Molecular Evidence for a Thymus-Independent Partial T Cell Development in a FOXN1\(^{-/-}\) Athymic Human Fetus

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Abstract

The thymus is the primary organ able to support T cell ontogeny, abrogated in FOXN1\(^{-/-}\) human athymia. Although evidence indicates that in animal models T lymphocytes may differentiate at extrathymic sites, whether this process is really thymus-independent has still to be clarified. In an athymic FOXN1\(^{-/-}\) fetus, in which we previously described a total blockage of CD4\(^\text{+}\) and partial blockage of CD8\(^\text{+}\) cell development, we investigated whether intestine could play a role as extrathymic site of T-lymphopoiesis in humans. We document the presence of few extrathymically developed T lymphocytes and the presence in the intestine of CD3\(^\text{+}\) and CD8\(^\text{+}\), but not of CD4\(^\text{+}\) cells, a few of them exhibiting a CD45RA\(^\text{+}\)/CD62L\(^\text{+}\) phenotype. The expression of CD3\(_\text{exp}^\text{+}\)T\(_\alpha\), RAG1 and RAG2 transcripts in the intestine and TCR gene rearrangement was also documented, thus indicating that in humans the partial T cell ontogeny occurring at extrathymic sites is a thymus- and FOXN1-independent process.

Introduction

The thymus supports a proper T cell ontogeny due to the presence of specialized epithelial cells, resulting in the export of naive CD4\(^{\text{+}}\)CD8\(^{\text{+}}\) T cells that follows the recruitment of progenitors from bone marrow [1].

Evidence indicates that T cells may also differentiate at extrathymic sites, as intestine and liver [2-6], where T cell populations may arise from preexisting precursor cells [7,8], even though it still remains to be demonstrated if the process is fully thymus-independent. In favor of a thymic independent differentiation process there is the evidence that a few T cells can be detected into the periphery in nude mice [9-11]. The T cell pool developed outside the thymus exhibits a peculiar phenotype [2] although not univocal in the different species. In fact, in mice, extrathymic T cells often exhibit the CD8\(^\text{+}\)/CD4\(^\text{+}\) homodimer, while in rats they may be CD8\(^\text{+}\)/CD4\(^\text{+}\) [12]. In human fetal intestine, T cells are characterized by a higher proportion of TCR\(\gamma\delta\) and CD8\(^\text{+}\)/CD4\(^\text{+}\) cells [13]. In addition, CD4 and CD8 double negative T cells (CD3\(^\text{–}\)CD4\(^\text{–}\)CD8\(^\text{–}\)) isolated from the intestine are generally considered of extrathymic origin [13]. In epithelium of the small intestine, lymphocytes may also express CD7 and CD2 in the absence of CD3 (CD2\(^\text{+}\)CD3\(^\text{–}\)CD7\(^\text{+}\)). In humans, the expression of RAG in the gut indicates that at this site a gene rearrangement process may take place, suggesting an active lymphopoiesis [14].

FOXXN1 is a developmentally regulated transcription factor, selectively expressed in epithelial cells of the skin and thymus, where it plays a necessary role for T lymphopoiesis [15-17] by inducing a proper epithelial cell differentiation and endothelial cell/thymic mesenchyme communication network [18]. FOXXN1 mutations lead to athymia [19,20] and result, in humans, in a SCID phenotype, referred as the human equivalent of the mice Nude/SCID syndrome [21-24]. During early prenatal life in humans, homozygous FOXXN1 mutation leads to a complete blockage of the CD4\(^\text{+}\) T cell maturation, while a few CD8\(^\text{+}\)TCR\(\gamma\delta\) cells, not expressing CD3\(_\text{e}\) molecule and not able to respond to a mitogenic stimulation, are found [25], thus suggesting an extrathymic site of lymphopoiesis for these cells. Here we studied the role of the intestine and liver as extrathymic sites of thymus-independent and FOXXN1-independent T lymphopoiesis in a FOXXN1\(^{-/-}\) athymic human fetus. We found the presence of a few T cells with a peculiar phenotype, indicative of the thymus-independent lymphopoiesis.

Results and Discussion

Detection of extrathymically derived T lymphocytes in the cord blood of FOXXN1\(^{-/-}\) fetus

The fetus analyzed in the present study was identified during a genetic counseling offered to heterozygous couples at risk for Nude/SCID disease, originated in the same geographic area where the first patients were identified [26]. The specific defect...
(R255X mutation in the FOXN1 gene) was searched on chorionic villi by direct sequencing.

In the absence of the thymus, few lymphocytes in CB co-express CD7^+CD2^+ (12% of CD3^- gated lymphocytes) in the FOXN1^+/− fetus, as compared to the control (17.2%) (Figure 1A). This population also comprises NK cells.

Extrathymic derived intraepithelial lymphocytes (IELs) are difficult to be univocally characterized, in that in mice they preferentially bear TCRγδ and express the CD8αβ [11,27,28], while in rats they express the CD8αβ heterodimer [12]. We previously described that in the FOXN1^+/− CBMCs, most of the CD8^+ cells were CD3^- [25], thus we looked at the CD8αβ^- cells on CD3^- gated lymphocytes. These cells were 1.3% in the FOXN1^+/− CBMCs and much more represented in the control (21.7%) (Figure 1A). Our data are in favor of a thymic dependence of such cells. In nude mice, a number of TCRγδ^+CD8αβ^- T IELs, lower than what found in euthymic mice, has also been reported [11].

The absence of CD4 molecule, would argue against the possibility that the CD3^-CD8αβ^- cells were dendritic cells (DC) [29]. Moreover, since the CD3^-CD8αβ^- cells were analyzed setting the gate on lymphoid cells, this would rule out the possibility that they were DCs of myeloid origin. In addition, the CD3^-CD8αβ^- cells are unlikely to be NK cells, in that they should express the CD8 with a dim intensity instead of a CD8 with a bright intensity, as in T cells, similarly to what found in Nute/SCID fetus.

In the FOXN1^+/− fetus, most of the rare CD3^-CD8αβ^- cells were CD4^- and CD8^- double negative (56.2% of CD3^- gated lymphocytes) as compared to the control (4.2% of CD3^- gated lymphocytes) (Figure 1A). A novel population of T cells with a similar phenotype, CD3^-B220^-CD4^-CD8^−, has also been identified in a nu/nu mouse, suggesting an extrathymic origin [30].

We previously documented in the FOXN1^+/− human fetus a considerable number of CD3ε^-CD8αβ^-TCRγδ^- cells, which also comprises cells with the CD8αβ heterodimer [25]. IELs may express only a partial CD3 complex bearing rare message of the ε

Figure 1. Detection of extrathymically derived T lymphocytes in the cord blood of FOXN1^+/− human fetus. (A) Flow cytometry analysis of CBMCs from WT (left dot plots) or FOXN1^+/− (right dot plots) fetuses (16 weeks of gestation). CD7 and CD2 together with the CD8α and CD8β expression patterns for the gated CD45^+CD3^- cells are shown. CD8α and CD4 expression is shown for the gated CD45^-CD3^- cells. Numbers indicate the frequency of the cells within the gate. Experiment was repeated two times. Data were obtained by gating first on viable cells and later on CD45^- cells. (B) RT-PCR analysis of CD3ε expression in CBMCs. The expression of CD3ε transcript in human skin fibroblasts (negative control), human thymus (positive control), CBMCs from WT or FOXN1^+/− fetuses is shown. Blanck, no cDNA. β-actin was used as loading control. Representative results from three independent experiments are shown. (C) Quantitative real-time PCR showing the expression of mRNAs encoding CD3ε (relative to β-actin) in skin fibroblasts (negative control), thymus (positive control) and CBMCs from WT or FOXN1^+/− fetuses (16 weeks of gestation). Representative results from two independent experiments are shown.

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chain although the T cell commitment is established by the presence of \( p \overline{\tau} \) transcript [31,32]. Thus, we evaluated the median fluorescence intensity (MFI) of CD3 signal, which was much lower in the FOXN1\(^{-/-}\) fetus than in the control (213 versus 1275 MFI, respectively), in keeping with the already reported dim signal in the same FOXN1\(^{-/-}\) fetus [25]. The presence in the FOXN1\(^{-/-}\) fetus of CD3\(^+\) cells, was however confirmed by the presence of the CD3\(\varepsilon\) transcript (Figure 1B). It should be noted that mRNA expression is almost equivalent in both FOXN1\(^{-/-}\) cells and wild type (Figure 1C). Thus, we cannot exclude that CD3\(^+\) cells are really CD3\(\varepsilon\) loci cells.

### Extrathymic sites of B-, NK- and T-lymphopoiesis in FOXN1\(^{-/-}\) SCID human fetus

Since in humans, intestine and liver are considered the main organs for extrathymic lymphopoiesis [2], we characterized the lymphocytes in these tissues. As expected on the T\(^{a+b}\)'NK\(^+\) phenotype of the human Nude/SCID [21], CD3\(^+\) cells and B cells (CD20) were normal in tissue sections (Figure 2A). The CD56 marker for NK cells revealed the presence of few and spread positive cells in the intestine sections but not in the liver (Figure 2A). Eventually, these findings confirm that, at 16 weeks of gestation, the development of mature B and NK cells is a thymus-independent process. Moreover, also the morphology of intestine and liver sections, evaluated through H&E staining, was normal (Figure 2B).

Within FOXN1\(^{-/-}\) intestine tissue, CD3\(^+\) cells were spread in the mucosa with a trend to aggregate in the crypts while in the control they formed clear aggregates (Figure 2B). In the liver of both FOXN1\(^{-/-}\) and control, CD3\(^+\) cells were present but spread (Figure 2B). Accordingly to what found in CB, CD4\(^+\) cells were absent in either intestine and liver of the FOXN1\(^{-/-}\) fetus, differently from the control (Figure 2B). A few CD8\(^+\) cells were detected in the FOXN1\(^{-/-}\) intestine similarly to the control (Figure 2B). Quantification in 5 random fields of the positive cells, stained as in Figure 2B, confirmed the absence of CD4\(^+\) cells and the presence of few CD8\(^+\) cells in both tissues of the FOXN1\(^{-/-}\) fetus (19.6\(\pm\)2 in FOXN1\(^{-/-}\) intestine versus 27.4\(\pm\)2 in WT intestine, \(p \leq 0.05\); 9.0\(\pm\)2 in FOXN1\(^{-/-}\) liver versus 14.0\(\pm\)4 in WT liver). No double positive (CD4\(^+\)/CD8\(^+\)) thymocytes were found by confocal microscopy in the FOXN1\(^{-/-}\) intestine (Figure 2C). When CD7\(^+\) cells were also stained for CD3, a few CD3\(^-\)/CD7\(^+\) cells were detected in the FOXN1\(^{-/-}\) fetus we demonstrated the presence of the CD3\(\varepsilon\) transcript through RT-PCR amplification of intestinal mRNA (Figure 2E). The quantitative PCR analysis revealed that the amount of this molecule in the intestine of FOXN1\(^{-/-}\) fetus was even higher than in the control (Figure 2F). Taken together these data suggest that a local production of T lymphocytes takes place in the intestine and liver in a thymus- and FOXN1-independent manner, even though we cannot completely exclude an early contribution of a thymus primordium to the production of T cells.

### Cells with naive phenotype can develop in the FOXN1\(^{-/-}\) human athymic fetus

The CD45RA molecule and the L-Selectin CD62L are expressed by naive lymphocytes in these tissues. As expected on the T\(^{a+b}\)'NK\(^+\) phenotype of the human Nude/SCID [21], CD3\(^+\) cells and B cells (CD20) were normal in tissue sections (Figure 2A). The CD56 marker for NK cells revealed the presence of few and spread positive cells in the intestine sections but not in the liver (Figure 2A). Eventually, these findings confirm that, at 16 weeks of gestation, the development of mature B and NK cells is a thymus-independent process. Moreover, also the morphology of intestine and liver sections, evaluated through H&E staining, was normal (Figure 2B).

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**Extrathymic sites of B-, NK- and T-lymphopoiesis in FOXN1\(^{-/-}\) SCID human fetus**

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**Identification of intestinal de novo lymphopoiesis in the FOXN1\(^{-/-}\) athymic fetus**

TCR gene rearrangement occurs at the T cell precursor stage and results in a functional antigen receptor. The process requires RAG1 and RAG2 recombination activity, which results in p\(\overline{\tau}2\) production. The fate of p\(\overline{\tau}2\)-expressing progenitors was found to include all \(\beta\) and most \(\gamma\) \(\delta\) T cells but to exclude B, NK, and thymic dendritic cells [32]. The expression of the surrogate TCR chain p\(\overline{\tau}2\) is upregulated during the DN3 stage of the T lymphocyte development, along with the expression of the RAG genes. p\(\overline{\tau}2\) expression is also found in pro-T cells at extrathymic sites of the T cell development in Nude mice [35]. As such, we evaluated the TCR repertoire of FOXN1\(^{-/-}\) CBMCs, most of them co-expressed both CD62L and CD3\(\varepsilon\) (0.8%), as compared to the 10.8% of the control (Figure 3B). In FOXN1\(^{-/-}\) CBMCs, 22.8% of CD3\(^+\) cells co-expressed both CD62L and CD45RA, similarly to the control (Figure 3C). The analysis of CD27 associated with CD45RA, as a further marker of a naive cell phenotype [33,34], revealed the presence of CD27\(^+\)/CD45RA\(^+\) cells (13.4% of CD3\(^+\) gated cells) in FOXN1\(^{-/-}\) CBMCs (Figure 3D). The immunofluorescence co-staining of CD45RA and CD3 molecules revealed in the intestine of the FOXN1\(^{-/-}\) fetus the presence of cells co-expressing both molecules (Figure 3E). This finding indicates that intestinal T lymphocytes also exhibit a naive phenotype.

**Cells with naive phenotype can develop in the FOXN1\(^{-/-}\) human athymic fetus**

The CD45RA molecule and the L-Selectin CD62L are considered markers of Recent Thymic Emigrants (RTE), thus being the hallmark of naive lymphocytes. In FOXN1\(^{-/-}\) CBMCs, 3.3% of CD45\(^+\) gated cells co-expressed CD3, with a dim intensity, and the CD45RA, differently from the control, in whom this population was 35.9%, almost all expressing CD3 with bright intensity (Figure 3A). On CD45\(^+\) gated cells, only a negligible number co-expressed the CD3 and CD62L markers (0.8%), as compared to the 10.8% of the control (Figure 3B). In FOXN1\(^{-/-}\) CBMCs, 22.8% of CD3\(^+\) cells co-expressed both CD62L and CD45RA, similarly to the control (Figure 3C). The analysis of CD27 associated with CD45RA, as a further marker of a naive cell phenotype [33,34], revealed the presence of CD27\(^+\)/CD45RA\(^+\) cells (13.4% of CD3\(^+\) gated cells) in FOXN1\(^{-/-}\) CBMCs (Figure 3D). The immunofluorescence co-staining of CD45RA and CD3 molecules revealed in the intestine of the FOXN1\(^{-/-}\) fetus the presence of cells co-expressing both molecules (Figure 3E). This finding indicates that intestinal T lymphocytes also exhibit a naive phenotype.
FOXN1^{−/−} patients, our results support the hypothesis that T cells do mature at extrathymic sites with an alternative lymphopoietic process, involving the same molecules implicated in intrathymic development, as pTα and RAGs. This process in humans is thymus- and FOXN1-independent. In summary, we document that a few T lymphocytes with a peculiar phenotype may develop in a thymus- and FOXN1-independent manner. We also report on the presence of intestinal CD3ε and CD8α, but not CD4ε, cells, a few of them showing a naïve phenotype. The expression of CD3ε, pTα, RAG1 and RAG2 transcripts in the intestine and TCR gene rearrangement, although abnormal, indicates that in humans a partial T cell ontogeny occurs at extrathymic sites in the Nude/SCID phenotype in a FOXN1-independent manner.

Materials and Methods

Fetus samples

Cord blood (CB) from the FOXN1^{−/−} fetus was obtained by cordocentesis at 16 weeks of gestation. Experiments using CB or fetal tissue samples were approved by the Institutional Ethical Committee for Biomedical Activities “Carlo Romano” at the “Federico II” University of Naples. Age-matched CB cells from the CEINGE bank were used as control. Fetus parents provided written informed consent.
Figure 3. Lymphocytes with naive phenotype in cord blood and intestine. Flow cytometry of CBMCs from normal and FOXN1−/− fetuses matched for gestational age (16 weeks of gestation). Dot plots show the expression pattern of the naïve cell markers. (A) Frequencies of CBMCs expressing both CD45RA and CD3 markers. (B) Frequencies of CBMCs expressing both CD62L and CD3 markers. (C) Frequencies of CBMCs coexpressing CD45RA and CD62L markers. (D) Frequencies of CBMCs coexpressing CD45RA and CD27 markers. Experiment in (A), (B), (C) and (D) was repeated two times. Data were obtained by gating first on viable cells and later on CD45+ cells (A and B) or finally also on CD3+ (C and D). (E) Confocal microscopy of fetal intestinal sections labeled with anti-human CD3 (red) and anti-human CD45RA (green). Representative results from three independent experiments with two samples are shown.

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Cell-surface staining and flow cytometry

In flow cytometry, FITC-, phycoerythrin (PE)-, allophycocyanin-cyanine 7 (APC-Cy7)-, peridinin chlorophyll protein (PerCP)- or PE-Cy7-coupled Abs were used on CB toward CD45 (2D1), CD7 (MT701), CD2 (RPA-2.10), CD3 (UCHT1), CD8α (SK-1), CD8β (2ST8.5H7), CD4 (L200), CD62L (SK11), CD45RA (HI100), CD27 (L128), CD45RO (UCHL-1), CD103 (Ber-ACT8) from BD Pharmingen, San Diego, CA or Beckman Coulter, Brea, CA. FACSCanto II flow cytometer and FACSDiva software (BD Bioscience, San Jose, CA) were used. For each sample, negative controls were stained with irrelevant Abs conjugated with the same fluorochrome [38]. The “fluorescence-minus-one” (FMO) controls have also been used to define precisely the cells that have fluorescence above background levels. Briefly, the samples have been stained with all of the reagents except one [38].

Histology

Intestine and liver tissue samples from a 16 weeks FOXN1<sup>−/−</sup> fetus or control were embedded in OCT compound and snapfrozen in liquid nitrogen or paraffin-embedded. The blocks were cut into serial 5-μm sections and mounted onto microscope slides for H&E staining and immunohistochemistry analysis. Immunodetections were performed by means of a Ventana automat (Ventana Medical Systems, Illkirch, France).

Immunohistochemistry

Tissue sections staining was performed on Benchmark XT platform (Ventana Medical Systems) with pre-diluted CD34, CD20, CD56, CD3, CD4 (Ventana-Confirm), CD8 (Cell Marque), 1:40 CD103 (Beckman Coulter, Brea, CA), 1:50 CD45RA (Dako, Denmark) and 1:25 CD62L Abs (Abcam, Cambridge, UK). Heat antigen retrieval was performed in buffer (CG1, Ventana) following the manufacturer instructions. The slides were incubated with primary Abs at 37°C for 32 min (CD34,
CD20, CD56, CD3, CD4 and CD8) or for 60 min (CD45RA, CD103, CD62L). Primary Ab was omitted for negative control. Nuclei were counterstained with hematoxylin. The reaction was detected by the ultraView Universal DAB Detection Kit, which utilizes a cocktail of enzyme labeled secondary Abs that locates the bound primary Ab. The complex is then visualized with hydrogen peroxide substrate and 3, 3′-diaminobenzidine tetrahydrochloride (DAB) chromogen, which produces a dark brown precipitate readily detected by light microscopy. Images were acquired by a microscope (DM 2500; Leica, Germany) at magnification 200 x or 100 x.

Confocal microscopy

Tissue samples were blocked with normal goat serum before staining and then treated with 1:50 of PerCP-labeled CD3 (BD Pharmingen, San Diego, CA) and 1:100 of FITC-labeled CD45RA Abs (BD Pharmingen, San Diego, CA) or 1:50 of PE-labeled CD4 (Beckman Coulter, Brea, CA) and 1:50 FITC-labeled CD8 (Beckman Coulter, Brea, CA) or 1:50 APC-labeled CD3 (Beckman Coulter, Brea, CA) and FITC-labeled CD7 (Beckman Coulter, Brea, CA). Images were acquired by a confocal microscope (LSM 510, Zeiss, Germany).

RNA and RT-PCR

Total RNA was isolated from normal human skin fibroblasts, normal human thymus, CB mononuclear cells (CBMCs) or intestinal frozen tissue using TRizol reagent (Invitrogen, Carlsbad, CA) and the Phase-lock gel columns (Eppendorf) by standard procedures. RNA was reverse transcribed by SuperScript III reverse transcription (Invitrogen, Carlsbad, CA). RT-PCR was performed using Taq polymerase (Roche, Germany). The following primers were used to amplify CD3ε: (forward) 5′-GATGCAGTCGGGCACTCACT-3′ and (reverse) 5′-TTGGGGGCAAGATGGTAATG-3′; or β-actin: (forward) 5′-GACAGGATGCAAGGAGAT-3′ and (reverse) 5′-TTGCTGATCCACATCTGCTG-3′. To avoid amplification of genomic DNA, the reverse primer for CD3ε was located on the 3′-4 exons junction.
Evaluation of TCR β-chain variable region (Vβ) spectratyping

TCR CDR3β sequencing of total mRNA isolated from intestine of the FOXN1+/− or control fetuses was performed after TCR β-chain amplification with a common reverse primer (CB3 primer) and 27 different forward primers (TCR Vβ gene family primers). PCR products were run on a CEQ 8000 automatic capillary sequencer (Beckman Coulter, Brea, CA) and fractionated on the size of the CDR3 region. Results were analyzed using CEQ 8000 software (Beckman Coulter, Brea, CA), which also gives the percentage contribution of a single family to the total TCR repertoire.

Quantitative real-time PCR

Real-time PCR was performed using the SYBR green detection reagent and analyzed with the Light Cycler480 system (Roche, Germany). Genes were normalized to the 18S reagent and analyzed with the Light Cycler480 system (Roche, Germany). Genes were normalized to 

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