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Chapter 4

Identification of a novel type of TB⁺SCID with a late double positive thymic arrest

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Abstract

More than 16 genes have been shown to be causative for severe combined immunodeficiency (SCID). However, around 20% of patients remain without molecular diagnosis. Here, we present data on a female patient with an atypical presentation of SCID whose initial diagnosis was hard to make. By transplantation of patient derived hematopoietic stem and progenitor cells in immunodeficient mice, we could demonstrate that this case was a T⁺B⁺NK⁺-SCID. Phenotypic analysis of the thymocytes of these mice demonstrated an arrest in T-cell development at the CD3⁻ to CD3⁻CD4⁺CD8⁻ double positive and the subsequent CD4⁻ and CD8⁻ single positive transition. Using whole exome sequencing, we identified a de novo heterozygous missense mutation in VPS4B that potentially could lead to SCID with atypical presentation.

Introduction

Severe combined immunodeficiency (SCID) is a congenital disorder that is characterized by a deficiency of T cells in peripheral blood (PB). This deficiency in T cells can be accompanied by defects in either B or NK cells or both and can be categorized in 2 groups: T⁺B⁺-SCID and T⁺B⁻-SCID. SCID affects approximately 1 in 100,000 live births and the patients present, mostly within their first year of life, with opportunistic infections and a failure to thrive. Currently, the best treatment option is a hematopoietic stem cell transplantation (HSCT) although clinical trials are ongoing with gene therapy approaches for SCID caused by mutations in either IL2RG or ADA. Patients suffering from SCID caused by a mutation in ADA also benefit from enzyme replacement therapy. Besides IL2RG and ADA, many genes can underlie this disease; up to now more than 16 have been described. However, a substantial fraction of patients without an identified disease-causing mutation is still remaining. Different fractions of SCID patients without a molecular diagnosis have been reported, ranging from 7 to 33%.

A new approach to search for disease-causing genes is whole exome sequencing. This can be done by sequencing the coding sequences of the genome of a group of patients, as has been shown for Bartter Syndrome and Miller Syndrome. Due to the rarity of SCID, especially the ones without a molecular diagnosis, it is very difficult to obtain a patient group for exome sequencing with the same molecular defect. In these cases whole exome sequencing of a single patient and both parents can be useful, in which the exome of the patient and both parents are compared to exclude inherited non-damaging single nucleotide variations (SNVs), instead of multiple patients and a control group. This approach has proven successful, for instance, by demonstrating that a mutation in CARD11 causes SCID.

Here, we report on a patient with atypical presentation of SCID. Using the novel approach of transplanting patient derived hematopoietic stem and progenitor cells (HSPCs) in the NOD/Scid-il2rg⁻/⁻ (NSG) mouse model, we could demonstrate that this patient has a hematopoietic cell-intrinsic defect and identified the stage of arrest in T-cell development. Using exome sequencing combined with whole exome sequencing analysis of patient and both parents we identified a de novo mutation in VPS4B that might be causative for T⁺B⁺NK⁺-SCID as it is predicted to act as dominant negative molecule.
Materials and methods

Human CD34+ bone marrow derived cells

Human bone marrow (BM) was obtained from healthy pediatric BM donors at the Leiden University Medical Center (LUMC, Leiden, The Netherlands). Informed consent was obtained from the parents for the use of leftover samples for research purposes. BM from the patient was obtained according to the guidelines of the Erasmus MC. The medical ethical committees of LUMC and of Erasmus MC approved this study and both served as institutional review boards. Informed consent was provided according to the Declaration of Helsinki. Mononuclear cells were isolated using Ficoll gradient centrifugation, frozen in fetal calf serum (FCS)/10% DMSO (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands and Sigma-Aldrich, St. Louis, MO, USA, respectively) and stored in liquid nitrogen. Cells were isolated and cultured as described previously11.

Mice

NOD.Cg-Pkdcscid Il2rgtm1Wjl/J (NSC) mice were obtained from Charles River Laboratories (Kent, United Kingdom) and bred in the animal facility at the Leiden University Medical Center. Experimental procedures were performed by the Ethical Committee on Animal Experiments of the LUMC. Female NSG mice were transplanted with CD34+ cells by intravenous injection within 24 hours after irradiation with 1.9 Gy using orthovoltage X-rays as described previously15-19. Mice transplanted with CD34+ cells derived from healthy pediatric bone marrow are historical controls, due to the rarity of this material, and have been described elsewhere11.

Flow cytometry

Analysis of lymphocyte populations in peripheral blood and composition of the B-cell precursor compartment in the BM of the patient was performed as previously described19.

Labeling of mononuclear cells of transplanted NSG mice has been described9. The following anti-human antibodies were used: CD3-PECy7 (UCHT1), CD4-APC-Cy7 (RPA-T4), CD5-FITC (UCHT1), CD7-PE-Cy5 (M-T701), CD8-PE-Cy7 (SK1), CD13-APC (WM15), CD16-PE (B73.1), CD19-APC-Cy7 (SJ25C1), CD20-PE (L27), CD23-APC (WM33), CD34-PE (8G12), CD45-V450, CD56-PE (UCHT2), CD7-PECy5 (M-T701), TCRβ-PE (T10B9.1A-31), streptavidin-PerCPCy5.5 (all from BD Biosciences, San Jose, CA) and CD10-biotin (eBioCB-CALLA) (eBioscience, San Diego, CA). Data were acquired on a Canto II (BD Biosciences) and analyzed using FlowJo software (Treestar, Ashland, OR, USA).

Sequence analysis

DNA was isolated from peripheral blood mononuclear cells from patient and both parents using the GeneElute™ Mammalian Genomic DNA Miniprep Kit (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer’s protocol. Sanger sequencing of known genes causative for SCID (ADA, RAG1, RAG2, IL2RG, CD3D, CD3E, CD3G, and CD3Z/CD247) was performed by PCR analysis (primers and amplification protocol are available upon request). The following primers were used for amplification and Sanger sequencing of the VPS48 gene: Forward 5’-ATCTGCGAGATGTGTTAC-3’, Reverse 5’-ATGAAAGACAGAAGTGTGC-3’. Amplification was performed using the following protocol: 2 minutes 94°C, followed by 35 cycles of 94°C 15 seconds, 60°C 30 seconds, 72°C 2 minutes, followed by 10 minutes at 72°C. Products were separated on a 2% gel and fragments were isolated using the Zymoclean Gel DNA Recovery Kit (Zymo Research Corporation, Irvine, CA, USA) for Sanger sequencing.

Whole exome sequencing

Exome sequencing was performed at GATC BioTech (Constance, Germany) using Agilent SureSelect post-capture and enrichment protocols together with Illumina HiSeq 2500 platform. Reads were aligned using an in-house developed GNU Makefile-based pipeline (‘Magpie’, https://git.lumc.nl/rig-framework/magpie), based on the GATK 2.7 best practices9. Briefly, each FASTQ pair was processed using Sickle (version 1.33, https://github.com/najoshi/sickle) with default settings to trim low-quality bases. Remaining reads were mapped to the hg19 human genome sequence using Burrows-Wheeler Aligner (BWA)-MEM (version 0.7.5)9. The resulting alignment was then compressed using the Samtools (version 0.1.19) suite9. Duplicates were removed using Picard Mark Duplicates (https://broadinstitute.github.io/picard/) and data was subsequently processed through the GATK tools RealignTargetCreator, IndelRealigner, BaseRecalibrator, and PrintReads according to the GATK version 2.7 best practices and using the provided GATK 2.5 data bundle. The resulting alignment file was then used as input for the GATK variant callers HaplotypeCaller and UnifiedGenotyper, followed by variant recalibration. The generated VCF file was annotated using the SE组装les web service (http://snps.gs.washington.edu/SeattleSeqAnnotation141) and various other publically available databases (see results).

Results

Clinical presentation of the patient

The patient was a girl born at gestational age of 39 weeks and 5 days with a birth weight of 4120 g. Her parents were non-consanguineous and the pregnancy was without complications. At the age of 5 months she was admitted to the hospital for failure to thrive. An auto-immune thyroiditis with hypothyroidism was detected and levothyroxine treatment was initiated. Nine months later, at the age of one year and two months, she developed a severe bronchiolitis secondary to a bocavirus and adenovirus infection. Because of respiratory failure she was admitted to the intensive care unit and subsequently developed progressive respiratory failure during mechanical ventilation, for which she was treated with extra-corporeal membrane oxygenation (ECMO) for 5 days. During ECMO two infusions of high dose methylprednisolon were given. Bronchoscopy showed trachomalacia of the right main bronchus. Immunological examination revealed a severe T-cell cytopenia (Fig. 1A), B-cell cytopenia (Fig. 1B) and a mild hypogammaglobulinemia (IgG: 2.5 g/L, IgA: 0.56 g/L, IgM: < 0.3 g/L). NK cell numbers were low at diagnosis but recovered quickly (Fig. 1C). Initially these abnormalities were thought to result from methylprednisolon treatment, but T-cell lymphopenia persisted, whereas B-cell and NK-cell lymphopenia recovered (Fig. 1A-C). At the time when B-cell numbers were spontaneously recovered, examination of the bone marrow showed no block in precursor B-cell development (Fig. 1D). Response to booster vaccination with tetanus, diphtheria and polio was normal, but no response could be detected to pneumococcal conjugate vaccine.

Atypical severe combined immunodeficiency

Mononuclear cells

Prkdc

https://broadinstitute.github.io/tm1Wjl

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Atypical severe combined immunodeficiency

During follow-up of the mice transplanted with patient-derived HSPCs, CD4+ cells could be detected (Fig. 3E), a molecule known to be upregulated upon SP was drastically decreased when patient single positive (SP) and CD8+ DP to SP transition while there might also be problems in TCR expression and signaling, 81 19 B treated with anti-epileptic drugs (levetiracetam) and haloperidol. 57 24 80 recurrent convulsions with multifocal epileptic activity and a delirium, for which she was post ECMO she developed intravenous immunoglobulin (IVIG) substitution, cotrimoxazol and valaciclovir prophylaxis. Because of suspected SCID she was treated with (intravenous and later subcutaneous) pathogenic. Subsequent SNP-array CGH analysis confirmed the chromosomal 46,XY and reported and abdominal ultrasound revealed the presence of a uterus, but ovaries or testes could not be genital organs had a normal female appearance, except for partial fusion of the labia minora, and abnormalities. Also in the RAG1 and RAG2 genes no pathogenic mutations could be detected (data not shown). On karyotyping examination the patient was chromosomally 46,XY. External patient derived HSPCs or control HSPCs (Fig. 3A). We then analyzed the thymus of transplanted transplanted with patient derived HSPCs, we examined T-cell developmental stages in thymi of As virtually no T cells could be detected in peripheral blood of both the patient and NSG mice transplanted with patient-derived HSPCs while they were present in UCB and BM transplanted control NSG mice (Fig. 2B, C). Taken together, the phenotype observed in mice transplanted with patient-derived HSPCs was consistent with a T8-SCID diagnosis. Suspicion of SCID

Due to the persisting T-cell lymphopenia there was a suspicion of SCID and therefore genetic and metabolic analysis for ADA and PNP deficiency was initiated, but this showed no abnormalities. Also in the RAG1 and RAG2 genes no pathogenic mutations could be detected (data not shown). On karyotyping examination the patient was chromosomally 46,XY. External genetic defects development for different types of SCID that were caused by different known underlying genetic defects14. During follow-up of the mice transplanted with patient-derived HSPCs, there was outgrowth of both myeloid and B cells in peripheral blood, but T cells were not detected at any of the time points, whereas in mice transplanted with normal control HSPCs T cells were readily detected (Fig. 2A). In addition, at the end of the experiment, T cells were hardly detectable in mice transplanted with patient-derived HSPCs while they were present in UCB and BM transplanted control NSG mice (Fig. 2B, C). Taken together, the phenotype observed in mice transplanted with patient-derived HSPCs was consistent with a T8-SCID diagnosis.

Arrest in T-cell development at DP stage

As virtually no T cells could be detected in peripheral blood of both the patient and NSG mice transplanted with patient derived HSPCs, we examined T-cell developmental stages in thymus of transplanted NSG mice. Total thymocyte counts did not differ between mice transplanted with patient derived HSPCs or control HSPCs (Fig. 3A). We then analyzed the thymus of transplanted NSG mice for the presence of the different stages of T-cell development. The percentage of both mature CD4+ single positive (SP) and CD8+ SP was drastically decreased when patient derived HSPCs were transplanted (Fig. 3B, C). Furthermore the percentage of CD3+ CD4+CD8+ double positive (DP) cells was lower while there was an increase in the percentage of CD3+ DP cells. The level of both CD3+ and TCRβ expression was decreased in the total thymocyte population as compared to mice transplanted with UCB derived HSPCs (Fig. 3D). In addition, no CD5+ expressing cells could be detected (Fig. 3E), a molecule known to be upregulated upon TCR signaling14. From these phenotypic data, we could confirm that this patient suffered from SCID. Together these data point to an arrest in development that is most pronounced at the CD3+ DP to SP transition while there might also be problems in TCR expression and signaling, as indicated by the increase in CD3+ DP and decrease of CD3+ DP (Fig. 3C).
NK cells were present both in PB and BM (Fig. 4A, B) and no aberrancies were detected within the B-cell compartment in the BM of transplanted NSG mice (Fig. 4C), further supporting that this patient is a T-B-NK-SCID.

Figure 4: No aberrancies in NK cells and B cells in NSG mice transplanted with patient derived HSPCs. A) Percentages and B) numbers of NK cells (CD16/56+CD13/33-) in peripheral blood and bone marrow of NSG mice. Percentages are within hCD45+ mononuclear cell fraction. C) B-cell developmental stages characterized by expression of CD20 and CD10 in bone marrow of NSG mice. Fractions are within hCD45+CD19+ mononuclear cell fraction.

Causative genetic defect
Since the arrest in T-cell development was most profound at the DP stage, percentages of CD3 expressing cells were lower and CD5hi expressing thymocytes were absent, mutations in one of the genes encoding the CD3 complex were suspected. Previous analysis had demonstrated that no abnormalities were present in ADA, PNP, RAG1, RAG2 or IL2RG. Therefore, we used Sanger sequencing to determine whether mutations were present in any of the genes encoding the different CD3 chains. No mutations were detected in CD3D, CD3E, CD3G or CD247 (CD3Z) (data not shown). As other SCID genes had been excluded during diagnosis, exome sequencing was performed on DNA of the patient and both parents. Trio-analysis was performed using the Modular GATK Pipeline (Magpie), which is a variant-calling pipeline based on GATK best practice recommendations to analyze multiple samples simultaneously. More than 177 million raw reads were obtained. Almost 164 million reads (92.5%) were uniquely aligned to hg19 using Burrows-WheelerAligner (BWA). Median exome coverage was 61-fold both for the exome of the mother and the patient and 57-fold for the reads obtained from the father. In the patient, 381712 SNVs were identified (Fig. 5A). SNVs were called using both HaplotypeCaller and UnifiedGenotyper from the Genome Analysis Toolkit (GATK). High-quality non-synonymous variants in exons were filtered out and sequentially we only filtered for SNVs that were not
present in either dbSNP (v137) or in the 1000 Genomes Project, leaving 29 variants. Genes that have been previously associated with SCID were checked manually because these could have been filtered out in the previous steps. These genes were also checked for the presence of compound heterozygous mutations, which were not detected. None of these known SCID genes was affected in any of the methods used. Using the trio analysis of patient and both parents, we selected SNVs that were not present in one of the parents as they were both asymptomatic for de novo autosomal dominant (AD) modeling. For autosomal recessive (AR) modeling, we filtered SNVs that were homozygous in the patient and heterozygous in at least one parent. X-linked modeling was comparable to AR modeling except that the mother was heterozygous for the SNV and that the SNV was not present in the father. These filtering steps resulted in 13 SNVs (Fig. 5A). Autosomal dominant (AD) de novo modeling resulted in a total of 3 SNVs, autosomal recessive (AR) in 9 SNVs and X-linked recessive inheritance pattern in 1 SNV. PolyPhen and SIFT databases were used to determine whether variations were predicted as benign and tolerated in which case we did not consider them as being causative of the phenotype observed in the patient. Hereafter, we were left with 1 de novo SNV with a possible AD inheritance pattern. This SNV is located in VPS4B (vacuolar protein sorting 4 homolog B, NM_004869.3, NP_004860.2) encoding a heterozygous G to A mutation (c.869G>A) resulting in a predicted amino acid change at position 290 from arginine to glutamine (p.Arg290Gln). The variant is located in the AAA-ATPase domain of the protein, which is needed for proper function of VPS4B. Validation by Sanger sequencing verified the heterozygous genotype of the patient for the mutation in VPS4B and the absence of the SNV in both parents (Fig. 5B).

### Figure 5: Detection of a de novo mutation in VPS4B

A) Filtering of data derived from exome sequencing of DNA from the patient and both parents. B) Sanger sequencing results of VPS4B gene in patient and both parents. Top; patient, middle; father, bottom; mother. AD; autosomal dominant, AR; autosomal recessive.

### Discussion

Up to now, more than 16 genes have been identified to be causative for SCID that give rise to different phenotypes based on absence and presence of B cells and NK cells besides the T-cell deficiency. However, there remains a large fraction of patients with an unidentified underlying genetic defect and in addition still new SCID causing genes are being identified. Here, we describe a young patient with atypical presentation of SCID for who no mutation could be detected in genes known to be causative for SCID using standard Sanger sequencing protocols.

Previously, we have optimized the NSG humanized mouse model for efficient and robust engraftment of human BM derived HSPCs while maintaining multilineage differentiation and lymphocyte functionality. In addition, we have used this model to determine the arrests in T-cell development for different types of SCID that were caused by a known underlying genetic defect. We also showed that the model faithfully recapitulates the arrests in B-cell development thereby validating this model for studies of human lymphoid development. Here, we describe the use of the NSG humanized mouse model for detection of SCID and determination of the stage of arrest in T-cell development. In addition, we confirmed that the patient with a suspected diagnosis of SCID indeed suffered from this disease with a T-B-NK- phenotype. When the patient was admitted to the intensive care unit, there was a drop in number of B cells that recovered to normal levels. In line with this, no aberrancies in B-cell development were observed in NSG mice transplanted with patient-derived HSPCs. We demonstrated that solely the T-cell lineage was affected and the arrest in T-cell development was found at the DP to SP transition with an accumulation of CD3+ DP at the expense of CD3+ DP. Arrests in T-cell development can also be studied using cocultures of HSPCs on OP9-DL1 stromal cells as this model is used to study normal T-cell development in vitro. However, it remains difficult to reach the more mature stages, i.e. true DP stages and beyond, using these cultures. Therefore it would have been impossible to identify the late stage of arrest in human T-cell development that was identified here using the humanized mouse model.

A heterozygous microdeletion on chromosome 14 was found using a diagnostic SNP array CGH approach, however, this feature was also present in the father and therefore presumed unlikely to be pathogenic (data not shown). Therefore, we used whole exome sequencing to determine the causative defect using a trio analysis to exclude inherited polymorphisms, which were presumed unlikely to be causative. This approach has been proven successful in studies where they, for instance, identified a mutation in CARD11 to be causative for SCID. Here, we filtered the data obtained with whole exome sequencing by using 2 different SNV callers, exclusion of SNVs present in either dbSNP or the 1000 Genomes Project and prediction of the possible effect of the SNV by PolyPhen and SIFT prediction tools. Furthermore, we excluded SNVs that were present in the parents. This step is crucial to come to disease-causing SNVs, because the genetic heterogeneity as well as low incidence of SCID make it impossible to analyze large numbers of phenotypically identical patient samples. Furthermore, it allowed us to screen for the presence of compound heterozygous mutations in genes known
to be associated with SCID, which were not found. In addition, by reporting single patient studies, other patients suffering from SCID without a known genetic cause can be screened for identified mutations as was also done for Bartter syndrome. The use of whole exome sequencing technology for the use of diagnosis in orphan diseases such as SCID remains challenging and time consuming due to single patient analyses caused by low prevalence, however, we demonstrate here that it is possible.

After filtering of the exome data, we have identified a de novo heterozygous missense mutation in VPS4B in the patient that most likely has caused the observed immunodeficiency. The heterozygous mutation leads to a change in amino acids from Arginine to Glutamine at codon 290 and was confirmed by Sanger sequencing. We hypothesize that this might translate into a dominant negative form of VPS4B (dnVPS4B) as multiple dnVPS4B sequence variants have indeed been described that interfered with the normal function of VPS4B protein. One described form of dnVPS4B has an amino acid change from the negatively charged glutamic acid to glutamine, which is uncharged and this mutation prevents binding of ATP.

The mutation described here leads to a change of the positively charged amino acid arginine to the uncharged glutamine. It is possible that this change leads to a disruption of the function of the AAA-ATPase domain similar to the described dnVPS4B mutant, which had a mutation in the same domain and acts as dominant negative molecule preventing VPS4B function. VPS4B binds to the Endosomal Sorting Complexes Required for Transport (ESCRT)-III complex that is involved in multivesicular endosome (MVE) biogenesis and the final steps of vesicle fission. It has been described that VPS4B mediates scission of microvesicles that contain TCRs from the T-cell plasma membrane at the immunological synapse. Furthermore, expression of a dnVPS4B disrupted the function of endogenous VPS4 resulting in an altered distribution of TCRs at the immunological synapse. In addition, we mined microarray data from sorted human thymus subsets and it was observed that the expression of VPS4B increased in the SP stage of human T-cell development (Fig. 6). It is likely that the mutated VPS4B identified in the current study functions as a dominant negative mutant that interferes with endogenous VPS4B function, which is normally needed to progress to the SP stage of T-cell development. Moreover, thymocytes from mice that are double knockouts for VPS4B function, which is normally needed to progress to the SP stage of human T-cell development (Fig. 6). It is likely that the mutated VPS4B identified in the current study functions as a dominant negative mutant that interferes with endogenous VPS4B function, which is normally needed to progress to the SP stage of T-cell development. Moreover, thymocytes from mice that are double knockouts for VPS4B identified by exome sequencing of the index patient and both parents. Functional experiments are needed to confirm whether the identified dominant negative mutant indeed leads to an arrest in T-cell development. Furthermore, the NSG humanized mouse model was demonstrated to be useful in the determination whether the patient indeed suffered from SCID. Due to the long duration of the transplantation experiment, this will not be useful to aid in diagnosis. However, by identification of VPS4B as a potential candidate gene for SCID and after confirmation by functional experiments, this could be included for screening of other patients suffering from SCID without a known genetic cause.

To conclude, here we have identified a gene that potentially could be causative for T<sup>B</sup>NK<sup>-</sup>SCID. A de novo G to A mutation at position c.869 resulting in an amino acid substitution from the negatively charged arginine to the uncharged glutamine that could result in a dnVPS4B protein was identified by exon sequencing of the index patient and both parents. Functional experiments are needed to confirm whether this alleviates the phenotype. Unfortunately, it is still a challenge to differentiate repaired IPS cells into HSPCs that can be used for transplantation purposes in e.g. humanized mouse models.

Figure 6: Expression of VPS4B in different stages of human T-cell development. Data was mined from microarray data of sorted populations from human thymus material.

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References


