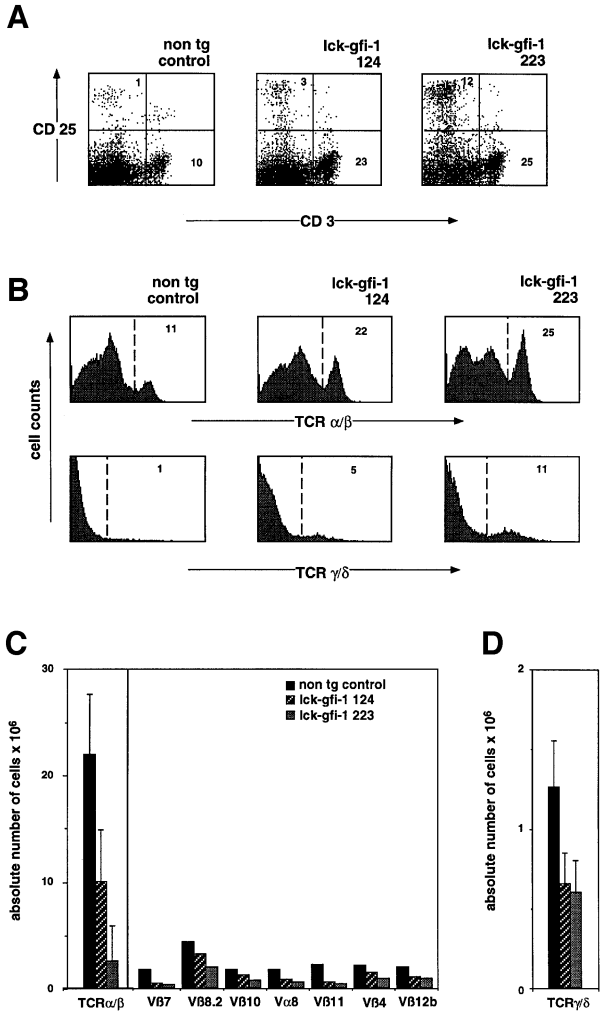

Fig. 3. Expression of TCR, CD25 and CD3 molecules on thymocytes from *lck-gfi-1* transgenics. (**A** and **B**) *lck-gfi-1* transgenic mice show normal expression levels of CD25, CD3 and of both types of TCR. Thymocytes from both transgenic lines and a non-transgenic control were isolated and stained with FITC- or PE-conjugated antibodies against CD3, CD25, the TCR β-chain common epitope (α/β TCR, common β-chain epitope, H57-597) or TCR γ/δ. Shown are values representative of each *lck-gfi-1* line and normal control mice. (**C**) Absolute numbers of TCR α/β-bearing cells are reduced in both lines of *lck-gfi-1* transgenic mice down to 10% of numbers in controls (line 223). A number of Vβ and Vα subtypes of variable chains can be detected on thymocytes of both *lck-gfi-1* transgenic lines. (**D**) The absolute numbers of TCR γ/δ cells are decreased in transgenic thymi to ~50% of the numbers found in normal littermate controls. For the absolute numbers of TCR α/β and TCR γ/δ cells, average values are shown with standard deviation from normal control animals ($n = 6$), from *lck-gfi-1* 124 mice ($n = 2$) and from the *lck-gfi-1* 223 line ($n = 4$).

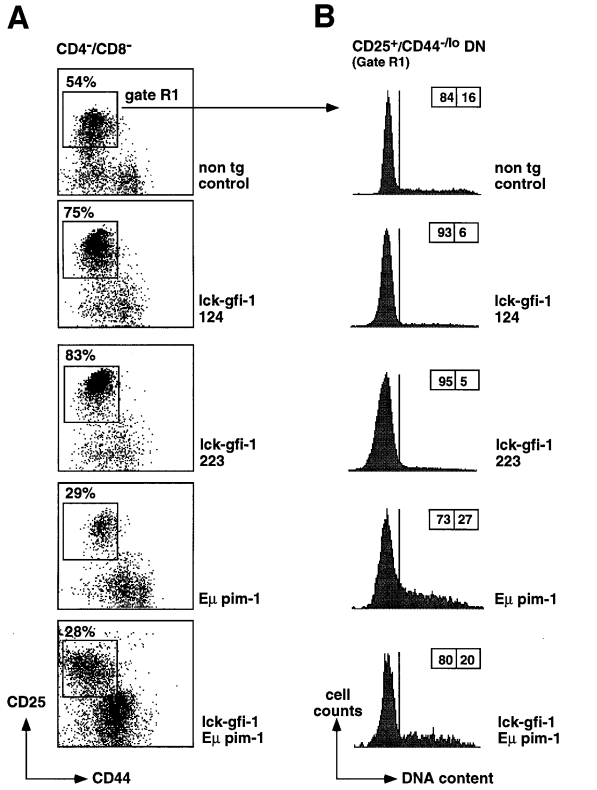

Fig. 4. Characterization of the CD4⁻/CD8⁻ DN subpopulations in normal and transgenic mice according to their expression of CD25 and CD44 surface markers. Gate R1: $CD25^+/CD44^{-,lo}$ cells. Thymocytes were depleted of CD4/CD8 positive cells with magnetic beads coupled with specific antibodies (Dynal), stained with FITC-labelled anti-CD25 and PE-labelled anti-CD44 antibodies and analysed for CD25 and CD44 expression on dual parameter dot plots. (**A**) Within the CD4⁻/CD8⁻ (DN) population, $CD25^+/CD44^{-,lo}$ cells (gate R1) are present at higher frequencies in *lck-gfi-1* transgenic mice at the expense of $CD25^-/CD44^{-,lo}$ cells compared with normal controls. This effect is more pronounced in the *lck-gfi-1* 223 line that has a higher transgene expression level. By contrast, *E*μ *pim-1* mice show a lower percentage of $CD25^+/CD44^{-,lo}$ cells compared with normal controls. Doubly Pim/Gfi-1 animals have similar numbers of $CD25^+/CD44^{-,lo}$ cells compared with *E*μ *pim-1* single transgenics. Data are representative of several *lck-gfi-1* transgenic mice (line 124, $n = 5$, line 223, $n = 8$), *E*μ*-pim-1* ($n = 6$), *E*μ*-pim-1/lck-gfi-1* 124 ($n = 3$) and non-transgenic controls ($n = 8$). (**B**) DN $CD25^+/CD44^{-,lo}$ cells were stained with the DNA dye Hoechst 33382 and analysed for cells in G_1/G_0 phase and in $S/G_2/M$ phase according to their DNA content. Cells from the $CD25^+/CD44^{-,lo}$ subset appeared to be arrested in G_1/G_0 in both *gfi-1* transgenic lines because they show only 3–5% of cells in $S/G_2/M$ phase compared with controls or *E*μ *pim-1* mice which showed more cells in $S/G_2/M$ (27%) compared with normal cells (16%).

to the expression of CD25 and CD44 surface markers. As the absolute number of DN cells is not significantly altered in *lck-gfi-1* transgenics compared with normal control mice (Figure 2C), the CD25/CD44-expressing subpopulations could be compared directly between transgenic and non-transgenic mice. Therefore, we prepared DN cells of *lck-gfi-1* and control animals by depleting them of CD4 and CD8 positive cells with antibody-coupled magnetic beads. The depleted cells were first checked by flow cytometry to be >99% CD4⁻/CD8⁻ and then stained for CD25 and CD44 surface markers. The results depicted in Figure 4A illustrate that expression of the *gfi-1* transgene correlated with a strong increase of $CD25^+/CD44^{-,lo}$ cells (75 and 83% for gate R1) at the expense of $CD25^-/CD44^-$ and $CD25^-/CD44^+$ cells within the DN population in both transgenic mouse lines compared with values obtained from normal controls (54% for gate R1). This suggested that overexpression of Gfi-1 can provoke a developmental block in pre-T-cell differentiation at transition from $CD25^+/CD44^{-,lo}$ cells to $CD25^-/CD44^{-,lo}$ cells, leading to a situation where almost all cells in the DN population are of the $CD25^+/CD44^{-,lo}$ type.

By contrast, in *E*μ *pim-1* transgenics, the relative numbers for the $CD25^+/CD44^{-,lo}$ cells dropped to 28% compared with normal controls (54% for gate R1; Figure

4A). Given that DN to DP transition is not disturbed in *Eμ pim-1* mice, this could indicate that high levels of Pim-1 can promote the development of $CD25^+/CD44^{-,lo}$ pre-T-cells towards $CD25^-/CD44^{-,lo}$ cells. Forced co-expression of a Gfi-1 transgene in double Pim-1/Gfi-1 transgenics did not alter this low percentage of $CD25^+/CD44^{-,lo}$ cells (29%; Figure 4A), indicating that the presence and expression of Pim-1 can overrule the effect of the Gfi-1 transgene. This is in agreement with the finding that the loss of $CD4^+/CD8^+$ DP cell numbers seen in *lck-gfi-1* mice is restored in *pim-1/gfi-1* double transgenic mice. However, it also suggests that high level expression of Pim-1 and of Gfi-1 disturbs pre-T-cell differentiation. We also observed that in *Eμ pim-1* transgenics the $CD25^-/CD44^{-,lo}$ DN cell population is underrepresented in comparison with non-transgenic mice (Figure 4A). Given that in *Eμ pim-1* transgenics a more rapid transition from the DN to the DP stage occurs, this could be interpreted as the result of a more rapid formation of DP cells from $CD25^-/CD44^{-,lo}$ DN cells leading to a smaller size of this particular DN subset.

To measure a potential influence of high levels of Gfi-1 and Pim-1 on cell cycle progression of $CD25^+/CD44^{-,lo}$ pre-T-cells undergoing β-selection, DN cells were prepared, stained with the DNA dye Hoechst 33382 as well as for CD25 and CD44 surface markers and then gated as described in Figure 4A. In both transgenic lines, Gfi-1 alone appeared to arrest $CD25^+/CD44^{-,lo}$ cells in the G_1/G_0 phase of the cell cycle (Figure 4B), pointing to an inhibitory effect of Gfi-1 on cell cycle progression. Results obtained with *Eμ pim-1* mice showed that high levels of Pim-1 alone promoted cell cycle progression in $CD25^+/CD44^{-,lo}$ cells evidenced by almost 2-fold more cells in $S/G_2/M$ phase in transgenics compared with normal controls (Figure 4B). Co-expression of both Pim-1 and Gfi-1 in double transgenic animals restored a normal proliferation rate in $CD25^+/CD44^{-,lo}$ DN cells (Figure 4B). The findings suggested that elevated levels of Gfi-1 negatively affects and Pim-1 positively affects cell cycle progression in $CD25^+/CD44^{-,lo}$ DN cells.

Gfi-1 and Pim-1 have antagonistic effects on the transition from E to L cells during β-selection

During β-selection, proliferation of DN thymocyte subsets and in particular the $CD25^+/CD44^{-,lo}$ DN cells occurs upon pre-TCR-mediated signaling in the L subset of the $CD25^+/CD44^{-,lo}$ DN subpopulation. To obtain a more precise picture and a possible explanation for why $CD25^+/CD44^{-,lo}$ cells in *lck-gfi-1* transgenic mice proliferate at a slower rate as seen in Figure 4, we gated the $CD25^+/CD44^{-,lo}$ DN subset on CD4/CD8-depleted cells as described in Figure 4A (gate R1) and analysed their size by analysing cell counts against forward scatter. The boundary between E and L type cells according to their size was done as described (Hoffmann *et al.*, 1996; Figure 5A, dashed line) and by comparing them with $CD25^+/CD44^{-,lo}$ DN pre-T-cells from Rag-2-deficient mice that only have E type cells (not shown). We found that the relative proportions of E-type smaller cells versus L-type cells within the $CD25^+/CD44^{-,lo}$ DN subpopulation were drastically altered by high levels of Gfi-1 (Figure 5). In both lines of *gfi-1* transgenic mice, the $CD25^+/CD44^{-,lo}$ DN L-subset is almost non-existent or significantly reduced (4–5% versus 15% in normal mice; Figure 5A) and cells of the E-type subset dominate this population. As E cells are arrested in G_1/G_0 and L cells proliferate at a very high rate (Hoffmann *et al.*, 1996; Figure 6), the latter account for the cells in $S/G_2/M$ phase seen in the histograms of $CD25^+/CD44^{-,lo}$ cells depicted in Figure 4B. This clearly explains the lower proliferation rate seen in $CD25^+/CD44^{-,lo}$ cells derived from *lck-gfi-1* transgenic mice. If Gfi-1 blocks the development of L cells from E cells, then a lower proliferation rate of the whole population of $CD25^+/CD44^{-,lo}$ cells must be the consequence.

Eμ pim-1 mice showed a higher proportion of L-cells than non-transgenic littermates (Figure 5A), which is in agreement with the higher rate of cell proliferation observed for the $CD25^+/CD44^{-,lo}$ subset in *Eμ pim-1* mice (Figure 4B). Here, the entire $CD25^+/CD44^{-,lo}$ subset, even the E subset, appeared to consist of larger cells (Figure 4B). The overexpression of both Pim-1 and Gfi-1 in *lck-gfi-1/Eμ pim-1* double transgenic mice restored the relative proportions of E and L cells in the $CD25^+/CD44^{-,lo}$ DN subpopulation approximately to those found in non-transgenic animals, but not the size of E cells (Figure 5A). These findings suggest that an elevated Gfi-1 expression can inhibit and high Pim-1 levels can promote the E to L transition within the $CD25^+/CD44^{-,lo}$ DN population.

To rule out that the observed effects are due to altered transgene expression in double *pim-1/gfi-1* transgenic mice, we analysed extracts from thymi of different wild-type and transgenic mice. The expression of the *pim-1* transgene did not affect transgenic Gfi-1 levels neither did Gfi-1 overexpression alter the expression levels of Pim-1 in double transgenic mice compared with the expression level of both transgenes in the respective single transgenic animals (Figure 5B and C).

Endogenous levels of Gfi-1 and Pim-1 are differentially regulated during pre-T-cell development

To be able to correlate the experimental findings in transgenic mice with a physiological function of Gfi-1 and Pim-1 during β-selection-associated pre-T-cell proliferation and development, we analysed the endogenous expression levels of both Gfi-1 and Pim-1 at different stages of pre-T-cell differentiation. Thymocytes from normal C57/Bl6 mice were depleted of CD4- and CD8-expressing cells by antibody-coupled magnetic beads (Dynal), the depleted cells were stained for CD25 and CD44 and were then sorted preparatively into $CD25^+/CD44^-$, $CD25^-/CD44^-$ cells by fluorescence-activated cell sorting (FACS) according to the gates depicted in Figure 6A. In addition, the $CD25^+/CD44^-$ subpopulation was sorted into the E and L subset according to their size by gating on forward scatter as previously described (Hoffmann *et al.*, 1996). As an additional control for correct sorting into E and L cells, the separation was done in comparison with thymocytes prepared from Rag-2-deficient mice that only contain the E subset (not shown). Moreover, correct sorting of $CD25^+/CD44^-$ into E and L cells was demonstrated by staining the subsets with propidium iodide. As expected, the E subset contained only 3% of cells in $S/G_2/M$ phase of the cell cycle whereas the L subset contained 80% cycling cells (Hoffmann *et al.*, 1996; Figure 6B). A further control for correct sorting of

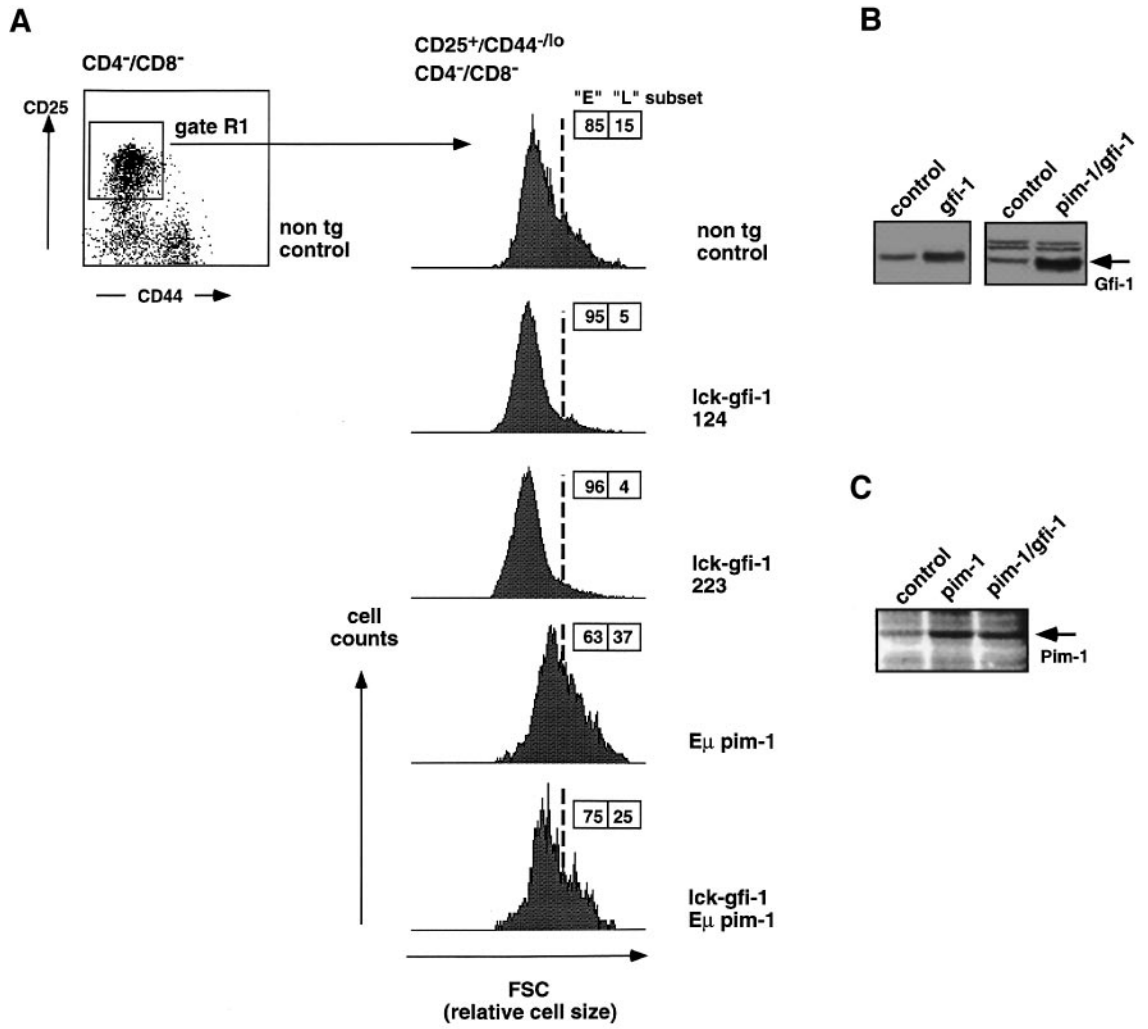

Fig. 5. Size distribution and E to L transition of $CD25^+/CD44^{-,lo}$ cells within the $CD4^-/CD8^-$ (DN) subpopulation. (**A**) DN cells were stained for CD25 and CD44 surface markers and gated for $CD25^+/CD44^{-,lo}$ DN cells as in Figure 4. The gated cells were analysed for cell size by forward angle light scattering. The boundary between E and L cells (dashed line) was set according to Hoffman *et al.* (1996) and by comparing the $CD25^+/CD44^{-,lo}$ DN cells with the same subset from Rag-2-deficient mice that have only the E subset (not shown). The large sized subset of $CD25^+/CD44^{-,lo}$ cells (L-type) is underrepresented and almost absent in both *lck-gfi-1* transgenic lines (4 and 5%) compared with negative control littermates (15%). In contrast, *Eμ pim-1* animals showed significantly more L cells (37%) than normal mice. The loss of L cells in *lck-gfi-1* transgenics is reversed in doubly *Eμ pim-1/lck-gfi-1* 124 animals (25%). Results are representative of several mice: *lck-gfi-1* transgenic lines (line 124, $n = 5$; line 223, $n = 8$), normal non-transgenic littermates ($n = 7$), *Eμ-pim-1* ($n = 6$) and *Eμ-pim-1/lck-gfi-1* 124 ($n = 3$). (**B** and **C**) Expression control by Western blotting of the Pim-1 and Gfi-1 transgenes in double transgenic mice. The expression level of Gfi-1 or Pim-1 is not altered in the presence of the respective second transgene.

the $CD25^+/CD44^-$ cells into E and L subset was provided by analysis of these sorted populations by Western blotting on the expression of the G_1-specific cell cycle inhibitor $p27^{KIP}$ (Figure 6C) that was, as was described (Hoffmann *et al.*, 1996), found to be present in E cells but down-regulated or absent in L cells.

Western analysis of the sorted subsets showed that endogenous Gfi-1 levels are readily detectable at about equal levels in both $CD25^+/CD44^-$ E and L subsets and at lower levels in $CD25^-/CD44^-$ cells (Figure 6C). In contrast, endogenous Pim-1 levels are low in E and L type cells but are strongly up-regulated in $CD25^-/CD44^-$ cells to a much higher level than found in $CD25^+/CD44^-$ E and L cells (Figure 6C). This demonstrates a differentially regulated expression of both Gfi-1 and Pim-1 during pre-T-cell differentiation and during β-selection-associated processes. Moreover, the unaltered endogenous expression levels of both Gfi-1 and Pim-1 in E and L cells suggest that the observed effects of both proteins on the E to L transition (see above) are very probably due to changes in the developmental programme of the cells and are less likely to be a consequence of altered cell cycle progression.

Discussion

The precise control of cell proliferation, differentiation and apoptosis is crucial for organ development and, in particular, pre-T-cell development in the thymus. Here, genomic reorganization, positive and negative selection and a number of signal transduction pathways initiating cell death and proliferation have to be coordinated precisely to ensure proper development of a T-cell repertoire that is able to sustain a T-cell-dependent immune response in

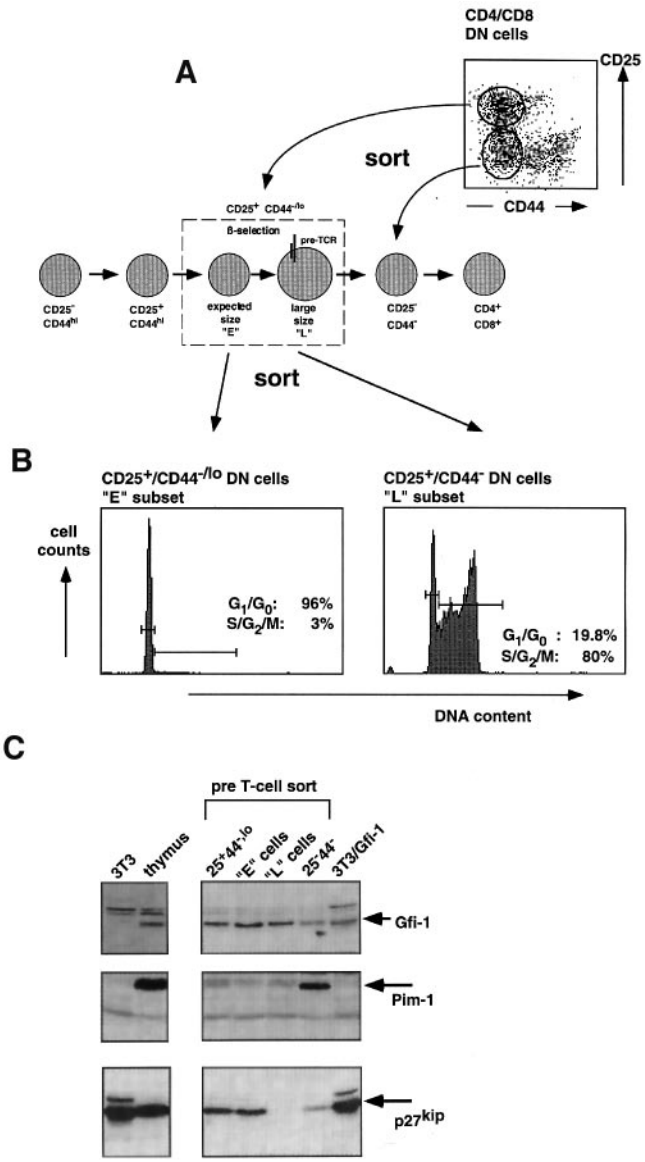

Fig. 6. Differential expression of Gfi-1, Pim-1 and $p27^{KIP}$ during thymic pre-T-cell development. (**A**) FACS plot of $CD4^{-}/CD8^{-}$ DN cells stained for the surface markers CD25 and CD44 and the gates used for the preparative sort of $CD25^{+}/CD44^{-,lo}$ DN cells and $CD25^{-}/CD44^{-}$ DN cells. Schematic representation of thymic T-cell differentiation in the DN subpopulation (see Introduction). (**B**) Cell cycle analysis by Hoechst staining of sorted E and L subsets from the $CD25^{+}/CD44^{-,lo}$ DN subpopulation (Hoffmann *et al.*, 1996). Whereas E cells are arrested in G_1/G_0, L cells are cycling with >80% in $S/G_2/M$ phase. (**C**) Expression of Gfi-1, Pim-1 and the G_1-specific cell cycle inhibitor $p27^{KIP}$ in sorted pre-T-cells. Shown are Western blots of lysates from the following sources: NIH 3T3 cells (no Gfi-1 expression), total thymus (positive control for Gfi-1), sorted $CD25^{+}/CD44^{-,lo}$ DN cells, sorted E subset of $CD25^{+}/CD44^{-,lo}$ DN cells, sorted L subset of $CD25^{+}/CD44^{-,lo}$ DN cells and sorted $CD25^{-}/CD44^{-,lo}$ DN cells. In the last lane, extracts of NIH 3T3 cells transfected with a Gfi-1 expression vector were included as an additional control for the identification of the Gfi-1 protein and to show that Gfi-1 itself does not alter the expression levels of Pim-1 or $p27^{KIP}$. Equal loading of the gel with protein extracts was controlled by Ponceau staining (not shown). Signal intensities of lanes 3–6 (pre-T-cell sort) can be compared directly because all protein extracts were prepared directly from sorted cells.

the periphery. Stringent control of proliferation and of developmental progression is of particular importance at the DN stage of pre-T-cell development to ensure that only those cells expand that have a functional TCR β-chain. In this respect, the regulation of developmental processes in the $CD25^{+}/CD44^{-,lo}$ DN population is critical for the process of β-selection to prevent 'useless' precursor cells with unproductive TCR β rearrangements from expanding and filling the thymus. Although the process of V(D)J recombination is in some aspects well described (for a review see Willerford *et al.*, 1996), the factors controlling cell proliferation, differentiation and signalling during the stages of pre-T-cell development when V(D)J recombination takes place remain to be fully elucidated. Here we present data that implicate the zinc finger protein Gfi-1 and the cytoplasmic serine/threonine kinase Pim-1 in the control of β-selection-associated processes that regulate the transition from DN to DP cells in thymic T-cells.

Effects of Gfi-1 levels on thymic cellularity and the DN to DP transition

The phenotype of transgenic mice that overexpress Gfi-1 specifically in thymic T-cells is surprisingly different from what would have been expected based on previous findings where Gfi-1 has been associated with higher proliferation and IL-2 independence or apoptosis (Gilks *et al.*, 1993; Grimes *et al.*, 1996a; Zörnig *et al.*, 1996). First and most remarkably, the size of the thymus and total thymocyte numbers are very low in *lck-gfi-1* transgenics, and the degree of this phenotype correlates with transgenic Gfi-1 expression levels. Elevated susceptibility to cell death has not been observed in *lck-gfi-1* mice, and thus does not appear to be responsible for the reduced thymic cellularity. A comparison of the absolute and relative cell numbers showed that the DP and the SP populations are most affected by overexpression of Gfi-1 in transgenic mice, but that the cellularity of the DN subset is not dramatically changed compared with non-transgenic littermates. This suggested that the development of T-cells is disturbed by high levels of Gfi-1 at the transition from DN to DP cells. This effect, provoked by overexpression of Gfi-1, could be explained by a potential activity of Gfi-1 as a repressor of TCR β expression which would lead to a similar phenotype in the *gfi-1* transgenics considering that TCR β-deficient mice have a disturbed DN to DP transition (Mombaerts *et al.*, 1992b). However, this is unlikely since in the *lck-gfi-1* transgenic mice the expression level of CD3 or the different TCR chains did not appear to be impaired. Further, TCR α/β cells with a number of Vβ and Vα subtypes as well as TCR γ/δ-bearing cells are clearly present, demonstrating that the process of V(D)J recombination *per se* is intact and cannot be accountable for the observed phenotype in *lck-gfi-1* mice. An alteration of lineage decision early in thymocyte development also seems an unlikely explanation for the observed reduced cellularity in *lck-gfi-1* transgenic animals because both TCR α/β and TCR γ/δ cell numbers are reduced in *lck-gfi-1* transgenic mice. However, lineage commitment may be altered to some degree if one considers that in the *lck-gfi-1* line TCR α/β cell numbers are reduced to 10% but TCR γ/δ numbers only to ~50% of wild-type levels (Washburn *et al.*, 1997). Therefore, it cannot be completely

ruled out that the observed phenotype in Gfi-1 transgenics is due to a disturbed lineage decision.

High Gfi-1 levels block β-selection-associated steps of pre-T-cell development

A distinct effect was provoked by Gfi-1 overexpression in transgenic animals: β-selection-associated proliferation that ensures the formation of L cells from E cells within the $CD25^+/CD44^{-,lo}$ DN population was strongly inhibited by high Gfi-1 levels. This became evident by analysing the size and cell cycle distribution of $CD25^+/CD44^{-,lo}$ DN cells and the finding that only E cells and almost no L cells are found in *lck-gfi-1* transgenic animals. These effects explain very well the general phenotype observed in *lck-gfi-1* transgenic mice, namely the reduced numbers of DP and SP cells in transgenic thymus and the loss of thymic cellularity in these mice. Gfi-1 overexpression could either block the proliferation of L cells or inhibit the progression of differentiation from E to L cells, i.e. block a developmental step. If the physiological role of Gfi-1 in these cells was the repression of cell cycle progression, one would expect to see lower endogenous Gfi-1 levels in normal L cells that are heavily cycling compared with normal E cells. However, this is not the case, as demonstrated here in Figure 6C, making it more likely that Gfi-1 functions as a regulator of the size of the $CD25^+/CD44^{-,lo}$ DN subpopulation and the developmental step that controls E to L transition. Clearly, as demonstrated here, the maintenance of certain Gfi-1 levels appears crucial for the correct developmental progression of pre-T-cells through these steps. Given the differential expression of Gfi-1 in DN subsets, it is conceivable that this zinc finger protein is indeed important for early T-cell differentiation associated with β-selection, although complete proof can only come from mutant mice deficient for Gfi-1.

The finding that co-expression of the serine/threonine kinase Pim-1 can counteract the effect of high Gfi-1 expression levels could suggest that both proteins regulate β-selection-associated E to L transition in opposite directions and that in addition to the level of Gfi-1, the Pim-1 level is also a critical factor in β-selection-associated pre-T-cell development. Furthermore, the analysis of endogenous Pim-1 protein levels demonstrated that its expression is also regulated differentially in the DN subpopulations but, similarly to Gfi-1, it is not altered at the E to L transition, implying that the ratio of Gfi-1 and Pim-1 levels is the critical parameter. Moreover, endogenous levels of Gfi-1 appear to be down-regulated in $CD25^-/CD44^-$ DN cells compared with $CD25^+/CD44^{-,lo}$ DN cells but, in contrast, Pim-1 levels are low in $CD25^+/CD44^{-,lo}$ DN cells and are strongly upregulated in the emerging $CD25^-/CD44^-$ DN population. Considering that the regulation of endogenous Gfi-1 and Pim-1 expression occurs in opposite directions in $CD25^+$ and $CD25^-$ DN subsets and the effect that constitutive expression of both proteins has on DN subpopulations, it is conceivable that they take part in the regulation of DN T-cell development not only at the E to L transition but also at the progression from $CD25^+$ to $CD25^-$ cells.

A number of experiments have shown that the presence of a functional TCR β-chain is crucial for the E to L transition during β-selection (Mombaerts *et al.*, 1992a,b; Shinkai *et al.*, 1992; Hoffmann *et al.*, 1996). The findings presented here clearly suggest that the relative levels of Gfi-1 and Pim-1 are important for precisely this step, namely the E to L transition, where the presence of a functional TCR β-chain is critical. We have shown that Gfi-1 overexpression does not affect expression of TCR subtypes and apparently does not affect rearrangement of TCR β-chain genes nor their correct expression. This offers the possibility that Gfi-1 and Pim-1 interfere at some point further downstream with pre-TCR-initiated signalling or other events to provoke the observed effects. Experiments in which *lck-gfi-1* transgenic mice were crossed with animals that are deficient in the TCR β-specific enhancer and do not express a TCR β-chain (Bouvier *et al.*, 1996) provided the first hint that the effect of Gfi-1 on DN to DP transition is independent of the presence of a TCR β-chain. Mice that are defective for the TCR β enhancer element still show considerable formation of DP cells that are TCR α/β negative. We found that the presence of the *lck-gfi-1* transgene in TCR β enhancer-defective mice completely abrogated the formation of these DP cells (T.Schmidt, T.Möröy and P.Ferrier, in preparation). This suggested that the effect of Gfi-1 on DN to DP transition is independent of the formation of a TCR β-chain and most probably affects signal transduction events downstream of TCR β, possibly the Ras/Raf/MAPK pathway (see below) or other signalling events necessary for the DN to DP transition. In a similar experiment, we have crossed the *Eμ pim-1* transgenic mice into $Rag\text{-}2^{-/-}$ mice that are not able to form TCR β-chains or any other TCR chain. These mice (*Eμ pim-1/Rag-2*$^{-/-}$) showed a variable but clear presence of DP cells which must have developed independently of a TCR β-chain in the Rag-2-negative background upon overexpression of Pim-1 (T.Schmidt, T.Möröy and P.Ferrier, in preparation). This suggested that the effect of Pim-1 on DN to DP transition is also independent of TCR β rearrangements or the presence of a TCR β-chain, and that Pim-1, similarly to Gfi-1, affects signal transduction events downstream of TCR β or other signalling events necessary for the DN to DP transition. Both experiments indicate that the differentiaton process of DN cells to DP cells is not regulated entirely by receptor complexes that contain a TCR β-chain but that additional mechanisms must exist that may well be regulated conversely by Gfi-1 and Pim-1.

To date, little is known about downstream factors that mediate pre-TCR signalling. Clearly, as the Ras/Raf/MAPK pathway is considered to be a strong candidate signalling pathway that is responsible for the production of L cells during β-selection (Crompton *et al.*, 1996; Swat *et al.*, 1996), one would have to propose that Gfi-1 or Pim-1 modulate signalling through this pathway. One argument for this view is clearly circumstantial, but nevertheless compelling. Rag-2- or Rag-1-deficient mice show a block in the DN to DP transition because they lack a functional TCR β-chain and their pre-T-cells cannot undergo β-selection and the associated proliferative steps; i.e. they cannot produce a $CD25^+/CD44^{-,lo}$ DN L subset (Mombaerts *et al.*, 1992a,b; Shinkai *et al.*, 1992; Hoffmann *et al.*, 1996). In this respect, $Rag^{-/-}$ mice are similar to the *lck-gfi-1* mice described here because the block of T-cell development in these animals occurs at the same

point as in *lck-gfi-1* mice, namely where β-selection takes place and where signalling from a pre-TCR is thought to promote the development of L cells from E cells. Further, it has been shown that expression of activated Ha-ras can restore the formation of DP cells in Rag-$2^{-/-}$ mice (Swat *et al.*, 1996) and that MAPK as a downstream target of Ras is responsible for the development of DN cells to DP cells (Crompton *et al.*, 1996), making it very likely that the Ras/Raf/MAPK pathway represents one of the pre-TCR-initiated signals necessary for β-selection-associated proliferation and differentiation, or at least can mimick it. Because Gfi-1 overexpression provokes a developmental block at exactly the same position as in Rag-deficient animals, the data presented here infer that high levels of Gfi-1 can interfere with pre-TCR signalling. Therefore, it is not unlikely that Gfi-1 is able to modulate Ras/Raf/MAPK-initiated signalling. Interestingly, it is now well established that IL-2 is able to induce signalling through Ras/Raf/MAPK as well (Satoh *et al.*, 1991; Graves *et al.*, 1992). Interference by Gfi-1 in Ras/Raf/MAPK signalling potentially could integrate the findings described here and the known effects of Gfi-1 with regard to IL-2 independence (Gilks *et al.*, 1993; Zörnig *et al.*, 1996). Although this would represent an attractive possibility for a framework in which Gfi-1 or Pim-1 could act, direct evidence for such an activity has to await additional experiments and in particular the elucidation of the molecular downstream targets of Gfi-1 and Pim-1.

Materials and methods

Generation of transgenic mice

A schematic representation of the construct used to generate *lck-gfi-1* transgenic mice is given in Figure 2A. The construct was obtained by inserting a 2 kb *Hin*cII fragment containing the murine *gfi-1* cDNA (Zörnig *et al.*, 1996) into the plck vector. The vector contained the proximal *lck* promoter and the genomic sequence of the hGH gene in a Bluescript background. The construct was freed from background sequences, purified and injected into fertilized mouse oocytes essentially as described (Hogan *et al.*, 1994). The fertilized mouse oocytes were derived from matings between (C57Bl/6×C3H) F1 mice. Successful integration of the injected DNA was monitored by Southern analysis of tail tip DNA as described (Hogan *et al.*, 1994). All transgenic mouse lines were maintained by breeding the obtained founders for three or more generations with inbred C57Bl/6 animals. Crossings with *Eμ pim-1* transgenics were performed as previously described (Zörnig *et al.*, 1996). Preparation of genomic DNA from tail tips and DNA blotting procedures were performed as described elsewhere (Sambrook *et al.*, 1989; Hogan *et al.*, 1994). The *gfi-1* probe was a 2 kb *Hin*cII cDNA fragment (Zörnig *et al.*, 1996). Preparation of RNA and Northern blotting was performed according to described procedures (Sambrook *et al.*, 1989).

Anti-Gfi-1 antibodies and affinity purification

A peptide consisting of AS 14–27 of the Gfi-1 protein was synthesized and coupled to keyhole limpet haemocyanin (KLH; Sigma) via glutaraldehyde. Coupling, subsequent immunization and antisera preparation from rabbits were carried out as described (Harlow and Lane, 1988). Antisera titres were tested by enzyme-linked immunosorbent assay (ELISA) against the Gfi-1 peptide. For recombinant protein expression in bacteria, the entire coding region of Gfi-1 was cloned into the His tag expression vector pQE-30 (Qiagen) by PCR (Vent Polymerase, NEB). His-tagged Gfi-1 was expressed and purified under denaturing conditions according to the manufacturer's instructions (Qiagen). Expression and purification were controlled by imunoblotting with an antibody directed against the RGS-His-tag (Qiagen). Approximately 300 μg of recombinant Gfi-1 protein were subjected to SDS–PAGE and transferred onto a Hybond C^+ membrane (Amersham). The area of the membrane loaded with recombinant Gfi-1 protein was detected by Ponceau staining, cut out and used for antisera affinity purification. First, the membrane was blocked with phosphate-buffered saline (PBS), 0.1% Tween-20, 5% skim milk (60 min, all steps at room temperature) and then incubated with antisera (1:50 dilution in PBS, 0.1% Tween-20, 5% skim milk) for 2 h. After washing with PBS/0.1% Tween-20 several times, bound antibody molecules were eluted with 900 μl of 200 mM glycine pH 2.2, 0.5% bovine serum albumin (BSA) (2 min) followed by immediate neutralization of the eluate in 100 μl of 1 M Tris pH 8.0. The eluate was used at a 1:200 dilution in immunoblots. Quality and specificity of the purified antibodies were monitored by immunoblotting with *in vitro* translated Gfi-1 protein.

Antibody staining procedures and cell cycle analysis

Single cell suspensions were prepared as described (Möröy *et al.*, 1993) at the time of autopsy from spleen, thymus or lymph nodes in PBS supplemented with 0.5% fetal calf serum (FCS) (staining solution). Cells were washed in this solution and incubated on ice for 30 min with antibodies directly conjugated with fluorochromes. Cells were washed twice in staining solution after the incubation and examined with a FACSCALIBUR (Becton-Dickinson). To analyse the DN population from thymus, CD4 and CD8 surface molecules were stained with the respective antibodies labelled with Tri-colour, and FITC-labelled anti-CD25 and PE-labelled anti-CD44. DP cells positive for Tri-colour staining were gated out, and the remaining population was analysed in a two-dimensional plot. Alternatively, CD4 and CD8 cells were depleted by magnetic beads coupled to the respective antibodies. Antibodies against TCR β- or α-chain subtypes or against TCR γ/δ were purchased from Pharmingen; all other antibodies used in FACS analysis were obtained from Medac. For propidium iodide labelling and cell cycle analysis, cells were washed once with cold PBS and fixed with ethanol overnight at 4°C. The cells were again washed and then incubated in 2 μg/ml propidium iodide (Sigma) in PBS/RNase (10 μg/ml) for 15 min. For the cell cycle analysis of DN subsets, thymocytes were depleted of $CD4^+$ and $CD8^+$ cells with magnetic beads (Dynal) and then stained with labelled antibodies against CD25 and CD44. The cells were again washed and then incubated in 2 μg/ml Hoechst 33382 (Sigma) in PBS for 30 min at 37°C. FACS analysis on the gated DN subsets was done by flow cytometry with a UV laser at 424 nm.

Immunoblotting

Extracts from thymi were loaded and separated on SDS–PAGE, transferred onto membranes and the Gfi-1 protein was detected with an anti-Gfi-1 rabbit antiserum using anti-rabbit Ig coupled to horseradish peroxidase. The enzymatic activity was detected using an ECL kit (Amersham) according to the specifications of the manufacturer. Equal and homogeneous transfer of proteins from the gel to membranes was controlled routinely by Ponceau staining of the membrane. NIH 3T3 cells were seeded on a 6 cm plate and transfected transiently with 8 μg of plasmid with Lipofectamine. After 2 days, cells were harvested in 1 ml of PBS, collected by centrifugation, and lysed in 100 μl of extraction buffer [50 mM HEPES pH 7.8, 20 mM NaF, 1 mM Na orthovanadate, 1 mM Na molybdate, 450 mM NaCl, 0.2 mM EDTA, 25% glycerol, 50 μg aprotinin, 50 μg leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 1% NP-40]. The lysate was cleared by centrifugation and the supernatant was separated by electrophoresis and transferred onto a nitrocellulose membrane. Untransfected cells were run as a control. The membrane was blocked overnight in PBS/0.1% Tween-20 with 5% skim milk and then incubated in blocking solution containing the respective antibodies for 2 h at 0.3 μg/ml. Blots were developed with appropriate secondary antibodies and ECL detection reagents (Amersham) as proposed by the manufacturer.

Histology

Thymi were fixed in 4% paraformaldehyde or in Carnoy's solution (60% ethanol, 30% chloroform, 10% acetic acid), embedded in paraffin and sectioned using a cryomicrotome. Sections were stained with haematoxylin/eosin.

Acknowledgements

We thank R.Perlmutter for the lck-hGH expression vector, A.Berns for the anti-Pim-1 antibody and *Eμ-pim-1* transgenic mice, and S.Korsmeyer for *Eμ bcl-2* transgenic mice. We are indebted to H.J.Fehling and Mark Dessing from the BII, Basel, for helping with the sorting of pre-T-cell subsets, for thoroughly reading the manuscript and for numerous helpful and important suggestions. We also thank K.Lennartz for help with cell

cycle analysis on the FACS. This work was supported by the Deutsche Forschungsgemeinschaft grant Mo 435/9-1, the graduate programme 'Normale und maligne Zellen' and the 'Fond der chemischen Industrie'.

References

Benoist,C. and Mathis,D. (1997) Positive selection of T cells: fastidious or promiscuous? *Curr. Opin. Immunol*., **9**, 245–249.

Bouvier,G., Watrin,F., Naspetti,M., Verthuy,C., Naquet,P. and Ferrier,P. (1996) Deletion of the mouse T-cell receptor β gene enhancer blocks αβ T-cell development. *Proc. Natl Acad. Sci. USA*, **93**, 7877–7881.

Crompton,T., Gilmour,K.C. and Owen,M.J. (1996) The MAP kinase pathway controls differentiation from double-negative to double-positive thymocyte. *Cell*, **86**, 243–251.

Dudley,E.C., Petri,H.T., Shah,L.M., Owen,M.J. and Hayday,A.C. (1994) T cell receptor β chain gene rearrangement and selection during thymocyte development in adult mice. *Immunity*, **1**, 83–93.

Fehling,H.J. and von Boehmer,H. (1997) Early αβ T cell development in the thymus of normal and genetically altered mice. *Curr. Opin. Immunol*., **9**, 263–275.

Fehling,H.J., Krotkova,A., Saint-Ruf,C. and von Boehmer,H. (1995) Crucial role of the pre-T-cell receptor α gene in development of αβ but not γδ T cells. *Nature*, **375**, 795–798.

Gilks,C.B., Bear,S.E., Grimes,H.L. and Tsichlis,P.N. (1993) Progression of interleukin-2 (IL-2)-dependent rat T cell lymphoma lines to IL-2-independent growth following activation of a gene (Gfi-1) encoding a novel zinc finger protein. *Mol. Cell. Biol.*, **13**, 1759–1768.

Godfrey,D.I. and Zlotnik,A. (1993) Control points in early T-cell development. *Immunol. Today*, **14**, 547–553.

Godfrey,D.I., Kennedy,J., Suda,T. and Zlotnik,A. (1993) A developmental pathway involving four phenotypically and functionally distinct subsets of $CD3^-CD4^-CD8^-$ triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *J. Immunol.*, **150**, 4244–4252.

Graves,J.D., Downward,J., Izquierdo,M., Rayter,S., Warne,P.H. and Cantrell,D. (1992) The growth factor interleukin-2 activates p21ras proteins in normal human T lymphocytes. *J. Immunol.*, **148**, 2417–2422.

Grimes,H.L., Chan,T.O., Zweidler-McKay,P.A., Tong,B. and Tsichlis,P.N. (1996a) The Gfi-1 proto-oncoprotein contains a novel transcriptional repressor domain, SNAG and inhibits G1 arrest induced by interleukin-2 withdrawal. *Mol. Cell. Biol*., **16**, 6263–6272.

Grimes,H.L., Gilks,C.B., Chan,T.O., Porter,S. and Tsichlis,P.N. (1996b) The Gfi-1 protooncoprotein represses Bax expression and inhibits T-cell death. *Proc. Natl Acad. Sci. USA*, **93**, 14569–14573.

Groettrup,M. and von Boehmer,H. (1993) A role for a pre-T-cell receptor in T cell development. *Immunol. Today*, **14**, 610–614.

Harlow,E. and Lane,D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hoffmann,E.S., Passoni,L., Crompton,T., Leu,T.M.U., Schatz,D.G., Koff,A., Owen,M.J. and Hayday,A.C. (1996) Productive T-cell receptor β-chain gene rearrangement: coincident regulation of cell cycle and clonality during development *in vivo*. *Genes Dev.*, **10**, 948–962.

Hogan,B., Beddington,R., Costantini,F. and Lacy,E. (1994) *Manipulating the Mouse Embryo*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Mallick,C.A., Dudley,E.C., Viney,J.L., Owen,M.J. and Hayday,A.C. (1993) Rearrangement and diversity of T cell receptor β chain genes in thymocytes: a critical role for the β chain in development. *Cell*, **73**, 513–519.

Maraskovsky,E., O'Reilly,L.A., Teepe,M., Corcoran,L.M., Peschon,J.J. and Strasser,A. (1997) Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant *rag-1*$^{-/-}$ mice. *Cell*, **89**, 1011–1019.

Mombaerts,P., Iacomini,J., Johnson,R.S., Herrup,K., Tonegawa,S. and Papaioannou,V.E. (1992a) RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*, **68**, 869–877.

Mombaerts,P. *et al.* (1992b) Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages. *Nature*, **360**, 2125–2231.

Möröy,T., Grzeschiczek,A., Petzold,S. and Hartmann,K.U. (1993) Expression of a pim-1 transgene accelerates lymphoproliferation and inhibits apoptosis in *lpr/lpr* mice. *Proc. Natl Acad. Sci. USA*, **90**, 10734–10738.

Murray,R., Suda,T., Wrighton,N., Lee,F. and Zlotnik,A. (1989) Interleukin-7 is a growth and maintenance factor for mature and immature thymocyte subsets. *Int. Immunol.*, **1**, 526–531.

Pearse,M., Wu,L., Egerton,M., Wilson,H., Shortman,K. and Scollay,R. (1989) A murine early thymocyte developmental sequence is marked by transient expression of the interleukin-2 receptor. *Proc. Natl Acad. Sci. USA*, **86**, 1614–1618.

Peschon,J.J. *et al.* (1994) Early lymphocyte expansion is severely impaired in interleukin-7 receptor-deficient mice. *J. Exp. Med.*, **180**, 1955–1960.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Satoh,T., Nakafuku,M., Miyajima,A. and Kaziro,Y. (1991) Involvement of p21 ras in signal transduction pathways from interleukin-2, interleukin-3 and granulocyte/macrophage colony stimulating factor but not from interleukin-4. *Proc. Natl Acad. Sci. USA*, **88**, 3314–3318.

Scheijen,B., Jonkers,J., Acton,D. and Berns,A. (1997) Characterization of pal-1, a common proviral insertion site in murine leukemia virus-induced lymphomas of c-myc and pim-1 transgenic mice. *J. Virol.*, **71**, 9–16.

Schmidt,T., Zörnig,M., Beneke,R. and Möröy,T. (1996) MoMuLV proviral integrations identified by *Sup-F* selection in tumors from infected *myc/pim* bitransgenic mice correlate with activation of the *gfi-1* gene. *Nucleic Acids Res*., **24**, 2528–2534.

Schmidt,T., Karsunky,H., Gau,E., Zevnik,B., Elsässer,H.P. and Möröy,T. (1998) Zinc finger protein GFI-1 has low oncogenic potential but cooperates strongly with pim and myc genes in T-cell lymphomagenesis. *Oncogene*, in press.

Selten,G., Cuypers,H.T. and Berns,A. (1985) Proviral activation of the putative oncogene Pim-1 in MuLV induced T-cell lymphomas. *EMBO J*., **4**, 1793–1798.

Sentman,C.L., Shutter,J.R., Hockenbery,D., Kanagawa,O. and Korsmeyer,S.J. (1991) Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell*, **68**, 879–888.

Shinkai,Y. *et al.* (1992) RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V (D)J rearrangement. *Cell*, **68**, 855–867.

Swat,W., Shinkai,Y., Cheng,H.-L., Davidson,L. and Alt,F.W. (1996) Activated *ras* signals differentiation and expansion of $CD4^+8^+$ thymocytes. *Proc. Natl Acad. Sci. USA*, **93**, 4683–4687.

van Lohuizen,M., Verbeek,S., Krimpenfort,P., Domen,J., Saris,C., Radaszkiewicz,T. and Berns,A. (1989) Predisposition to lymphomagenesis in *pim-1* transgenic mice: cooperation with c-*myc* and N-*myc* in murine leukemia virus-induced tumors. *Cell*, **56**, 673–682.

von Freeden-Jeffrey,U., Vieira,P., Lucian,L.A., McNeil,T., Burdach,S.E. and Murray,R. (1995) Lymphopenia in interleukin (IL)-7 gene-deleted mice indentifies IL-7 as a non-redundant cytokine. *J. Exp. Med*., **181**, 1519–1526.

von Freeden-Jeffrey,U., Solvason,N., Howard,M. and Murray,R. (1997) The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression. *Immunity*, **7**, 147–154.

Washburn,T., Schweighoffer,E., Gridley,T., Chang,D., Fowlkes,B.J., Cado,D. and Robey,E. (1997) Notch activity influences the αβ versus γδ T cell lineage decision. *Cell*, **88**, 833–843.

Willerford,D.M., Swat,W. and Alt,F.W. (1996) Developmental regulation of V (D)J recombination and lymphocyte differentiation. *Curr. Opin. Genet. Dev.*, **6**, 603–609.

Zörnig,M., Schmidt,T., Karsunky,H., Grzeschiczek,A. and Möröy,T. (1996) Zinc finger protein GFI-1 cooperates with MYC and PIM-1 in T-cell lymphomagenesis by reducing the requirements for IL-2. *Oncogene*, **12**, 1789–1801.

Zweidler-McKay,P.A., Grimes,H.L., Flubacher,M.M. and Tsichlis,P.N. (1996) Gfi-1 encodes a nuclear zinc finger protein that binds DNA and functions as a transcriptional repressor. *Mol. Cell. Biol.*, **16**, 4024–4034.

Received June 2, 1998; revised July 6, 1998; accepted July 24, 1998