Combined immunodeficiency with CD4 lymphopenia and sclerosing cholangitis caused by a novel loss-of-function mutation affecting IL21R

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Running title:
Extending the phenotype of IL21R-deficient patients

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Materials and Methods

Subjects

This study has been approved by the responsible local ethics committee of the Medical University of Vienna, Austria. Biological material was obtained upon informed consent in accordance with the Declaration of Helsinki.

Genetic analyses

Targeted enrichment of the selected 356 PID-associated genes was performed using the HaloPlex target enrichment system (Agilent) according to the manufacturer’s instructions. In brief, 200 ng of genomic DNA was distributed and digested by 8 pairs of restriction enzymes. The DNA fragments were pooled and hybridize with custom-made probes hybridizing to exons and 25 bp flanking regions of the selected genes. The hybridized DNA fragments were circularized, indexed and amplified. The quality of the library was inspected using 2100 Bioanalyzer (Agilent) prior to NGS. A multiplexed 100 bp paired-end-read exome sequencing (ES) was carried out on Illumina HiSeq2000 Sequencer running on HiSeq Control Software (HCS) 1.4.8, Real Time Analysis Software (RTA) 1.12.4.2. The data analysis was carried out using Burrows-Wheeler Aligner to align the reads to the human genome 19 (hg19). Insertion/deletion realignment was performed as well as GATK (Genome Analysis Toolkit) base quality score recalibration. For single nucleotide variant (SNV) and Insertion/Deletion calling, Unified Genotyper and GATK Variant quality score recalibration was performed. The variants were called and the variant effects predicted using SnpEff software(1). Known variants were excluded and the lists were filtered for nonsense, missense and splice-site variants within the exons of the targeted genes.

Variant validation by capillary sequencing was performed according to standard methods as previously described(2).
**Flow cytometry**

Flow cytometry analysis of peripheral blood mononuclear cells (PBMCs) was performed on a BD LSR Fortessa. PBMCs from the patient and a healthy control subject were isolated using Ficoll density gradient centrifugation and stained for 30 minutes at 4°C with mouse anti-human antibodies: CD3-allophycocyanin (APC)-H7 (clone SK7, BD Biosciences), CD4-BV605 (clone RPA-T4, BD Biosciences), CD8-FITC (clone HIT8a, BD Biosciences), CD19-PerCP-Cy5.5 (clone HIB19, eBioscience), CD25-phycocerythrin (PE, clone M-A251, BD Biosciences), CCR7-CF594 (clone 150503, BD Biosciences), CD31-APC (clone WM59, eBioscience), CXCR5-APC (clone 51505, BD Biosciences), CD27-PE (clone M-T271, BD Biosciences), CD10-APC (clone SN5c, eBioscience), CD38-PECy7 (clone HIT2, BD Biosciences), CD56-PECy5 (clone NKH1, Beckman Coulter) and CD21-PE (clone Bly4, BD Biosciences). Excess antibody was washed away by adding FACS staining buffer (FSB: 1x PBS + 2% fetal calf serum), centrifugation and resuspension in FSB prior to acquisition on LSR Fortessa.

**Intracellular staining**

The peripheral blood mononuclear cells were incubated for 1 hr in RPMI medium supplemented with 10% fetal calf serum in a 37°C incubator prior to stimulation with 10 ng/ml IL-21 (eBioscience, 14-8219-80) or 50 ng/ml IL-10 for 30 minutes at 37°C. Cells were immediately fixed with 1 volume BD Fix buffer (BD Biosciences) for 10 min at 37°C. After washing with FSB the cells were permeabilized by the dropwise addition of 1 ml of cold 60% methanol while vortexing. The permeabilized cells were incubated on ice for 30 min prior to staining with fluorochrome-conjugated antibodies. Phosphorylated STAT3 was detected by FACS using the AF647-conjugated antibody against phospho-Y705 residue (clone 4/P-STAT3, BD Biosciences).

**T-cell proliferation**

To monitor T-cell proliferation the PBMCs were were resuspended in PBS to a concentration of 5x10^6 cells/ml labeled with 1 µl of 1 µM violet proliferation dye (BD Biosciences) for 15 min at 30°C. After washing with PBS the cells were seeded in 200 µl RPMI medium supplemented with 10% FCS and 1 µg/ml anti-CD3 (clone OKT3, eBioscience) and anti-CD28 (clone CD28.2, eBioscience) into each well of a 96-well plate at a concentration of 1x10^6 cells per ml. The T-cell activation and proliferation capacity was measured by flow cytometry on day 1 and 3 after stimulation.
**Supplementary Figure 1. Genetic and phenotypic analysis of IL21R mutation.** (A) The pedigree of the index patient. Chromatograms display heterozygous deletion of the guanidine residue in parents and a homozygous deletion in the patient. (B) Schematic representation of the truncated IL21R compared to the wild type protein. The positions of the mutation and the frame-shift induced stop codon, as well as the amino acid length of the mutant and wild type protein are indicated. (C) Flow cytometry analysis of the activation markers CD69, CD95 and CD25 in CD4 (left) and CD8 T cells (right) upon *in vitro* stimualtion with anti-CD3 and anti-CD28 antibodies. (D) Flow cytometry analysis of the naïve and memory T-cell populations. (E) Flow cytometry analysis of the immature transitional B-cell populations in a healthy donor and the patient. HD, healthy donor.
Supplementary references
