

**Extending the clinical and immunological phenotype of human interleukin-21 receptor deficiency**

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## Supplementary information

### Methods

#### Patient

Samples from patient and controls were obtained after parental written consent. The study was approved according to the Declaration of Helsinki by the local review board and Ministry of Health (0306-10-HMO).

#### Molecular genetics

For whole exome analysis exonic sequences were enriched in the DNA sample of the patient using SureSelect Human All Exon 50Mb Kit (AgilentTechnologies, Santa Clara, CA). Sequences were determined by HiSeq2000 (Illumina, San Diego, CA) and 100-bp were read paired-end. Reads alignment and variant calling were performed with DNAnexus software (Palo Alto, CA) using the default parameters with the human genome assembly hg19 (GRCh37) as a reference.

The exome analyses of the DNA of the patient yielded 62.67 million confidently mapped reads, respectively. Following alignment to the human reference genome (hg19), 139,433 variants were noted, respectively. The average coverage of the exons was  $\times 71.79$  and the % of regions with less than 10 reads was 8.08%. We removed variants which were called less than X8, which were off-target, heterozygous, synonymous, MAF>0.1% in dbSNP or >1% at the Hadassah in house database as well as those predicted benign polymorphism by Mutation Taster software<sup>1</sup>. 12 variants were selected by this process (Supplementary Table I), but only *IL21R* was known as an immunologically relevant gene.

#### Signalling assays

Phosphorylation of STAT1 (pY701), STAT3 (pY705) and STAT5 (pY694) was determined by intracellular flowcytometry. Peripheral blood mononuclear cells (PBMCs) were stimulated with the respective cytokines (200ng/ml IL-21, Miltenyi Biotech, Bergisch Gladbach Germany; 200ng/ml IL-2, Biotest AG, Dreieich, Germany; 200ng/ml IFN- $\gamma$ , Bioferon, Laupheim, Germany; 200ng/ml IL-6, Immunotools, Friesoythe, Germany) for 15 min or were left untreated. Fixation and permeabilization were performed with the BD Phosflow Intracellular Staining kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Subsequently, cells were stained with the respective antibodies and analysed by flow cytometry on a LSRFortessa (BD Biosciences).

## Determination of intracellular cytokines

PBMCs were stimulated with 50ng/ml Phorbol myristate acetate (PMA) and 0.75µg/ml ionomycin for 4 hours in the presence of Golgiplug (BD Biosciences). Intracellular IL-17, IL-4 and IFN-γ were measured using the BD staining kit for intracellular cytokines in combination with indicated surface markers and cells were analysed by flow cytometry.

## Activation assays

All *in vitro* cultures were performed in RPMI/10%FCS. The up-regulation of IL-21R was determined after stimulation of PBMCs with 10µg/ml anti-CD40 (R&D Systems, Minneapolis, MN) for 2 days. The up-regulation of ICAM-1, CD25, CD86 and CD69 on B cells was determined after *in vitro* stimulation with 10µg/mL F(ab')<sub>2</sub> anti-IgM (Southern Biotech) and 10µg/ml anti-CD40 with or without 100ng/ml IL-21 for 36 hours. T cells were stimulated for 16 hours with 0.5µg/ml anti-CD3 (OKT3, kindly provided by B. Blumenthal, University of Freiburg), 1µg/ml anti-CD28 with or without 100ng/ml IL-21. Subsequent to stimulation, cells were stained with the respective antibodies. For all *in vitro* stimulations DAPI was added before analysis to exclude dead cells.

For lymphocyte proliferation assays PBMCs were labelled with 0.5µM CFDA/SE (life technologies). T cells were stimulated for 5 days with 0.5µg/ml plate-bound anti-CD3 and 0.5µg/ml anti-CD28. B cells were stimulated with 100U/ml IL-4 (Immunotools)/ 5µg/ml anti-IgM/ 10µg/ml anti-CD40 or with 100ng/ml IL-21/ 10µg/ml anti-CD40 for 7 days. Plasmablast differentiation of isolated B cells of the patient and healthy cordblood was performed as described previously<sup>2</sup>. On day 9 cells were analysed by flowcytometry and IgM, IgG and IgA levels were determined in the supernatants by ELISA.

Natural killer (NK)-cell and cytotoxic T-lymphocyte (CTL) degranulation and cytotoxicity were performed as described previously<sup>3</sup>. Degranulation was determined by the up-regulation of CD107a, cytotoxicity was measured in a <sup>51</sup>chromium-release assay. The assays were performed in the absence or presence of 100ng/ml IL-21.

## Antibodies used in this study:

CD19 APC-Cy7, CD21 PE-Cy7, CD38 PerCp-Cy5.5, CD19 Brilliant Violet 421, CD28 PerCP-Cy5.5, CD25 PerCP-Cy5.5, CD86 APC, CD45RA APC-Cy7, CD16 PerCp-Cy5.5 (BioLegend Inc.; San Diego, CA); IgG Alexa Fluor 700, CD27 Horizon V450, CD10 Brilliant Violet 605,

CD31 PE, CD4 Pacific Blue, STAT1 Alexa Fluor 647, STAT3 PE-CF594, STAT5 Alexa Fluor 647, CD27 Brilliant Violet 605, CD8 APC, CD69 FITC, ICAM-1 PE, CD127 Alexa Fluor 647, IL-21R PE, IgG1 PE, IFN- $\gamma$  FITC, CD8 Pacific Blue, CD107a PE, CD56 APC, CD10 APC (BD Biosciences, San Jose, CA); IgD FITC, IgA PE, IgM PE (Southern Biotech, Birmingham, AL); CD45RA FITC, CD19 PE-Cy7, CD8 PE, CD4 PE-Cy7, CD79a PE and CD3 PE-Cy7 (Beckman Coulter); CCR7 PE (R&D Systems, Minneapolis, MN); IL-17 PE, IL-4 APC (eBioscience Inc, San Diego, CA); IgM Alexa Fluor 647 (Jackson ImmunoResearch Laboratories, Suffolk, UK); TdT FITC, IgM FITC (Dako, Glostrup, Denmark).

#### Polymerase chain reaction (PCR)

IL-21R transcript expression was examined in EBV lines of the patient and two healthy controls. After RNA isolation (RNeasy Plus Mini Kit, Qiagen, Venlo, Netherlands) and reverse transcription (Superscript II reverse Transcriptase, Life Technologies, Carlsbad, CA) PCR was performed with primer pairs for IL-21R forward CATGGAGAGGATTGTCATCTGTC, reverse CAGAAATTCAGGGACCAAGTCAT and GAPDH forward GAAGGTGAAGGTCCGAGTC; reverse: GAAGATGGTGATGGGATTC.

#### Histology

2 $\mu$ m slides were taken from formalin fixed, decalcified (50% citric acid and 50% formic acid) paraffin embedded bone marrow of the patient and a control. H&E and Giemsa were stained routinely. For immunohistochemistry, the slides underwent heat mediated antigen retrieval with TRS (Dako target retrieval solution, Glostrup, Denmark) at pH 6.1. The CD38 antibody (clone SPC32, Leica Biosystems / Novocastra, Newcastle Upon Tyne, UK) was diluted 1:100 and detected by a horseradish peroxidase catalyzed brown chromogen reaction (Dako Autostainer Link®). Photos were taken with an Olympus BX 51 microscope (Olympus, Hamburg, Germany) with the AxioCam MRc microscope camera (Carl Zeiss, Oberkochen, Germany).

## Literature

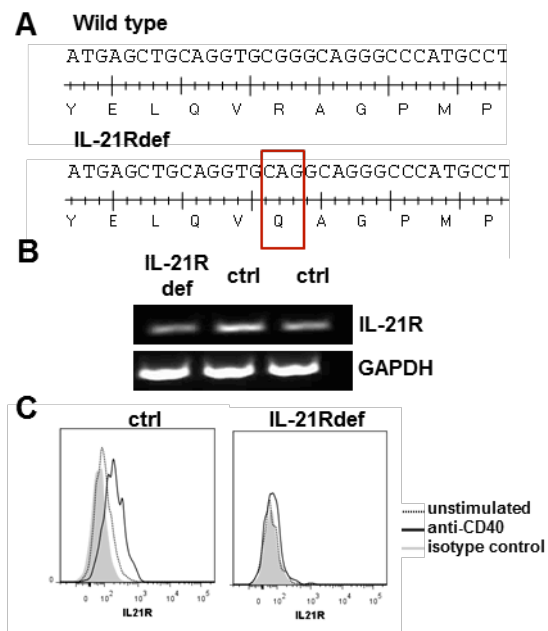
- 1 Schwarz, J. M., Rodelsperger, C., Schuelke, M. & Seelow, D. MutationTaster evaluates disease-causing potential of sequence alterations. *Nature methods* 7, 575-576, doi:10.1038/nmeth0810-575 (2010).
- 2 Stepensky, P. et al. Deficiency of caspase recruitment domain family, member 11 (CARD11), causes profound combined immunodeficiency in human subjects. *J Allergy Clin Immunol* 131, 477-485 e471, doi:10.1016/j.jaci.2012.11.050 (2013).
- 3 Jessen, B. et al. Subtle differences in CTL cytotoxicity determine susceptibility to hemophagocytic lymphohistiocytosis in mice and humans with Chediak-Higashi syndrome. *Blood* 118, 4620-4629, doi:10.1182/blood-2011-05-356113 (2011).

Chromosome	nucleotide	ref	mut	known variants	gene	EVS	AA change
1	158627472	G	T	rs115877891	SPTA1	0.000907	A-867-E
3	43122763	G	A		POMGNT2	0	P-54-L
3	45812898	A	G	rs147760034	SLC6A20	0.000615	F-212-S
4	122254003	T	C	rs144384829	QRFPR	0.000154	H-257-R
6	160510197	G	A	rs142990243	IGF2R	0.001	V-2127-M
7	103183253	C	T		RELN	0	S-2199-N
11	12495366	G	A	rs138341373	PARVA	0	E-111-K
12	133384738	C	A	rs146678000	GOLGA3	0.000231	S-306-I
13	39586234	C	G	rs367810608	PROSER1	0.000077	A-900-P
16	27455957	G	A		IL21R	0	R-201-Q
16	48130784	C	G		ABCC12	0	R-1023-T
17	80545038	T	C	rs185405224	FOXK2	0.000077	I-559-T

**Supplementary Table 1** Predicted pathogenic homozygous mutations

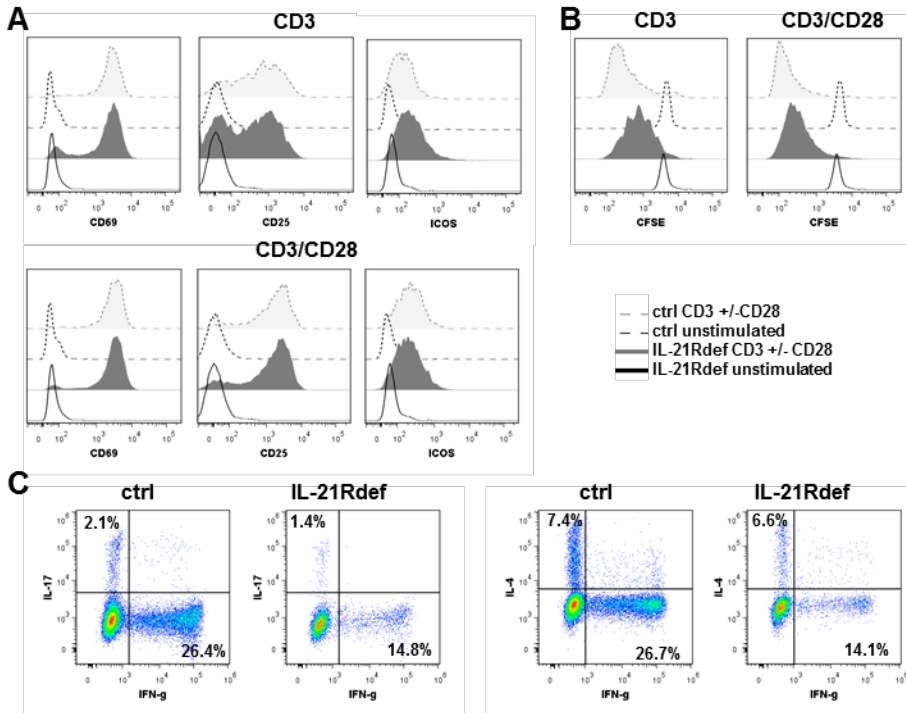
The table summarizes the predicted pathogenic homozygous mutations remaining after filtering the exome output as described in the text., ref reference sequence, mut mutation, EVS exome variants, AA amino acid.

## Supplementary Figures



### Supplementary Figure 1 Molecular characterization of IL-21R deficiency

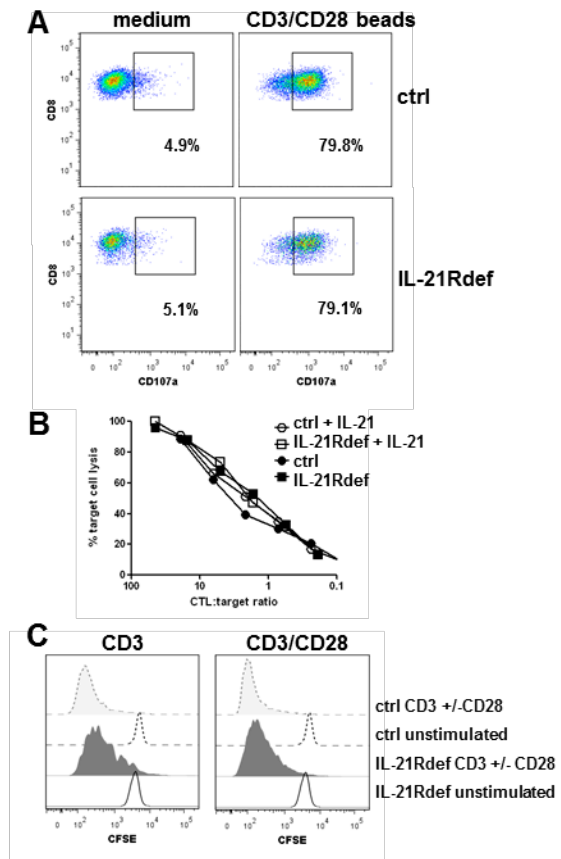
**A** Regular (upper row) and mutated (lower row) nucleotide and amino acid sequences of *IL-21R* are shown, surrounding the mutation c.G602A, p.Arg201Gln (red box). **B** *IL-21R* expression in EBV lines of the IL-21R-deficient patient (IL-21Rdef) and two healthy controls (ctrl) compared to *GAPDH* expression. **C** Flowcytometric detection of IL-21R protein expression on CD27<sup>neg</sup> naïve B cells with and without stimulation through anti-CD40.



**Supplementary Figure 2** CD4 T-cell function in IL-21R deficiency

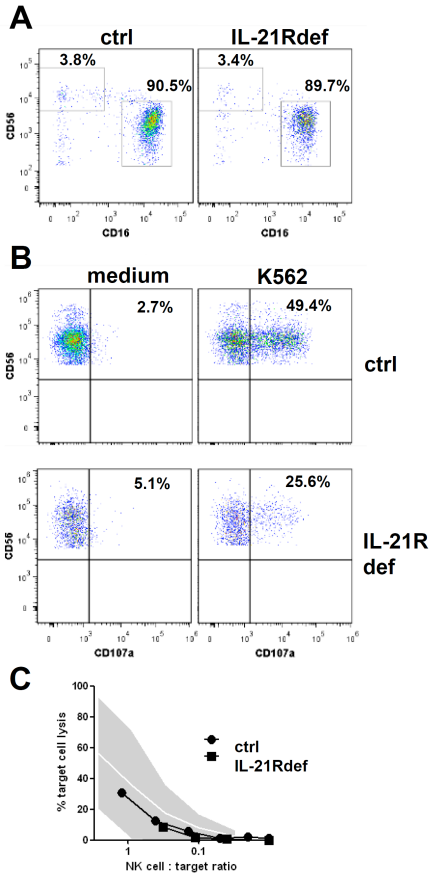
**A.** Up-regulation of CD69, CD25 and ICOS on CD45RA<sup>pos</sup> naïve CD4 T cells after stimulation with anti-CD3 (top) or anti-CD3/anti-CD28 (bottom). **B** Proliferation of CD4 T cells after stimulation with anti-CD3 (left) or anti-CD3/anti-CD28 (right) **C** IFN- $\gamma$ , IL-4 and IL-17 production of CD45RA<sup>neg</sup> memory CD4 T cells after PMA/ionomycin stimulation.





**Supplementary Figure 3** CD8 T-cell cytotoxicity function in IL-21R deficiency

**A** CD8 T-cell degranulation visualized by the up-regulation of CD107a with and without stimulation by CD3/CD28 beads. **B** Cytotoxic activity of CD8 T cells was determined in a <sup>51</sup>Cr-release killer assay using L1210 target cells. **C** CD8 T-cell proliferation on day 5 after stimulation as indicated.



**Supplementary Figure 4** NK cell phenotype and function in IL-21R deficiency

**A** Percentage of CD16<sup>neg</sup>/CD56<sup>high</sup> and CD16<sup>pos</sup>CD56<sup>dim</sup> NK cells of the day control and the IL-21R-deficient patient after gating on CD3<sup>neg</sup>/CD56<sup>pos</sup> lymphocytes. **B** NK-cell degranulation is shown by the up-regulation of CD107a in a control and the IL-21R-deficient patient in cultures without (left) or with K562 target cells (right). Cells are gated on CD3<sup>neg</sup>/CD56<sup>pos</sup> NK cells. **C** NK-cell cytotoxicity was determined in a <sup>51</sup>Cr-release assay for a healthy control and the IL-21R-deficient patient.