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FOXN1 mutation abrogates prenatal T-cell development in humans

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Summary

The transcription factor FOXN1 is implicated in the differentiation of thymic and skin epithelial cells and its alterations are responsible for the Nude/SCID phenotype. During a genetic counseling program offered to couples at risk in the community where a high frequency of mutated FOXN1 has been documented, the identification of a human *FOXN1*$^{-/-}$ fetus gave the unique opportunity to study T-cell development in utero. A total blockage of CD4^{+} T-cell maturation and a severe impairment of CD8^{+} cells were documented. The evaluation of the variable-domain β-chain (Vβ) families' usage among T lymphocytes revealed that the generation of TCR diversity occurred at some extent in the *FOXN1*$^{-/-}$ fetus, although it was impaired compared to the control. A few non-functional CD8^{+} cells, mostly bearing TCRγδ in the absence of CD3 were found. FOXN1 is crucial for in utero T-cell development in humans. The identification of a limited number of CD8^{+} cells suggests an extrathymic origin for these cells, thus implying a FOXN1-independent lymphopoiesis.

Key words: Severe combined immunodeficiency, T-cell development, FOXN1

Abbreviations*:*

SCID: severe combined immunodeficiency
FOX: forkhead box
HPC: hematopoietic precursor cell

FOXN1 is referred to human gene
Foxn1 is referred to murine gene
FOXN1 is the protein

The genetic study of human severe combined immunodeficiencies (SCID) has unraveled important issues concerning the rules that govern lymphocyte development and function.[1] The thymus has been long and unanimously considered the unique primary lymphoid organ where ontogeny of T cells, which are the essence of the cellular immune system, occurs.[2] The epithelial component of the thymic stroma is essential for T-cell development.[3] In humans, the DiGeorge syndrome (DGS) has been long considered the prototype of an athymic disorder, even though in these patients some mature type T cells are present suggesting the presence of a thymic rudiment or the presence of an extrathymic site of lymphopoiesis. In mice and rats, spontaneous mutations in forkhead box-N1 (*Foxn1)* transcription factor gene, mapping on chromosome 11, revealed that Foxn1 is essential for thymic and skin epithelial differentiation and thymopoiesis.[4] This model is referred as the Nude phenotype due to hairlessness.[5] **These nude mutant animals develop an abnormal thymus, resulting in a severe and selective T-cell deficiency and an overall severely impaired immune system.[6] In particular, thymus-dependent lymphoid cells in the paracortex of lymph nodes and periarteriolar regions of spleen are lacking.[7] These mice also fail to develop an increased cellularity of lymph nodes draining a local injection of PHA.[8]** The human equivalent of the mouse Nude/SCID has been first described in 2 sisters, originated from a geographically isolated community in the south of Italy, with the R255X homozygous mutation in *FOXN1* gene.[9] This mutation leads to a complete absence of a functional protein similar to the previously described rat and mouse Foxn1 mutations.[10] In humans, *FOXN1* is located on chromosome 17. In the village where the first patients originated, the identification of a number of heterozygotes for the same mutation suggested the need to offer to the population a prenatal diagnosis program for the disease.

We report on an athymic Nude/SCID fetus, who gave the unique opportunity to gain further insights into the prenatal ontogeny of T lymphocytes in humans.

METHODS AND RESULTS

During the genetic **counseling** program a fetus at risk was identified. At 11 postmenstrual weeks of gestation, prenatal diagnosis was performed through villocentesis**. Genomic DNA was extracted by standard procedures from chorionic villi and processed.** ***FOXN1*** **gene analysis was performed according to a polymerase chain reaction (PCR) assay and direct sequencing as previously described.**[11] *FOXN1* gene sequence analysis revealed the homozygous C-to-T transition at nucleotide position 792, leading to the nonsense R255X mutation. This finding led to interruption of the pregnancy. Protein expression analysis by immunohistochemistry on fetal skin fragments revealed the absence of the FOXN1 protein as expected on the basis of the molecular alteration.

The concomitant assembly of CD4 and CD8 molecules on the thymocyte surface or their individual expression in conjunction with CD3, are markers of discrete stages of T-cell development. In the immunophenotype study, cord blood mononuclear cells (CBMCs) were isolated from a Nude/SCID and a control human fetus, matched for the same gestational age, by density gradient centrifugation over Ficoll-Hypaque (Biochrom). **Cells were stained with the appropriate antibody (CD45-APC, CD3-PerCP, CD19-PerCP, CD16CD56-PE-Cy7, CD8α-PE-Cy7, CD4-FITC, TCRαβ-FITC, TCRγδ-PE, CD45RA-PE) (BD Pharmingen) at 4°C for 30 min, washed and finally analyzed using a FACSCanto II flowcytometer Becton Dickinson.** The evaluation of the Nude CBMCs revealed that the lack of the thymus led to a very low number of $CD3^+$ cells, 3.5% versus 25.4% of the control. However, near all these cells in the $FOXN1^{-/-}$ fetus were dim (fig 1A, B), **suggestive of a lower number of CD3 molecule per cell, indicative of a maturation arrest.** In the $FOXN1^{-/-}$ fetus, 1.3% of the $CD45^+$ lymphocytes gated cells expressed CD4, while in the control fetus, this population was higher (23.4%). Of note, no $CD4^+$ cells co-expressed CD3, 0.3% compared to 22% of the control fetus (fig 1A). Differently, in the $FOXN1^{-/-}$ fetus, we found a considerable number of $CD8\alpha^+$ (10.3%), although CD8 cells co-expressing CD3 were considerably low (1.5% of the $CD45^+$ lymphocytes gated cells), whereas $CD3^+CD8^+$ in the control were 5.9% (fig 1B). Of note, within the $CD3^+CD8^+$ subset, 1.1 and 4.5% of the $CD45^+$

gated lymphocytes displayed a naive phenotype, as assessed by the expression of CD45RA isoform, in the *FOXN1*$^{-/-}$ fetus and in the control, respectively (fig 1C). Moreover, in the control, 2.4% of CD3^{+} CBMCs were double positive for CD4^{+} and CD8^{+} markers**.** No double positive T cells were found in the *FOXN1*$^{-/-}$ fetus.

Since T cells originate from a common multipotent hematopoietic precursor cell (HPC), to exclude a direct involvement of HPC in the alterations observed, CD34^{+} cells were evaluated and found comparable to the control fetus, accounting for 1% of the CD45^{+} hematopoietic cells. Consistently with this finding, no abnormalities in the development of B (65%) and Natural Killer (NK) (25%) cells were found in the *FOXN1*$^{-/-}$ fetus (**fig 1D)**.

T cells are also distinguished by their cell surface T-cell receptors (TCRs). A substantial reduction of T cells bearing TCRαβ was observed in the *FOXN1*$^{-/-}$ fetus (1.5% versus 37.1% of the control) (**fig 1E**). Differently, in the *FOXN1*$^{-/-}$ fetus 6.5% of the CD45^{+} gated lymphocytes expressed TCRγδ, but the majority of these cells were CD3^{-} cells (4.9%) (**fig 1F**). All these cells co-expressed the CD8αβ heterodimer (**fig 1G)**. In the control, TCRγδ cells were 3.7% of the CD45^{+} gated lymphocytes and only a minority was CD3^{-} (1.1%) (**fig 1F**).

The cell proliferation of CBMCs derived from Nude/SCID and control fetuses was determined by incorporation of tritiated thymidine for 72 hours of culture after stimulation with 8 μg/mL phytohemagglutinin (PHA) or anti-CD3 mAb (1 ng/mL or 0.1 ng/mL), previously precoated on tissue culture plates for cross-linking. As expected, *FOXN1*$^{-/-}$ CBMCs showed an absent proliferative response after stimulation with anti-CD3 or phytohemagglutinin (PHA), compared with the control cells (**fig 1H**).

Since antigen receptor gene rearrangement process represents a hallmark of an ongoing development in T lymphocytes, phenotypical studies in the *FOXN1*$^{-/-}$ and in the control fetuses were implemented by a high-troughput analysis of TCR repertoire. T cells were separated into CD4^{+} and CD4^{-} cells by magnetic sorting with coated beads (Dynabeads). For spectratyping analysis, TCR CDR3β sequencing was performed after TCR variable-domain β-chain (Vβ) amplification with a

common reverse primer (CB3 primer) and 27 different forward primers (TCR Vβ gene family primers). Results were analyzed using CEQ 8000 software (Beckman Coulter). The evaluation of the Vβ families' usage revealed that the generation of TCR diversity was consistently impaired in the *FOXN1*$^{-/-}$ fetus, differently from the control of the same gestational age (fig 2A). While in the control 26 of the 27 families were expressed in both subsets, in the *FOXN1*$^{-/-}$ fetus only few families were expressed. Furthermore, 2 of them (Vβ 6.1 and 6.2) in the CD4^{+} subset accounted for the 73% of the entire repertoire (fig 2A, B), while in the CD4^{-} subset only 1 family (Vβ 6.1) had a Gaussian profile (fig 2A). Moreover, in this subset, the Vβ 25 family exhibited an oligo-monoclonal profile (fig 2A). **A contamination of maternal cells, which may have crossed the placenta and engrafted into the fetus in utero,[12] was ruled out by evaluating 15 highly polymorphic autosomal short tandem repeat loci through multiplex-PCR.**

Thus, even though the number of T lymphocytes is very low in the *FOXN1*$^{-/-}$ fetus, these data provide evidence that in this model of congenital athymia TCR gene rearrangement, although altered, occurs at some extent.

DISCUSSION

Our results provide an evidence of the crucial role of FOXN1 in the early prenatal stages of T-cell ontogeny also in humans, in that its alteration leads to a total blockage of CD4^{+} T-cell maturation and a severe impairment of CD8^{+} cells with an apparent bias toward γδ T-cell production. The different FOXN1-dependence of the CD4^{+} or CD8^{+} cells maturation is similar to what occurs in the case of loss of the nuclear high-mobility group (HMG) box protein TOX that leads in mice to a selective blockage of CD4 differentiation but not of CD8.[13] Foxn1 is expressed in all thymic epithelial cells (TECs) during initial thymus organogenesis and broadly during fetal stages. Both fetal TEC differentiation and maintenance of the thymus microenvironment require a "crosstalk" between TECs and developing thymocytes.[14] Of note, in mice homozygous for the *Foxn1* gene

mutations TECs fail to differentiate leading to a blockage of thymopoiesis and severe immunodeficiency.[15]

Despite an extensive knowledge about the thymus role to foster T-cell development is available, some still unexplained evidence in human athymic conditions suggests that in-depth information of this process is still to be achieved and, in particular, the involvement of different non-lymphoid tissues in T-cell ontogeny. In human Nude/SCID, the absence of thymic tissue results in a severe T-cell immunodeficiency. Since FOXN1 is selectively expressed in the thymus and skin, skin epithelial cells could play a role for a productive T-cell ontogeny, as previously shown in in vitro models.[16]

Our results demonstrate that FOXN1 is crucial for in utero T-cell development and not for B and NK-cell differentiation in humans. The identification of a limited number of $CD8^+$ cells, bearing TCRγδ, suggests a different origin for these cells, **even though it is not known if these cells are derived from a remnant thymus. However, this seems unlikely since at the autoptic evaluation no thymic tissue at all was found. These cells are, however, unable to sustain a productive immune response into the periphery.**

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FIGURE LEGENDS

Figure 1 (A, B) Dot plots represent the expression of CD3 and CD4 or CD3 and CD8 surface markers in the *FOXN1*$^{-/-}$ fetus and in the cord blood of a control of the same gestational age. (C) Expression of CD8 and of CD45RA on CD3^{+} gated cells in the *FOXN1*$^{-/-}$ and control cord blood. (D) Dot plots represent the expression of CD19 and CD16/56 surface markers in the *FOXN1*$^{-/-}$ fetus. (E, F) Dot plots represent the expression of CD3 and TCRαβ or CD3 and TCRγδ on cord blood cells of the *FOXN1*$^{-/-}$ and the control fetuses. (G) Dot plot represents the expression of CD8 and TCRγδ in the CD3^{-} cells in the *FOXN1*$^{-/-}$ fetus. (H) Proliferation of CBMCs from *FOXN1*$^{-/-}$ and control fetuses following PHA stimulation or CD3 cross-linking. Incorporation of tritiated thymidine was evaluated after four days of culture.

Figure 2 (A) Analysis of T-cell receptor Vβ families repertoire in the CBMCs of *FOXN1*$^{-/-}$ and control fetuses. Results were obtained using primers that amplify CDR3 regions from the TCR Vβ genes indicated. Vβ families were considered normal when showing 5 or more peaks in a gaussian distribution. (B) The percentage of TCR Vβ expression within CD4^{+} and CD4^{-} cells in the *FOXN1*$^{-/-}$ fetus. Arrows on the x axis depict TCR Vβ segments and arrows on the y-axis show the percentage of Vβ mRNA expression.

FIGURE 1

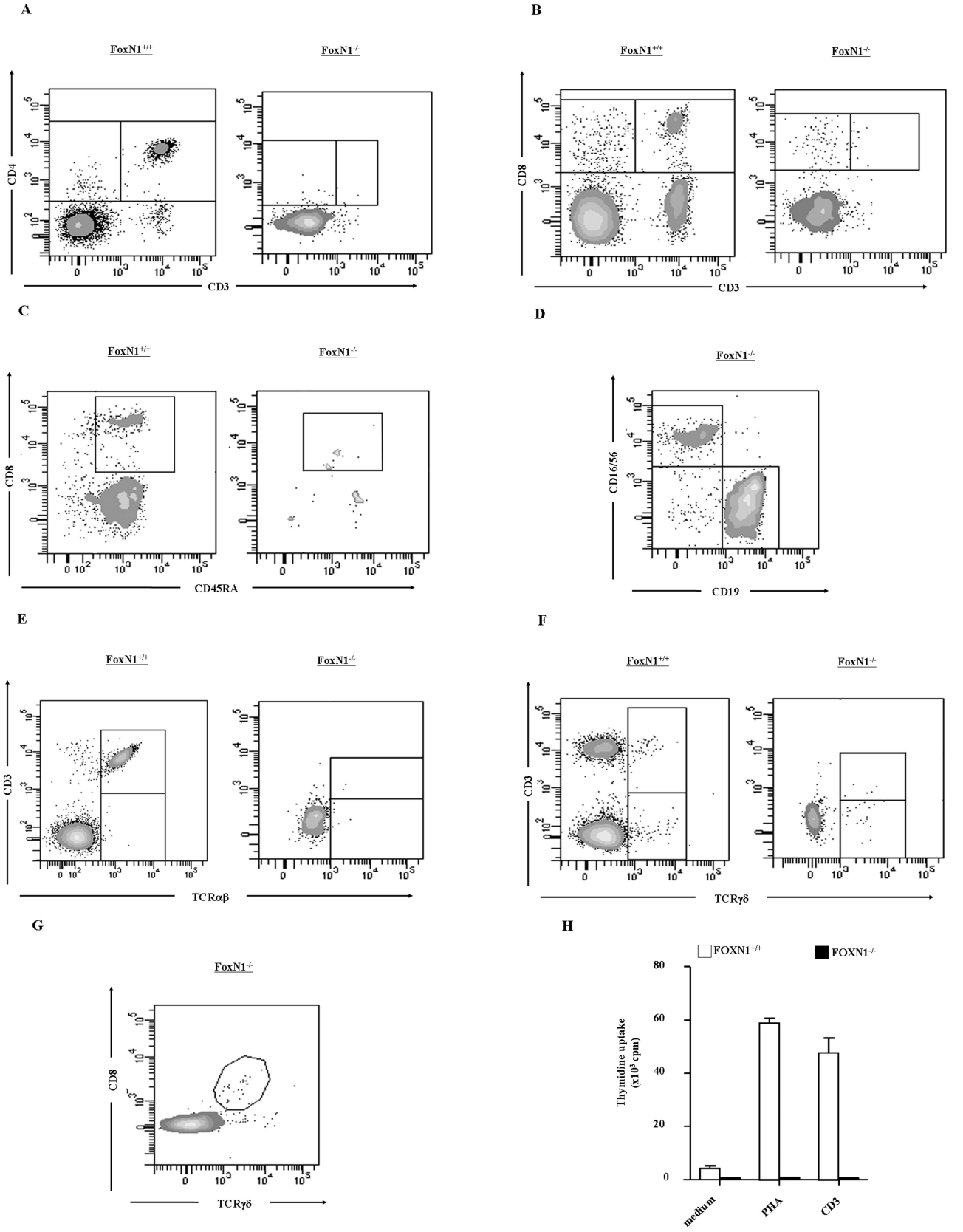
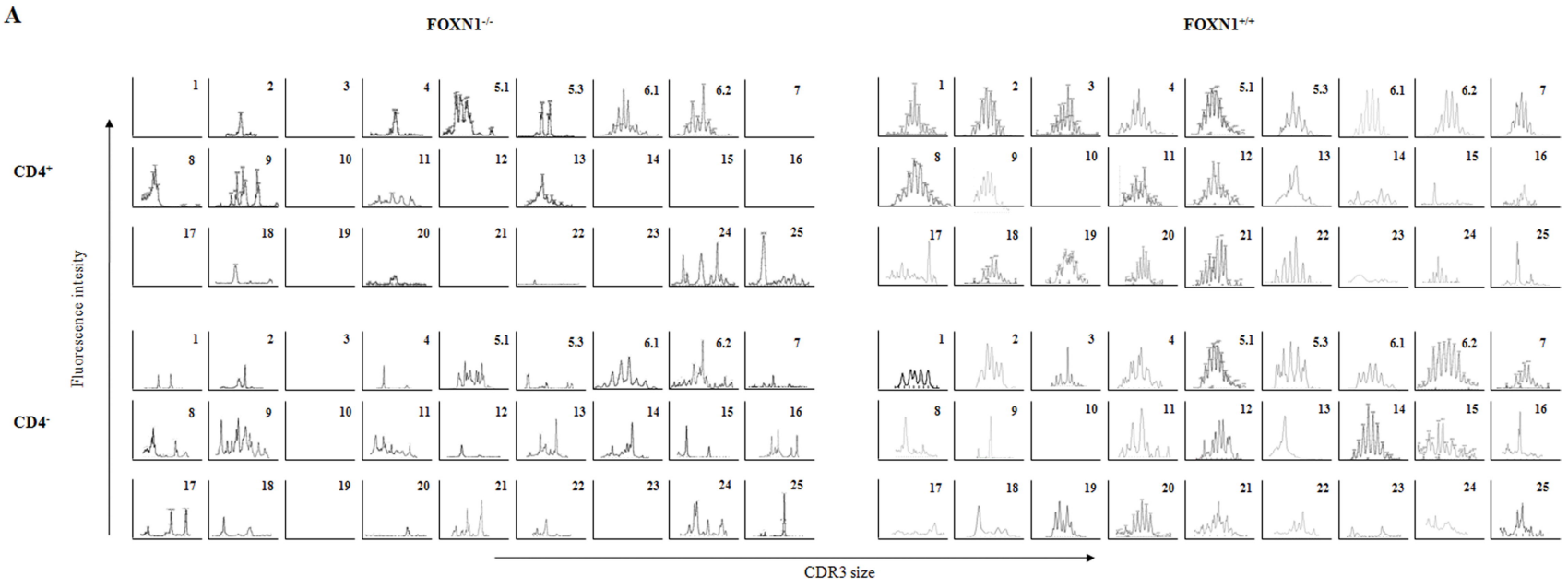
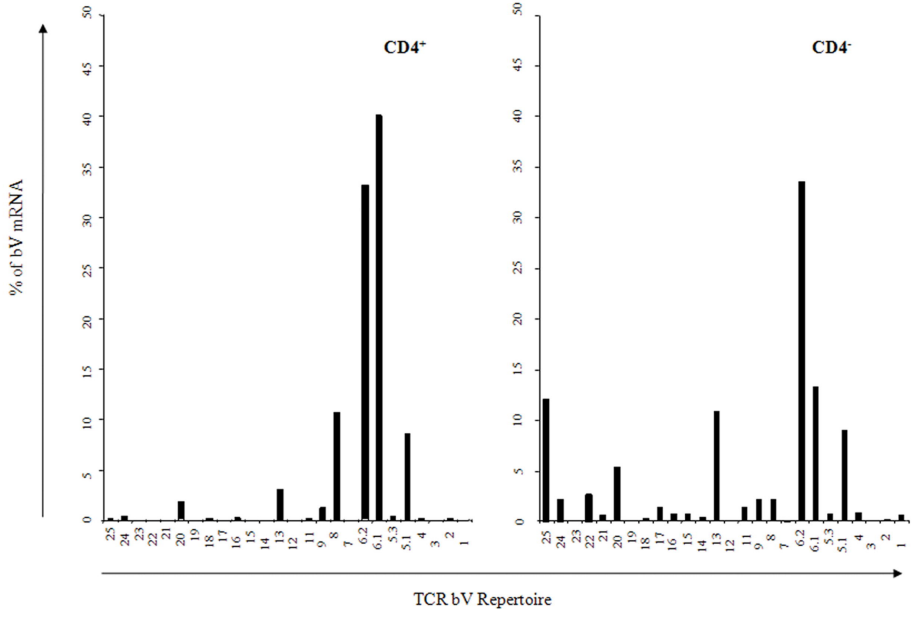

A
FoxN1+/+
FoxN1-/-
CD4
CD3
B
FoxN1+/+
FoxN1-/-
CD8
CD3
C
FoxN1+/+
FoxN1-/-
CD8
CD45RA
D
FoxN1-/-
CD16/56
CD19
E
FoxN1+/+
FoxN1-/-
CD3
TCRαβ
F
FoxN1+/+
FoxN1-/-
CD3
TCRγδ
G
FoxN1-/-
CD8
TCRγδ
H
FOXN1+/+
FOXN1-/-
Thymidine uptake (x10³ cpm)
80
60
40
20
0
medium
PHA
CD3

FIGURE 2

A


FOXN1-/-
FOXN1+/+
CD4+
CD4-
Fluorescence intesity
CDR3 size

B


CD4+
CD4-
% of bV mRNA
TCR bV Repertoire