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Selectively impaired development of intestinal T cell receptor $\gamma\delta^+$ cells and liver $CD4^+$ $NK1^+$ T cell receptor $\alpha\beta^+$ cells in T cell factor-1-deficient mice

T cell factor-1 (Tcf-1) is a transcription factor that binds to a sequence motif present in several T cell-specific enhancer elements. In Tcf-1-deficient (Tcf-$1^{-/-}$) mice, thymocyte development is partially blocked at the transition from the $CD4^-8^+$ immature single-positive stage to the $CD4^+8^+$ double-positive stage, resulting in a marked decrease of mature peripheral T cells in lymph node and spleen. We report here that the development of most intestinal $TCR\gamma\delta^+$ cells and liver $CD4^+$ $NK1.1^+TCR\alpha\beta^+$ ($NK1^+T$) cells, which are believed to be of extrathymic origin, is selectively impaired in Tcf-$1^{-/-}$ mice. In contrast, thymic and thymus-derived (splenic) $TCR\gamma\delta^+$ cells are present in normal numbers in Tcf-$1^{-/-}$ mice, as are other T cell subsets in intestine and liver. Collectively, our data suggest that Tcf-1 is differentially required for the development of some extrathymic T cell subsets, including intestinal $TCR\gamma\delta^+$ cells and liver $CD4^+$ $NK1^+$ T cells.

1 Introduction

T cell factor-1 (Tcf-1) was initially identified as a transcription factor that binds a sequence motif occurring in the enhancer elements of a number of genes including CD3ε and TCRα [1–3]. To determine whether Tcf-1 plays a role in T cell development, the Tcf-1 gene has recently been disrupted by homologous recombination [4]. In the resulting Tcf-1-deficient (Tcf-$1^{-/-}$) mice, thymocyte development is partially arrested at the transition from $CD8^+CD3^{low}$ (immature) single-positive to $CD4^+8^+$ double-positive cells. As a result of the blockage, T cell numbers in lymph nodes and spleen are decreased tenfold and threefold, respectively.

In addition to the well-characterized intrathymic pathway of T cell differentiation, extrathymic pathways of T cell development have been suggested to exist. In particular, both gut epithelium and liver are believed to be major sites of extrathymic T cell differentiation in the mouse [5–11]. Using thymectomized RAG-deficient mice reconstituted with bone marrow cells from athymic (nude) mice, thymus-independent development of $CD4^-8\alpha^+\beta^-$ T cells in the gut (which include most $TCR\gamma\delta^+$ cells and a subset of $TCR\alpha\beta^+$ cells) has been clearly demonstrated to occur [9]. Extrathymic development in the liver is more controversial [12]; however, recent data indicate that a subset of $TCR\alpha\beta^+$ cells including cells bearing the NK1.1 marker ($NK1^+T$ cells) can also develop from bone marrow precursors in the liver or spleen of thymectomized radiation bone-marrow chimeras [11]. Collectively, these data suggest that several distinct lineages of T cells can develop independently of the thymus.

In the present report, we investigated whether such putatively thymus-independent subsets of T cells can develop normally in Tcf-$1^{-/-}$ mice. We find that most intestinal $TCR\gamma\delta^+$ cells and liver $NK1^+T$ cells are absent in Tcf-$1^{-/-}$ mice, whereas other T cell subsets (including $CD4^-8\alpha^+\beta^-$ $TCR\alpha\beta^+$ IEL) are present in normal numbers in these tissues. Our data thus imply that Tcf-1 is differentially required for the development of some (but not all) extrathymic T cell lineages.

2 Materials and methods

2.1 Mice

The line of Tcf-$1^{-/-}$ mice used in this study has a deletion in exon VII, which encodes an essential part of the sequence-specific HMG box as described [4]. Tcf-$1^{-/-}$ and Tcf-$1^{+/-}$ mice were bred and maintained in animal facilities of the Swiss Institute for Experimental Cancer Research. They were healthy and fertile. C57BL/6 (B6) mice were purchased from Harlan Olac (Bicester, GB). All mice were used between 6–10 weeks of age.

2.2 Cell preparation

To obtain intraepithelial lymphocytes (IEL), small intestine dissected free of Peyer's patches was opened longitudinally, washed with PBS and cut into 5-mm pieces [13]. Subsequently, these pieces were incubated in PBS supplemented with 2 mM dithiolthreitol and 4 mM $NaHCO_3$, with stirring for 20 min at 37 °C. The supernatant, containing IEL and epithelial cells, was pelleted and washed with 5 % FCS PBS, and fractionated by discontinuous (40 % and 80 %) Percoll gradient centrifugation for 10 min at 900 × g. The interface was harvested and washed with 5 % FCS in PBS. To obtain liver mononuclear cells (MNC), the liver was pressed through stainless steel mesh and suspended in 50 ml PBS [14]. After being washed once with PBS, the cells (including MNC and hepatocytes) were fractionated by discontinuous (40 % and 80 %) Percoll

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Abbreviations: B6: C57BL/6 **DN:** Double negative **IEL:** Intraepithelial lymphocytes **Lef-1:** Lymphoid enhancer-binding factor-1 **MNC:** Mononuclear cells **NK1⁺:** NK1.1⁺ T cell receptor α/β **Tcf-1:** T cell factor-1

Key words: T cell receptor $\gamma\delta^+$ cell / $CD4^+NK1^+$ T cell receptor $\alpha\beta^+$ cell / Extrathymic pathway / T cell factor-1

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gradient centrifugation for 10 min at 900 × g. The interface was harvested, washed with 5 % FCS in PBS and used for experiments.

$CD4^-8^-$ thymocytes or heat-stable antigen $(HSA)^{low}$ thymocytes used as a source of thymic $\gamma\delta^+$ cells or thymic $NK1^+T$ cells, respectively, were obtained by treatment of whole thymocytes with IgM mAb RL172.4 (anti-CD4) and 3.168.1 (anti-CD8), or B2A2 (anti-HSA) in the presence of rabbit complement. Recovered cells were then purified by Ficoll-Isopaque gradient centrifugation.

2.3 Antibodies and flow cytometric analysis

The following mAb conjugates were used in this study: GL-3-FITC (anti-TCRδ); H57-597-PE (anti-TCRβ, Pharmingen, San Francisco, CA); H35-17.2-FITC (anti-CD8β), 53-6.7-PE or GK1.5-PE (anti-CD8α or anti-CD4, Caltag Laboratories, San Francisco, CA); GK1.5-FITC or -biotin (anti-CD4); 53-6.7-FITC (anti-CD8α); PK136-biotin (anti-NK1.1, Pharmingen, San Diego, CA) and M1/69-FITC (anti-HSA). All samples were further stained with streptavidin Tri-color and analyzed by FACScan and Lysis II program (Becton Dickinson).

3 Results and discussion

3.1 Selective reduction of intestinal $TCR\gamma\delta^+$ cells in $Tcf\text{-}1^{-/-}$ mice

We first compared the proportions of $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ cells among IEL in $Tcf\text{-}1^{-/-}$, $Tcf\text{-}1^{+/-}$ and C57BL/6 (B6) mice (Fig. 1 and Table 1). The total number of MNC recovered per intestine were comparable in the three groups (2.8×10^6 in $Tcf\text{-}1^{-/-}$, 2.4×10^6 in $Tcf\text{-}1^{+/-}$, and 2.9×10^6 in B6 mice). Surprisingly, intestinal $TCR\gamma\delta^+$ cells were dramatically reduced in $Tcf\text{-}1^{-/-}$ mice, resulting in a relative increase of $TCR\alpha\beta^+$ cells (Fig. 1A). Among $TCR\alpha\beta^+$ IEL, $CD8\alpha^+\beta^-$ and $CD8\alpha^+\beta^+$ subsets were distributed equally in $Tcf\text{-}1^{-/-}$ and $Tcf\text{-}1^{+/-}$ mice (Fig. 1B). Furthermore, a small subset of $CD4^+8^-$ IEL was detectable at similar levels in mutant and control mice (Fig. 1C). The $CD4^-8\alpha^+\beta^-$ subset of IEL has been well-characterized as thymus-independent, using thymectomized irradiated bone marrow- or fetal liver-reconstituted chimeras and congenitally athymic nude mice [6–9]. These cells bear homodimeric CD8αα and are either $TCR\alpha\beta^+$ or $TCR\gamma\delta^+$. Very recently, $CD4^-8\alpha^+\beta^-$ IEL have been demonstrated preferentially to use FcεRIγ instead of CD3-ζ and/or -η chain, thus allowing them to develop normally in mutant mice lacking the latter polypeptides [15–17]. Although CD8αα T cells of both TCRαβ and TCRγδ lineages share many characteristics as thymus-independent IEL, the developmental requirement for Tcf-1 was TCRγδ-lineage specific, since $TCR\alpha\beta^+$ cells expressing homodimeric CD8αα were present in normal numbers in $Tcf\text{-}1^{-/-}$ mice.

3.2 Comparison of $TCR\gamma\delta^+$ subsets in small intestine, thymus and spleen of $Tcf\text{-}1^{-/-}$ mice

Since the majority of thymus-independent intestinal $TCR\gamma\delta^+$ cells are selectively absent in $Tcf\text{-}1^{-/-}$ mice, we

Table 1. IEL subsets in $Tcf\text{-}1^{-/-}$ mice

Subsets[a)]	Proportion of total IEL		
	$Tcf\text{-}1^{-/-}$	$Tcf\text{-}1^{+/-}$	B6 ($Tcf\text{-}1^{+/+}$)
$TCR\alpha\beta^+$	81.0 ± 6.3	50.2, 42.4	35.5 ± 6.3
$TCR\gamma\delta^+$	5.1 ± 1.2	32.1, 32.9	41.3 ± 6.1
$CD4^-8\alpha^+\beta^-$	25.1 ± 3.1	45.9, 46.8	53.5 ± 6.7
$CD4^-8\alpha^+\beta^+$	55.5 ± 4.8	36.2, 33.3	14.9 ± 1.7
$CD4^+8^-$	5.4 ± 2.2	4.2, 3.6	4.0 ± 0.8
$CD4^+8\alpha^+\beta^-$	3.1 ± 1.7	3.6, 1.5	4.0 ± 1.8

a) Subsets were defined as described in Figure 1. Data are expressed as mean ± SD of three to five mice except where indicated for individual mice.

determined whether or not thymic and splenic $TCR\gamma\delta^+$ cells are present in these mice. As shown in Fig. 2, whereas intestinal $TCR\gamma\delta^+$ cells were decreased seven- to eightfold in $Tcf\text{-}1^{-/-}$ mice, both thymic and splenic $TCR\gamma\delta^+$ cells appear to develop normally. In fact, the mean absolute number of thymic $TCR\gamma\delta^+$ cells was slightly increased in $Tcf\text{-}1^{-/-}$ mice (4.3×10^5) compared to control B6 mice (2.2×10^5). Several lines of evidence suggest that the majority of splenic $TCR\gamma\delta^+$ cells are derived from the thymus in adult mice [18–21]. Using direct thymocyte labeling techniques, Kelly et al. [21] demonstrated that thymic $TCR\gamma\delta^+$ cells are exported to the spleen. In addition, while $TCR\gamma\delta^+$ IEL preferentially use Vγ7 [22, 23] and are phenotypically $Thy1^-$ [5, 6], HSA^- ([24], T. Ohteki and H. R. MacDonald, unpublished data) and $CD28^-$ [13], $TCR\gamma\delta^+$ cells in adult thymus and spleen use

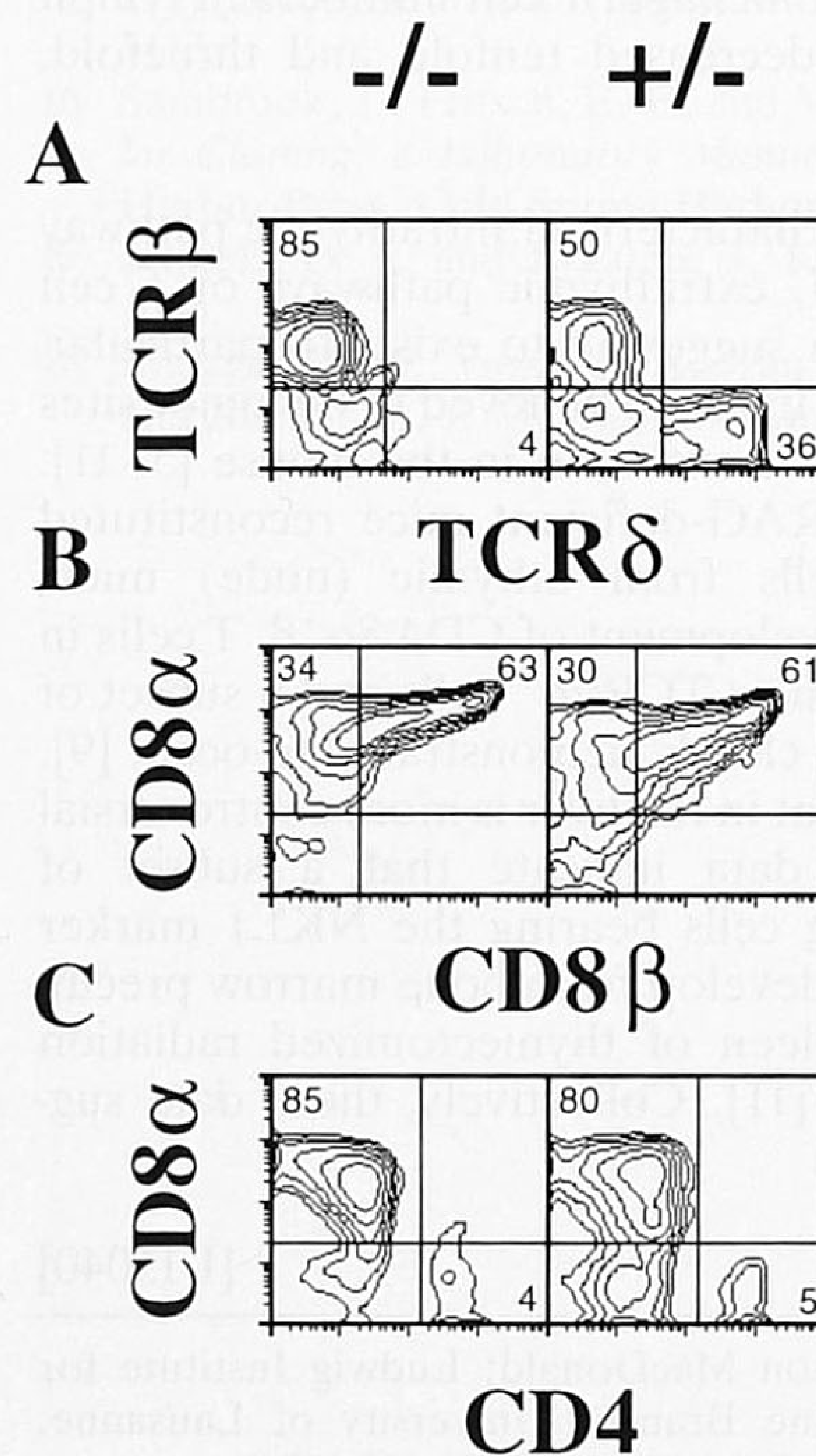

Figure 1. T cell subsets in $Tcf\text{-}1^{-/-}$ and $Tcf\text{-}1^{+/-}$ IEL. (A) Proportion of $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ cells in total IEL. Cells were stained with H57-597-PE (anti-TCRβ) and GL-3-FITC (anti-TCRδ) (B) Proportion of $CD8\alpha^+\beta^-$ and $CD8\alpha^+\beta^+$ cells among gated $TCR\alpha\beta^+$ IEL. Cells were stained with 53.6.7-FITC (anti-CD8α), H57-597-PE (anti-TCRβ) and H35-17.2-biotin (anti-CD8β) plus streptavidin Tri-color. (C) Proportion of $CD4^+8\alpha^-$ and $CD4^-8\alpha^+$ cells in total IEL.

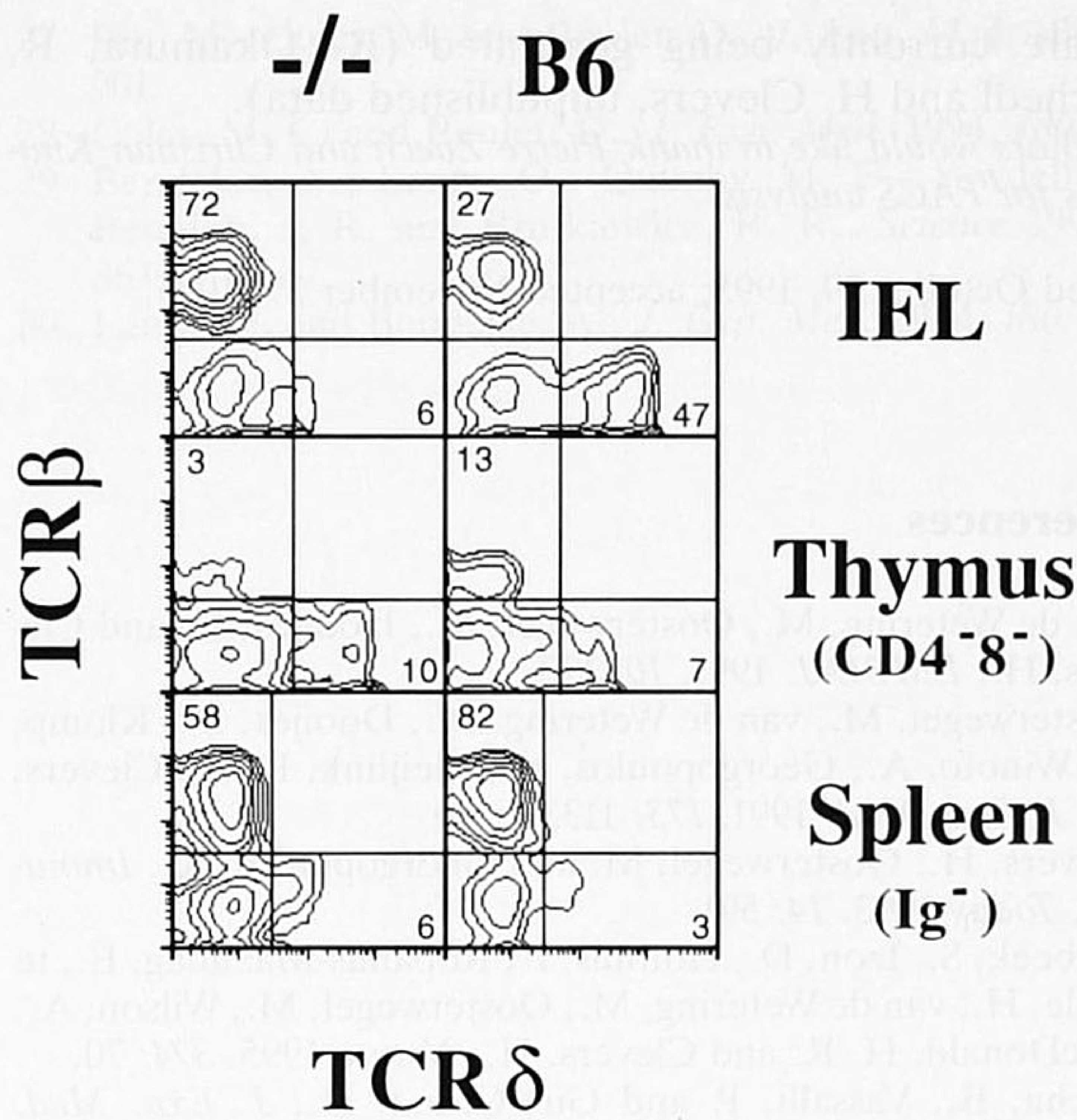

Figure 2. TCRγδ$^+$ cells in small intestine, thymus and spleen of Tcf-1$^{-/-}$ and control B6 mice. Total IEL, CD4$^-$8$^-$ (DN) thymocytes and Ig$^-$ spleen cells were stained with H57-597-PE (anti-TCRβ) and GL-3-FITC (anti-TCRδ).

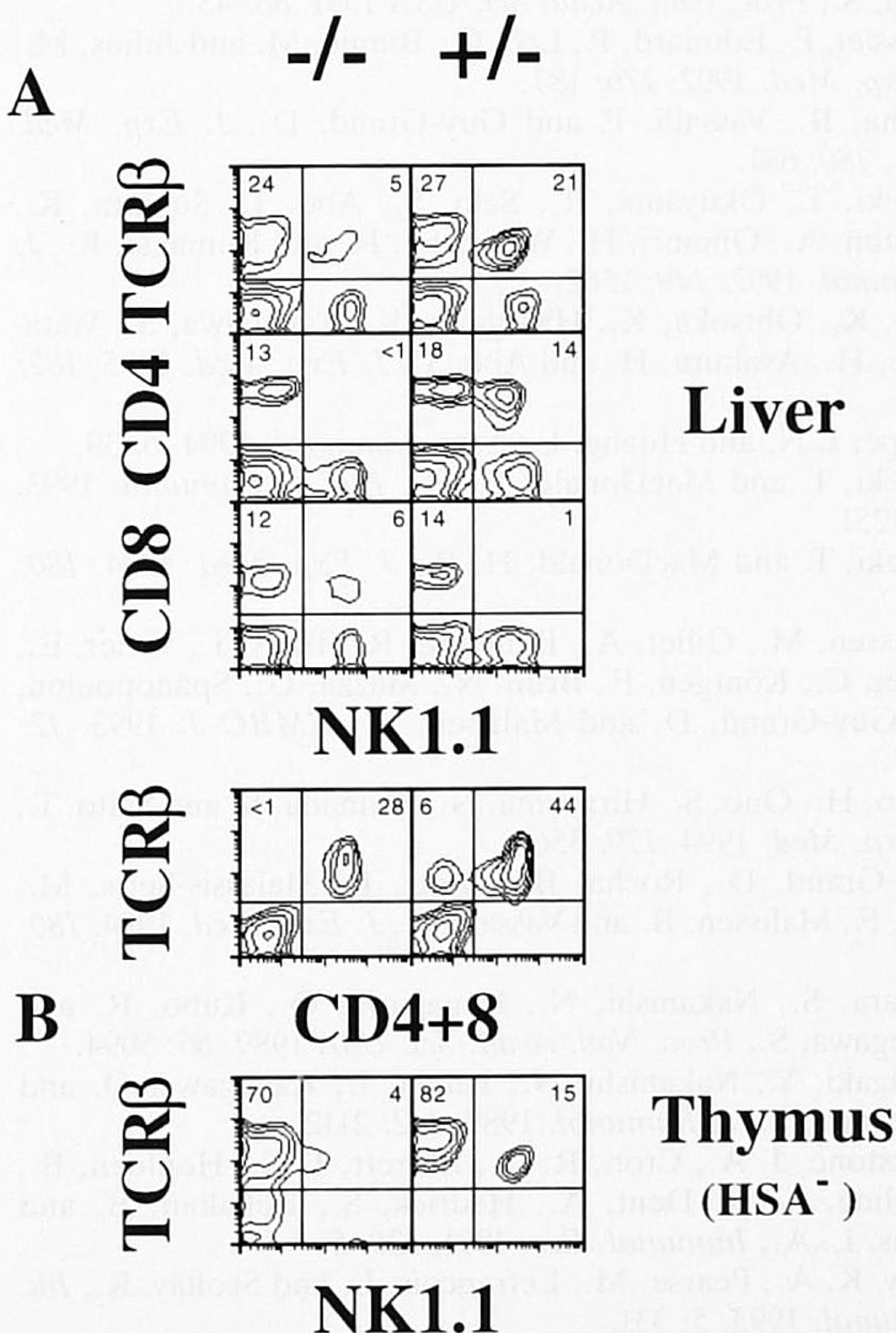

Figure 3. Altered NK1$^+$T cell subsets in the liver Tcf-1$^{-/-}$ mice. (A) Total liver MNC were stained with H57-597-PE (anti-TCRβ) or GK1.5-PE (anti-CD4) or 53.6.7-FITC (anti-CD8) and PK136-biotin (anti-NK1.1) plus streptavidin-Tri-color. To detect the DN TCRαβ$^+$ (mainly NK1$^+$) subset, cells were stained with GK1.5-FITC (anti-CD4), 53.6.7-FITC (anti-CD8) and H57-597-PE (anti-TCRβ). (B) HSA^{low} thymocytes were stained with H57-597-PE (anti-TCRβ) and PK136-biotin (anti-NK1.1) plus streptavidin-Tri-color.

Table 2. Altered NK1$^+$ T cell subsets in the liver of Tcf-1$^{-/-}$ mice

Subsets[a)]	Proportion of total liver MNC Tcf-1$^{-/-}$	Tcf-1$^{+/-}$	B6 (Tcf-1$^{+/+}$)
TCRαβ$^+$NK1.1$^+$	5.1 ± 0.8	17.2, 21.2	20.2 ± 2.3
TCRαβ$^+$NK1.1$^-$	30.1 ± 6.2	35.0, 27.5	26.6 ± 3.8
CD4$^+$NK1.1$^+$	0.3 ± 0.1	10.3, 13.8	14.7 ± 2.0
CD4$^+$NK1.1$^-$	12.9 ± 3.2	22.0, 17.9	15.9 ± 2.3
CD8$^+$NK1.1$^+$	6.8 ± 1.7	1.3, 1.2	0.9 ± 0.2
CD8$^+$NK1.1$^-$	14.2 ± 3.8	14.1, 9.9	10.2 ± 1.3
TCRαβ$^+$CD4$^-$8$^-$	1.0 ± 0.1	5.5, 6.3	5.7 ± 0.9

a) Subsets were defined as described in Fig. 3. Data are expressed as mean ± SD of three to five mice except where indicated for individual mice.

mainly Vγ4 [18–20] and the majority of thymic TCRγδ$^+$ cells are Thy1$^+$, HSA$^+$ [25] and CD28$^+$ [13]. Taken together, our data suggest that Tcf-1 is required for the development of most thymus-independent intestinal TCRγδ$^+$ cells, but not for thymic or thymus-derived TCRγδ$^+$ cells. It should be noted, however, that the phenotype of thymic TCRγδ$^+$ cells is somewhat abnormal in Tcf-1$^{-/-}$ mice, since (in contrast to control Tcf-1$^{+/-}$ mice) the majority are HSA$^-$ (data not shown).

3.3 Impaired development of liver NK1$^+$T cells in Tcf-1$^{-/-}$ mice

Mouse NK1$^+$T cells require β2-microglobulin-associated (presumably CD1) molecules on hematopoietic cells for their development and are found primarily in liver and

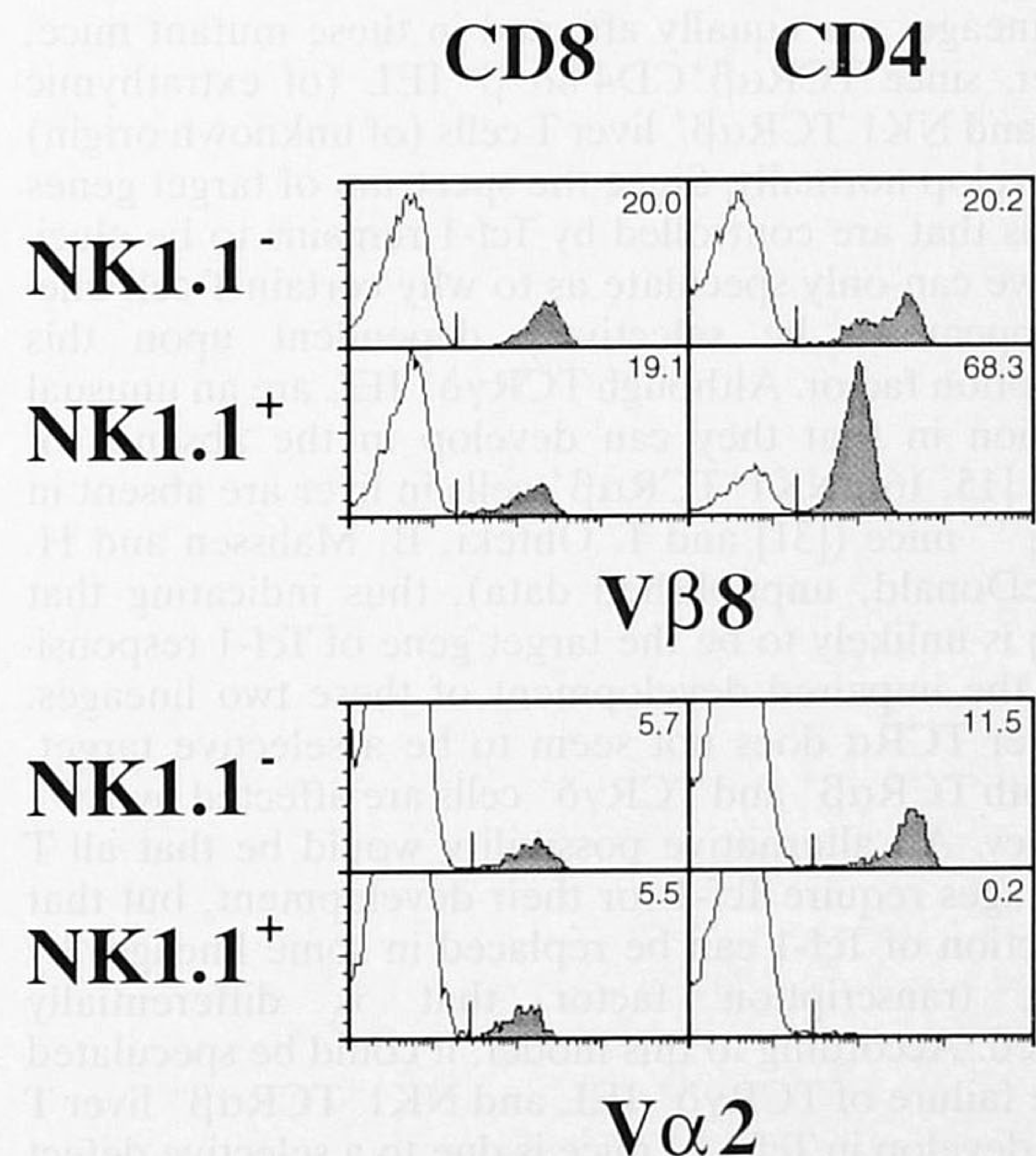

Figure 4. Vβ8 and Vα2 expression among liver NK1$^+$T cell subsets. Total liver MNC from Tcf-1$^{-/-}$ or control (B6) mice were stained with F23.1-FITC (anti-Vβ8) or B20.1-FITC (anti-Vα2), 53.6.7-PE (anti-CD8) or GK1.5-PE (anti-CD4) and PK136-biotin (anti-NK1.1) plus streptavidin-Tri-color. Histograms for CD8$^+$ and CD4$^+$ subsets are gated on Tcf-1$^{-/-}$ or B6 liver MNC, respectively (see Fig. 3).

thymus [14, 26–28]. The NK1$^+$T cell subset comprises CD4$^+$8$^-$ and DN cells, but not CD4$^-$8$^+$ cells in normal B6 mice [14, 26]. Total numbers of MNC obtained per liver were 8.8×10^6 in Tcf-1$^{-/-}$, 5.9×10^6 in Tcf-1$^{+/-}$ and 4.0×10^6 in B6 mice. Interestingly, total liver NK1$^+$T cells were significantly reduced and further analysis revealed that CD4$^+$8$^-$ and DN NK1$^+$T subsets were absent in Tcf-1$^{-/-}$ mice (Fig. 3A and Table 2). A similar reduction in NK1$^+$T cells was observed in the thymus of Tcf-1$^{-/-}$ mice (Fig. 3B). Surprisingly, CD4$^-$8$^+$NK1$^+$T cells, which are rarely seen in Tcf-1$^{+/-}$ and B6 mice, were expanded in the liver of Tcf-1$^{-/-}$ mice, thus accounting for the residual NK1$^+$T cells (Fig. 3A and Table 2). As we and others have reported, NK1$^+$T cells in both liver and thymus have a biased TCR repertoire that is skewed to Vβ8, Vβ7 and Vβ2 in combination with a highly conserved TCRα chain consisting of a Vα14-Jα281 rearrangement [14, 26–30]. Consistent with these reports, CD4$^+$8$^-$NK1$^+$T cells in control B6 liver express up to 70 % Vβ8 and no detectable Vα2 (Fig. 4). In contrast, the CD4$^-$8$^+$NK1$^+$T cell subset found in Tcf-1$^{-/-}$ liver express normal levels of Vβ8 and Vα2 (which is similar to NK1$^-$CD4$^-$8$^+$cells both in Tcf-1$^{-/-}$ and in B6 mice). Thus, the novel CD4$^-$8$^+$ NK1$^+$ subset arising in Tcf-1$^{-/-}$ mice shows no apparent TCR bias and may therefore be selected independently of the CD4$^+$8$^-$ and DN subsets of NK1$^+$T cells found in normal mice.

4 Concluding remarks

In conclusion, we find that the development of two unrelated (and putatively extrathymic) T cell lineages, intestinal TCRγδ$^+$ cells and liver NK1$^+$TCRαβ$^+$ cells, is selectively impaired in Tcf-1-deficient mice. Not all extrathymic T cell lineages are equally affected in these mutant mice, however, since TCRαβ$^+$CD4$^-$8α$^+$β$^-$ IEL (of extrathymic origin) and NK1$^-$TCRαβ$^+$ liver T cells (of unknown origin) both develop normally. Since the spectrum of target genes in T cells that are controlled by Tcf-1 remains to be elucidated, we can only speculate as to why certain T cell lineages appear to be selectively dependent upon this transcription factor. Although TCRγδ$^+$ IEL are an unusual population in that they can develop in the absence of CD3ζ/η [15, 16], NK1$^+$TCRαβ$^+$ cells in liver are absent in CD3ζ/η$^{-/-}$ mice ([31] and T. Ohteki, B. Malissen and H. R. MacDonald, unpublished data), thus indicating that CD3ζ/η is unlikely to be the target gene of Tcf-1 responsible for the impaired development of these two lineages. Moreover TCRα does not seem to be a selective target, since both TCRαβ$^+$ and TCRγδ$^+$ cells are affected by Tcf-1 deficiency. An alternative possibility would be that all T cell lineages require Tcf-1 for their development, but that the function of Tcf-1 can be replaced in some lineages by another transcription factor that is differentially expressed. According to this model, it could be speculated that the failure of TCRγδ$^+$ IEL and NK1$^+$TCRαβ$^+$ liver T cells to develop in Tcf-1$^{-/-}$ mice is due to a selective defect in expression of such a putative redundant factor in these lineages. In this context, the expression patterns of Tcf-1 and lymphoid enhancer-binding factor 1 (Lef-1) are almost completely overlapping, and their DNA-binding characteristics are identical [32]. It is therefore likely that Tcf-1 and Lef-1, at least in part, will perform redundant functions. To test this notion, Tcf-1/Lef-1 double knock-out mice are currently being generated (R. Okamura, R. Grosschedl and H. Clevers, unpublished data).

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