A reversion of an *IL2RG* mutation in combined immunodeficiency providing competitive advantage to the majority of CD8+ T cells

Taco W. Kuijpers,[1] Ester M.M. van Leeuwen,[2] Barbara H. Barendregt,[3,4] Paul Klarenbeek,[2,5] Daan J. aan de Kerk,[2] Paul A. Baars,[2] Machiel H. Jansen,[2] Niek de Vries,[2,5] René A.W. van Lier,[2,*] and Mirjam van der Burg[3]

**Current address: Sanquin Blood Supply, Amsterdam, The Netherlands*

[1]Emma Children's Hospital, Academic Medical Center (AMC), Amsterdam; [2]Department of Experimental Immunology, AMC; [3]Department of Pediatrics, Erasmus MC, University Medical Center Rotterdam, Rotterdam; [4]Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam; [5]Department of Clinical Immunology and Rheumatology, AMC, The Netherlands

©2013 Ferrata Storti Foundation. This is an open-access paper. *doi:10.3324/haematol.2012.077511*

Online Supplementary Design and Methods

Samples

Heparinized peripheral blood samples were collected from the patient longitudinally. PBMC were isolated using standard density gradient centrifugation techniques by use of Lymphoprep (Nycomed, Pharma, Oslo, Norway). In some instances, peripheral blood mononuclear cells (PBMC) were cryopreserved until use. The patient's samples were analyzed simultaneously with PBMC obtained from healthy (age-matched) control donors. All participants provided written consent after being fully informed about the study. The study was approved by the Medical Ethical Committee of the Academic Medical Center in Amsterdam which acts according to the principles of the Declaration of Helsinki principles (version Seoul 2008).

Immunofluorescent staining and flow cytometry

Absolute numbers of T cells, B cells and NK cells were determined in whole blood with Multitest six-color reagents according to the manufacturer's instructions using a FACSCanto II and FACS Diva software (BD Biosciences, Erembodegem, Belgium). For analysis of isolated PBMC, cells were resuspended in phosphate-buffered saline (PBS), containing 0.5% (w/v) bovine serum albumin and 0.01% sodium azide. Two hundred PBMC were incubated with saturating concentrations of fluorescently label conjugated monoclonal antibodies. The following directly conjugated reagents were used for flow cytometry: CD4-PE, CD8-PerCP-Cy 5.5, CD3-APC, CD16-PE, CD56-PE, CD19-PerCP-Cy 5.5, CD20-APC, CD25-PE, streptavidin-APC, CD122-biotin and CD132-biotin from BD Biosciences, CD45-RD1 from Beckman Coulter (Mijdrecht, The Netherlands), and CD27-FITC from Sanquin (Amsterdam, The Netherlands). Cells were analyzed using a FacsCalibur flow cytometer and CellQuest software (BD Biosciences).

Proliferation assays

PBMC were resuspended in PBS at a final concentration of 5-10x106 cells/mL. PBMC were labeled with 0.5 μM (final concentration) of 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Paisley, UK) in PBS for 8 to10 min at 37°C under constant agitation. Cells were washed, resuspended in culture medium: IMDM supplemented with 10% fetal calf serum (BioWhittaker, Verviers, Belgium), antibiotics and $3.57x10^{-4}$ % (v/v) β-mercaptoethanol (Merck, West Point, PA, USA) and subsequently cultured for 6 days. T cells were stimulated with anti-CD3 (clone 1XE) ± anti-CD28 (clone 15E8) monoclonal antibodies (both from Sanquin, Amsterdam, The Netherlands) at saturating concentrations and/or with specific cytokines at an optimal dose for control cells (for IL-2: 100 U/mL, IL-7 and IL-15: 10 ng/mL), as described before. For antigen-specific T-cell stimulation tetanus toxoid (RIVM, Bilthoven, The Netherlands), cytomegalovirus (CMV), varicella-zoster virus (VZV) (both Microbix Biosystems, Toronto, Canada) and *Candida albicans* (Hal Allergy, Leiden, The Netherlands) antigens were used at a final concentration of 15 fL/mL, 20 μL/mL (CMV and VZV) and 25 μg/mL, respectively, previously defined as the optimal effective dose for stimulation of $CD4^+$ T cells.

For B-cell proliferation, labeled PBMC containing a fixed number of B cells ($2x10^5$ per well) were cultured and stimulated with different combinations of anti-IgM, anti-CD40 (both from Sanquin), 20 ng/mL IL-21 from Invitrogen (Paisley, UK), 200 μg/mL CpG ODN 2006 from Invivogen (Toulouse, France), 50 U/mL IL-2. Supernatants were tested for secreted IgM and IgG with an in-house ELISA using polyclonal rabbit anti-human IgG and IgM reagents and a serum protein calibrator all from Dako (Heverlee, Belgium).

IL2RG gene sequence analysis

Genomic DNA was isolated from total peripheral blood leukocytes and sorted lymphocyte subsets with the Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) or with a direct lysis method using detergents and proteinase K in case of cell numbers below 500,000. The coding exons of the *IL2RG* gene (NCBI NM_000206) were amplified by polymerase chain reaction and sequenced using the BigDye Terminator v1.1 cycle sequencing kit according to the manufacturer's instructions (Applied Biosystems) and a 3130 genetic analyzer (Applied Biosystems). Short tandem repeat analysis was performed with the PowerPlex16 kit (Promega, Madison, WI, USA).

Online Supplementary Table S1. Percentage reversion in various sorted lymphocyte subsets.

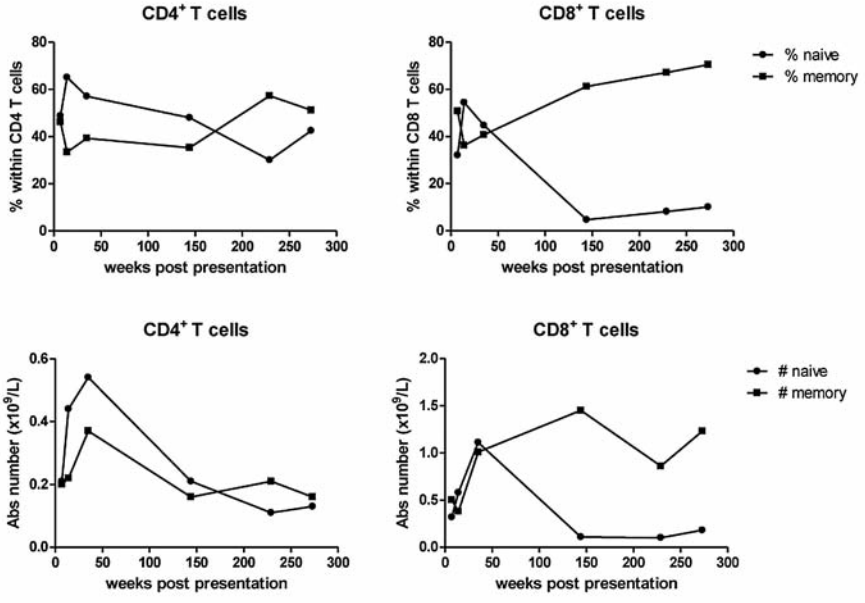
	Year of sampling		
	2005	2006	2007
T		50	
B		0	
NK		0	
leukocytes		10	
naïve CD4	0		0
memory CD4	5		10
effector CD4			40
naïve CD8	5		40
memory CD8	25		90
effector CD8			80
TCRαβ			60
TCRγδ			20

T-cell receptor repertoire analysis by high throughput sequencing

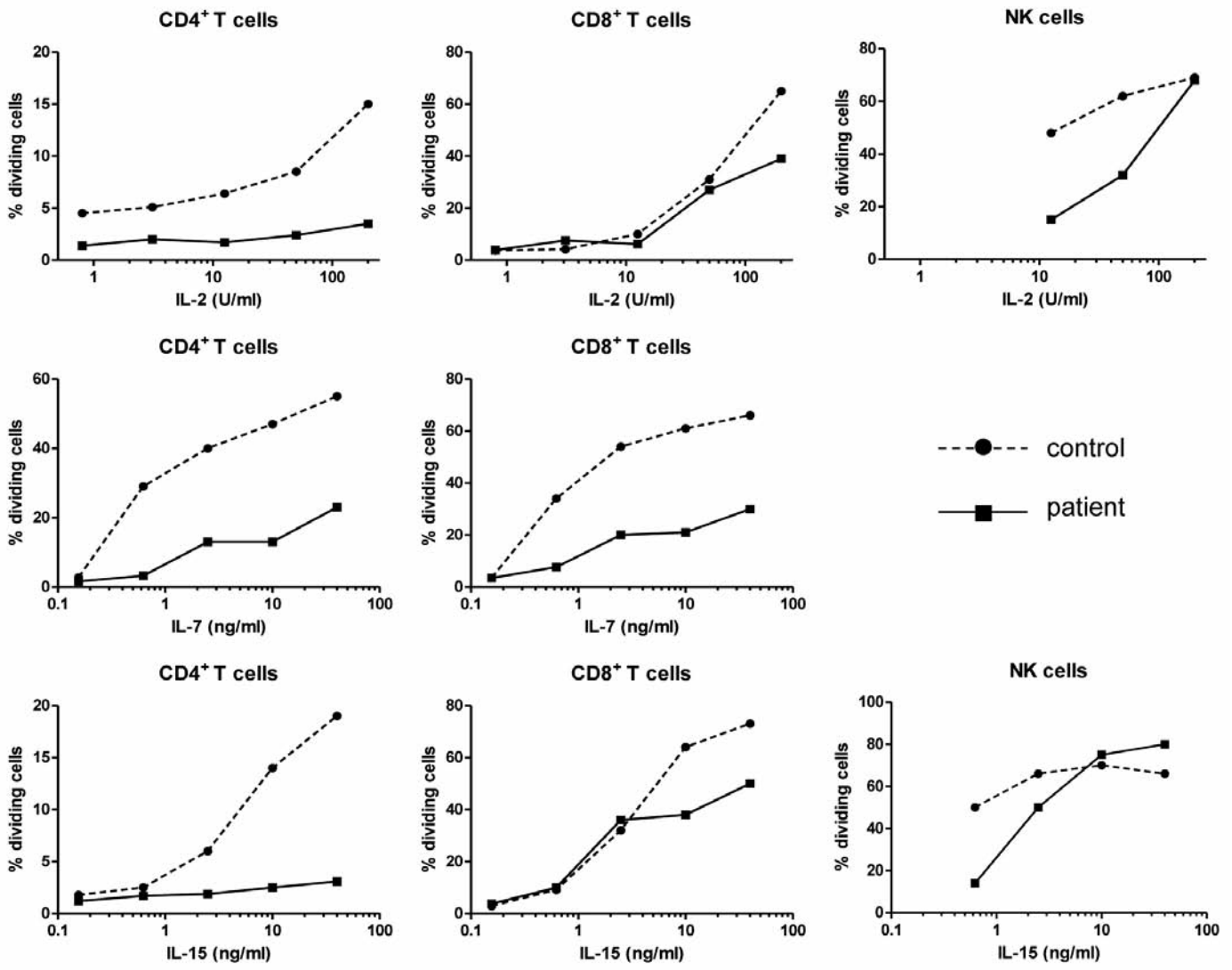
The T-cell receptor repertoire of sorted cell populations was analyzed as previously described. The complementarity determining region (CDR)-3 of the TCR-β-chain was used as clonal tag to identify individual clones. Briefly, mRNA was isolated using the RNeasy Mini System (Qiagen, Venlo, the Netherlands). cDNA was synthesized with Superscript RT-III and oligo-dT primers according to the manufacturer's protocol (Invitrogen Life Technologies, Breda, NL). In the first step of linear amplification cDNA was amplified using a modified version of the Vβ primerset described by Dongen *et al.* All V-primers were tailed with the primerB sequence needed for sequencing according to the 454 Titanium protocol for Amplicon sequencing (Roche Diagnostics, Mannheim, Germany). In this article we use the HUGO nomenclature according to Folch and Lefranc.

In the first step of linear amplification the cDNA is amplified in the presence of 5 pmol of each of the 23 V-primers (Biolegio, Nijmegen, the Netherlands), 1x buffer B (Solis BioDyne, Tartu, Estonia), 1mM $MgCl_2$, 0.1 mM dNTPs and 3U of Hotfire (Solis BioDyne) in a volume of 20 μL using a T-Professional thermocycler (Biometra, Goettingen, Germany). Amplified products were purified using AMPure XP SPRI-beads (Agencourt Bioscience, Beverly, USA).

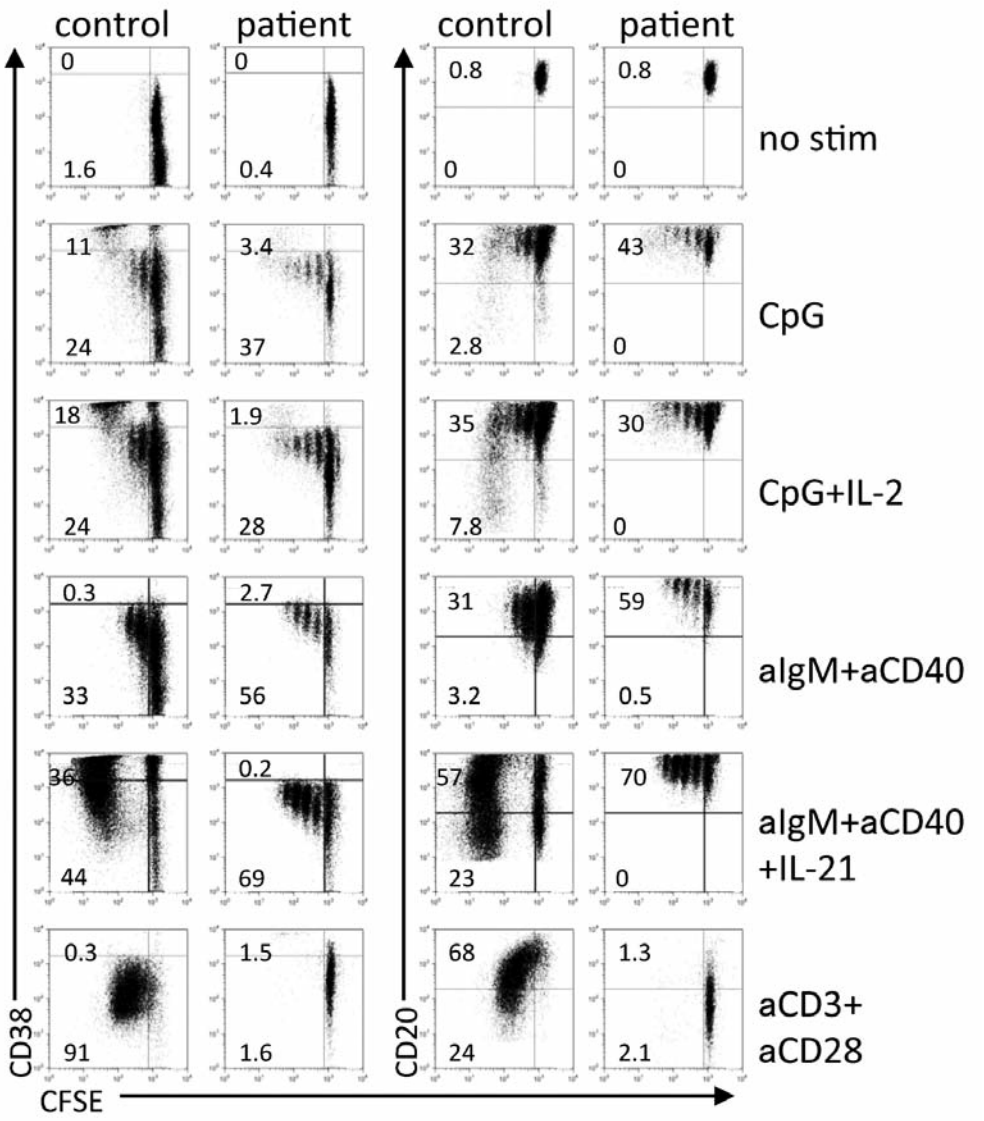
After linear amplification a generic PCR was performed to prepare the samples for sequencing. In this second reaction primerB is used as generic forward primer and a generic primer specific for the TCR-β constant gene segment (CTCAAACACAGCGACCTC) as reverse primer. The reverse primer is tailed with a multiplex identifier and primerA as described in the amplicon sequencing manual. After amplification, the samples are purified using the AMPure beads and quantified using fluorospectometry and prepared according to the manufacturer's protocol for Amplicon Sequencing on a Roche Sequencer FLX using the Titanium platform. Over 40,000 (bead-bound) TCR sequences were analyzed.



Online Supplementary Figure S1. Numbers and differentiation of $CD8^+$ T cells. Changes in percentages and absolute numbers of naïve ($CD45RA^+CD27^+$) and memory ($CD45RA^-CD27^+$) $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells over time.



Online Supplementary Figure S2. Decreased proliferation of T and NK cells in response to CD132 cytokines. Proliferation of $CD4^+$, $CD8^+$ T cells and NK cells upon different concentrations of IL-2, IL-7 and IL-15, all cytokines that signal via CD132, the common γ chain of the receptor. Circles: control cells, squares: patient. The percentage of dividing cells indicated is after 6 days of culture.



Online Supplementary Figure S3. Defective B-cell proliferation *in vitro*. Proliferation of CFSE-labeled CD19^{+} B cells after culture with the indicated combinations of stimuli. CD38 up-regulation and CD20 down-regulation indicates differentiation into plasmablasts. Numbers in the corresponding quadrant indicate percentages.