Deficiency of CARD11 causes profound combined immunodeficiency in humans

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Funding: This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 620 (K.W.), and by the Federal Ministry of Education and Research (BMBF 01 EO0803) (K.W., H.E., B.G.). P.S. was supported by a Dahlia Greidinger Cancer Research Fund. A.E. was supported by a Major Initiative Award from the Clive and Vera Ramaciotti Foundation and a Career Development Fellowship from the NHMRC (APP1035858). The authors are responsible for the contents of this publication.
ABSTRACT

Background: Profound combined immunodeficiency may present with normal numbers of T and B cells, therefore the functional defect of the cellular and humoral immune response is often not recognized until the first severe clinical manifestation. Here we report a patient of consanguineous descent presenting at 13 months of age with hypogammaglobulinemia, *Pneumocystis jirovecii* pneumonia and a suggestive family history.

Objective: To identify the genetic alteration in a patient with combined immunodeficiency and characterize human CARD11 deficiency.

Methods: Molecular, immunological and functional assays were performed.

Results: The immunological characterization revealed only subtle changes in the T- and NK-cell compartment while B-cell differentiation though normal in number was distinctively blocked at the transitional stage. Genetic evaluation revealed a homozygous deletion of exon 21 in *CARD11* as the underlying defect. This deletion abrogated protein expression and activation of the canonical NF-κB pathway in lymphocytes after antigen receptor or PMA stimulation, while CD40 signaling in B cells was preserved. The abrogated activation of the canonical NF-κB pathway was associated with severely impaired up-regulation of ICOS, OX40, cytokine production, proliferation of T cells and BAFF receptor expression on B cells.

Conclusion: Thus in CARD11 deficiency the combination of impaired activation and especially up-regulation of ICOS on T cells together with severely disturbed peripheral B-cell differentiation apparently leads to a defective T-B co-operation, probably germinal center formation and clinically results in severe immunodeficiency. This report discloses the crucial, non-redundant role of the canonical NF-κB activation and specifically CARD11 in the antigen specific immune response in humans.
Clinical implications
CARD11 deficiency presents as combined immunodeficiency despite normal lymphocyte counts. Diagnostic clues are hypogammaglobulinemia, B-cell phenotype and lack of T-cell proliferation to anti-CD3/28. The immunodeficiency can be successfully corrected by HSCT.

Capsule summary
Abrogation of antigen-receptor induced NF-κB signaling in a patient with CARD11 deficiency causes profound combined immunodeficiency due to a complex developmental and functional dysregulation of the adaptive immune system.

Key words
CARD11, human, combined immunodeficiency, hypogammaglobulinemia, pCID, transitional B cell, NF-κB, BAFF-R, ICOS, germinal center

Abbreviations used
BAFF-R receptor of the B-cell-activating factor
BCR B-cell receptor
CARD11 caspase recruitment domain family, member 11
CFSE carboxyfluorescein diacetate, succinimidyl ester
CID combined immunodeficiency
ENU N-ethyl-N-nitrosourea
GC germinal center
ICOS inducible T-cell co-stimulator
NK cell natural killer cell
PBMC peripheral blood mononuclear cells
PHA phytohemagglutinin
PMA Phorbol 12-Myristate 13-Acetate
SNP single nucleotide polymorphism
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INTRODUCTION

Combined immunodeficiency disorders (CID) affecting the cellular and humoral immune response in humans, predispose patients to common bacterial and opportunistic infections. Severe forms (SCID) present in the first year of life and are caused by genetic defects affecting either the recombination of the antigen receptor or receptors of essential cytokines. In recent years, several less severe forms of CID have been discovered, presenting later in life with opportunistic infections or alterations of the T-cell homeostasis. While some of these patients suffer from hypomorphic mutations of known SCID associated genes, increasingly new defects are discovered altering the signaling of the T- or B-cell receptor (TCR/BCR) without affecting T- and B-cell numbers, but affecting their function. Thus, CD3γ-deficiency often presents with normal T- and B-cell counts. Abrogation of TCR induced calcium response in Orai1- or STIM1-deficient children permits normal differentiation of T and B cells, but not their antigen specific response. Other defects preferentially affect T-cell subpopulations while total T cell counts can be normal. Therefore key signaling molecules downstream of the antigen receptors are prime candidates underlying profound combined immunodeficiency (pCID).

CARD11 belongs to the family of membrane-associated guanylate kinases (MAGuK) which play a crucial role in the differentiation of neuronal as well as immunological tissues as scaffold proteins facilitating the assembly of clusters of signaling molecules at sites of cell-cell contact. Through its interaction with BCL10 and MALT1, CARD11 is essential in the activation of the canonical NF-κB pathway in lymphocytes. In mice with a targeted deletion of Card11 or carrying a hypomorphic allele the antigen-independent development of T and B cells in thymus and bone marrow, respectively, was normal, but peripheral activation-dependent proliferation and differentiation of lymphocytes were altered. Serum immunoglobulin levels were low and after vaccination Card11-mutant mice mounted only poor responses to T-dependent and -independent antigens.
Here we report a female patient with a homozygous deletion of exon 21 in \textit{CARD11} presenting at the age of 13 months with disturbed B-cell differentiation, hypogammaglobulinemia and \textit{Pneumocystis jirovecii} pneumonia.
METHODS

All investigations were performed after informed consent by the parents. The study was approved by the institutional review board (ITB 0306-10-HMO) according to the declaration of Helsinki.

All animal studies were approved by the animal experimentation ethics committee of the Australian National University.

For additional material and methods see Online Repository.

Signaling assays

For detection of phosphorylated p65 and IκBα, peripheral blood mononuclear cells (PBMCs) were incubated for 2 hours at 37°C. Cells were either left untreated or stimulated with 20 µg/ml F(ab')2 anti-IgM (Southern Biotech) for 35 min and with 200 ng/ml PMA (Sigma) or recombinant CD40 ligand (CD40L) for 15 minutes at 37°C. Phosphorylation of ERK1/2 was determined upon stimulation with 20 µg/ml F(ab')2 anti-IgM or 200 ng/ml PMA for 5 minutes at 37°C. Fixation and permeabilization was performed with the BD Biosciences®Phosflow intracellular staining kit following the manufacturer's instructions. Subsequently, cells were stained with antibodies against cell surface markers and intracellular proteins and analyzed by flow cytometry.

Intracellular calcium mobilization was measured as described previously. 2 x 10⁶ PBMCs were labeled with Indo-1 (Invitrogen). Subsequent cell surface staining allowed gating on CD27neg/IgGneg/IgAneg non-memory B cells. Baseline acquisition for 45 seconds was followed by stimulation with 10 µg/ml F(ab')2 anti-IgM. Ionomycin (Sigma) was added after 8 minutes as a loading control.

Determination of intracellular cytokines

For determination of intracellular cytokines, BD staining kit for intracellular cytokines was used following the manufacturers’ instructions. IL-2 production was detected after stimulation of PBMCs with 100 ng/ml PMA and 0.75 µg/ml ionomycin in the presence of the protein
transport inhibitor GolgiStop® for 13 hours at 37°C. IL-17, IL-4 and IFN-γ were detected after stimulation of cells with PMA (50 ng/µl) and 1 µg/ml ionomycin for 4 hours at 37° in the presence of 500 U/ml IL-2 and GolgiPlug®. Subsequent to the stimulation cell surface staining was performed. Cells were fixed and permeabilized following the manufacturer’s instructions and stained for intracellular cytokines.

Activation assays
To determine up-regulation of activation markers on B cells, in-vitro stimulation of 5 x 10^5 PBMCs with 15 µg/ml F(ab')2 anti-IgM and optimal amounts of recombinant CD40L was performed. Cells were harvested after two days and stained with the corresponding antibodies.

To investigate T cell specific up-regulation of activation markers, 5 x 10^5 T cells were separated and immediately stimulated via CD3/CD28 beads for 18 hours at 37° (Invitrogen). For activation of mouse T cells, 1.5 x 10^5 splenocytes were cultured for 18 hours in 96 well plates pre-coated with 1 µg/ml anti-CD3 or 1 µg/ml anti-CD3 and 10 µg/ml anti-CD28 (all BD Pharmingen). Subsequent to stimulation, cells were washed and stained with the appropriate antibodies.

Plasmablast differentiation
PBMCs of the patient and human cord blood cells (6.25 x 10^5 cells) were stimulated with CD40L and IL-21 for 9 days in Iscove’s medium supplemented with 10 % FCS, 1 µg/ml insulin, 2.5 µg/ml apo-transferrin, 0.1 % fatty acid supplement, 1% non-essential amino acids, 2 mM glutamine, and 1 µg/ml reduced glutathione. CD40L and IL-21 were prepared as described. The phenotype was determined by flowcytometry.

Immunoglobulin concentrations
Ig concentrations were determined by sandwich ELISA. In brief, 96 well plates were coated with anti-human Igmix (Jackson ImmunoResearch) in bicarbonate buffer. Bound
immunoglobulins were detected by alkaline-phosphatase-conjugated anti-human IgM, IgG or IgA (Jackson ImmunoResearch), respectively. Immunoglobulin concentrations were calculated from human IgM, IgG, or IgA standards (N Protein Standard SL; Siemens) processed in parallel.

T-cell proliferation
PBMCs were labeled with 0.5 μM CFSE (Invitrogen) following standard protocols. Cells were left untreated or stimulated with 1 μg/ml anti-CD3 (OKT-3, eBioscience) and 1 μg/ml anti-CD28 (BD Biosciences) or 2.5 μg/ml PHA (Remel) for 5 days at 37°C. Cells were harvested, stained for CD4 and CD8 and subsequently analyzed on a Navios flowcytometer (Beckman coulter).

Mouse strain
The unmodulated mouse strain carrying a hypomorphic allele of Card11 has been described previously\textsuperscript{10}. All mice were on a C57BL/6 or B10.BR background.
RESULTS

Case report

At first presentation we evaluated a nine-month old girl born to consanguineous parents of Palestinian descent with upper respiratory tract infection, hypogammaglobulinemia and a remarkable family history (Fig1). She is the youngest of six children. The firstborn sister failed to thrive and died without diagnosis at three-months-of-age due to progressive respiratory failure. The fourth child, male, presented at six-months-of-age with meningitis and recurrent pneumonias. At 15 months he developed progressive respiratory distress with high fever and died within a few days. The only recorded laboratory anomaly was a panhypogammaglobulinemia. Three other siblings are healthy with normal immunoglobulin levels. The index patient had a normal medical history until the age of six months when she developed an upper respiratory tract infection. At that time her laboratory examinations revealed significantly reduced IgG levels (IgG 0.88 g/l (normal range for age: 2.17-9)), but normal IgA (0.35 g/l (0.11-1.06)) and IgM (0.65 g/l (0.35-1.26)). Three months later laboratory tests demonstrated panhypogammaglobulinemia but otherwise a normal immune phenotype, PHA proliferative response, TREC (5255 copies per 0.5 μg DNA, normal for age: >400 copies) and T-cell repertoire. She was started on monthly infusions of intravenous immunoglobulins. At the age of thirteen months she presented with fever and severe dyspnea due to *Pneumocystis jirovecii* pneumonia. After successful treatment the patient was put on prophylaxis with trimethoprim-sulfamethoxazole and because of the suspicion of pCID she was listed for allogeneic bone marrow transplantation. In parallel, a genetic diagnosis by whole exome sequencing and SNP array was sought.

*Immune phenotype of CARD11 deficiency*

White blood cell counts at the time of the immunological characterization were still unremarkable (Table1). Flowcytometric analysis of PBMCs depicted normal total CD4 T-cell counts, CD45RA<sup>pos</sup> naïve, CD45RO<sup>pos</sup> memory CD4 T cells and CD45RA<sup>pos</sup>/CD31<sup>pos</sup> recent thymic emigrants (RTE) (Table1). However, the absolute and relative counts of regulatory T
Stepensky 12

cells (Tregs) (CD4<sup>pos</sup>/CD25<sup>pos</sup>/CD127<sup>neg</sup>) were reduced, compared to age matched reference values (Fig2A).

Among CD8 T cells, absolute numbers and distribution of naïve (CD27<sup>pos</sup>/CD45RA<sup>pos</sup>/CCD7<sup>pos</sup>) and central memory (CD27<sup>pos</sup>/CD45RA<sup>neg</sup>/CCD7<sup>pos</sup>) were within the normal range. Effector memory (CD27<sup>neg</sup>/CD45RA<sup>neg</sup>/CCD7<sup>neg</sup>) and terminally differentiated T cells (CD27<sup>neg</sup>/CD45RA<sup>pos</sup>/CCD7<sup>neg</sup>) were reduced according to age-matched reference values in the literature. Also numbers of natural killer T cells (NKT cells) and CD4/CD8 double negative α/β T cells were normal, only 10.1% γ/δ T cells were slightly above the normal range.

In contrast to the T-cell compartment, the distribution of patient’s B cell subpopulations was strongly distorted (Table 1). Total CD19<sup>pos</sup> B cells were normal in absolute and relative numbers compatible with the previous KREC analysis (4411 copies per 0.5 μg DNA, normal for age: >100 copies). The analysis of subpopulations revealed a striking expansion of transitional CD10<sup>pos</sup>/CD38<sup>hi</sup>/IgD<sup>pos</sup> B cells while all subsequent populations were reduced compared to age-matched reference values (Fig2B). Interestingly, BAFF-R expression was decreased on naïve (CD10<sup>neg</sup>/CD38<sup>pos</sup>/IgD<sup>pos</sup>) and transitional B cells of the patient compared to the corresponding subpopulations in an age matched control (Fig2C).

CD3<sup>neg</sup>/CD16/CD56<sup>pos</sup> natural killer cells (NK cells) were normal (Table1). Both CD56<sup>bright</sup>/CD16<sup>neg</sup> and CD56<sup>dim</sup>/CD16<sup>pos</sup> subpopulations were present.

**Molecular characterization of CARD11 deficiency**

Based on parental consanguinity, we assumed a founder mutation transmitted in an autosomal-recessive manner. DNA SNP array resulted in the identification of multiple homozygous regions comprising thousands of genes. We therefore opted for whole exome sequencing in the patient DNA sample. While analyzing our data we heard of a child with similar phenotype and a mutation in CARD11 (Greil et al submitted). Comparing uncovered exons in several same-batch exome analysis we noted that exon 21 of CARD11 was entirely uncovered in the patient sample, but was well covered (~x65) in the control samples.
With CARD11 as a good candidate, we first sequenced cDNA from the patient with CARD11-specific primers. Fragments including exon 21 were about 140 bp shorter than the expected size (Fig3A). Sanger sequencing of these PCR products revealed a complete lack of exon 21 (136 bp) in the patient’s cDNA sequence. All cDNA PCR products without exon 21 were normal in size and sequence. Sequencing DNA between exons 20 and 22 in the patient detected a homozygous 1,377 bp genomic deletion including the entire sequence for exon 21 (Fig3B). Interestingly, the deletion is flanked by a stretch of 36 completely identical bases. Both parents and two healthy siblings were tested heterozygous for this large deletion.

While CARD11 protein expression was readily detectable in EBV lines of controls, no protein could be detected in the patient’s EBV line (Fig3C). Since the antibody was targeted against the N-terminal amino acid 1-221, the deletion of exon 21, encoding amino acid 902-946, must result in a complete loss of CARD11 protein expression.

**Signaling in CARD11-deficient T and B cells**

The integrity of the canonical NF-κB signaling pathway was addressed by measuring the degradation of IκBα and the phosphorylation of p65 following stimulation of PBMCs with anti-IgM, CD40L and PMA, respectively.

Strikingly, degradation of IκBα and phosphorylation of p65 following anti-IgM and PMA stimulation, as it was seen in B cells of healthy donors (HD), was completely absent in the patient’s B cells. In contrast, stimulation with CD40L induced degradation of IκBα and phosphorylation of p65 comparable to the healthy control (Fig4A).

In line with this, we observed an abolished phosphorylation of p65 and degradation of IκBα in the patient’s T cells following PMA stimulation, confirming the non-redundant critical role of CARD11 in PKC-dependent signaling in B and T cells (Fig4B). Given the complex interactions of the diverse signaling pathways downstream of antigen receptor, we also examined the integrity of other pathways. Anti-IgM and PMA stimulation resulted in a normal phosphorylation of ERK1/2 in the patient’s B cells (Fig4C). The same was true for T cells.
after PMA treatment (Fig4D), confirming data from mice that this signaling pathway is not affected by the deletion of CARD11. Also calcium mobilization in B cells following anti-IgM stimulation was undistinguishable from healthy control cells (Fig4E).

**B-cell function in CARD11 deficiency**

Upon stimulation for 48 hours via the BCR, expression of ICAM-1 and CD25 wasn’t induced in the patient’s B cells, while stimulation of PBMCs with CD40L resulted in a comparable up-regulation of both activation markers in the patient’s and control B cells, confirming their dependence on NF-κB upon BCR stimulation. The regular up-regulation of CD69 and CD86 upon stimulation with anti-IgM and CD40L excluded an overall activation defect (Fig5A).

In addition, patient’s B cells developed into plasmablasts after stimulation with CD40L and IL-21 comparable to cells from human cord blood (Fig5B), demonstrating the capacity of terminal B-cell differentiation despite CARD11 deficiency. Moreover, CARD11-deficient plasmablasts produced similar amounts of IgG and IgA in-vitro comparable to cord blood controls, while IgM levels were even increased (Fig5C).

**T- and NK-cell function in CARD11 deficiency**

In order to investigate the impact of CARD11 deficiency on cytokine production and activation, we determined IL-2, IL-4, IFN-γ and IL-17 production following stimulation with PMA/ionomycin. While the percentage of IL-2, IL-17 and IFN-γ producing CD4 T cells was reduced compared to controls, IL-4 producing CARD11-deficient CD4 T cells were within the normal range (Fig6A). Up-regulation of CD69, CD25, CD40L, OX40 and ICOS was determined on CD4 T cells (Fig6B). Following TCR/CD28 stimulation, CD69 and CD40L up-regulation were within normal ranges while the increase of OX40, CD25 and ICOS expression was strongly impaired in CARD11-deficient CD4 T cells compared to healthy controls. Finally, proliferation of CARD11-deficient CD4 T cells after CD3/CD28 stimulation was completely abrogated in-vitro, while CD4 T cells proliferated after stimulation with PHA, although slightly less than control PBMCs (Fig6C). Equivalent results were observed for CD8 T cells (data not shown).
After PMA/ionomycin stimulation of PBMCs less NK cells produced IFN-γ in CARD11 deficiency (Fig6D).

**Defective BAFF-R expression and up-regulation of activation markers in murine Card11 deficiency**

Proposing a crucial role of CARD11 in early peripheral B cell differentiation and the germinal center induction through ICOS, we went back to our Card11-mutant mouse model\(^{10}\). In these mice, both mature and immature B cell subsets expressed lower levels of BAFF-R, most strongly affecting the T2 transitional B cell stage (Fig7A).

Since the up-regulation of most co-stimulatory molecules on murine Card11-deficient T cells after anti-CD3/CD28 activation had not been described previously we investigated the up-regulation of CD69, CD25, CD40L, OX40 and ICOS in Card11-mutant mice compared to control littermates. T cells from naive Card11-mutant mice showed impaired up-regulation of all determined activation markers, especially in response to anti-CD3/CD28 stimulation, rendering the severe deficiency in up-regulation of co-stimulatory molecules a key contributing factor to the disturbed T-dependent responses in CARD11 deficiency (Fig7B).
DISCUSSION

Here we report a child with a new form of profound combined immunodeficiency due to a homozygous deletion in CARD11. The manifestation of the first opportunistic infection in the context of hypogammaglobulinemia, consanguinity and a positive family history prompted a genomic screening for a causal defect. This search was significantly accelerated with the finding of CARD11 deficiency in a second child by Greil et al (Greil et al manuscript submitted). These two children demonstrate that complete CARD11 deficiency presents rather early in life with pCID and not with common variable immunodeficiency (CVID) as had been suspected and previously tested unsuccessfully\(^\text{16}\).

From a clinical standpoint it is important to notice, that pCID in CARD11 deficiency will be missed by testing for TREC/KREC copy numbers in newborn screening and by checking lymphocyte counts later in life since numbers of T, B and NK cells may be normal as in our child. The essential diagnostic clues are: Missing T-cell proliferation to anti-CD3/CD28 stimulation and the abnormal expansion of late transitional B cells, the lack of mature B cells and hypogammaglobulinemia. Card11-targeted mouse strains demonstrated that the defective activation of NF-kB and JNK after antigen receptor and PMA stimulation is the responsible pathomechanism in CARD11 deficiency\(^\text{10,17-19}\). Similarly, we found defective canonical NF-kB activation, along with intact ERK and calcium activation in B and T cells of our patient, endorsing the selective impact of human CARD11 deficiency on the antigen receptor pathway.

Despite severely disturbed proliferation after TCR stimulation, the differentiation of peripheral T-cell subpopulations is normal, except of Tregs\(^\text{20,21}\). The differentiation and maintenance of Tregs depend on proper IL-2 signals\(^\text{22,23}\). We could demonstrate that CARD11-deficient T cells failed to produce normal amounts of IL-2 and the up-regulation of the IL-2 receptor α-chain (CD25) after TCR stimulation was diminished. Thus, reduced IL-2 production and IL-2 receptor expression may contribute to Treg deficiency in humans, in addition to the previously demonstrated direct role of CARD11 in IL-2 receptor signaling\(^\text{23}\). IFN-γ production was more strongly affected than IL-4 production in T cells of our patient.
This is in contrast to recent reports in mice demonstrating a crucial role of CARD11 in Th2-cell differentiation\textsuperscript{24}. Interestingly, also IL-17 was severely reduced demonstrating a complex dysregulation of the effector function of the T-cell compartment. It remains speculative whether this functional defect contributed to the susceptibility for \textit{Pneumocystis jirovecii} infection in our patient, since IFN-\(\gamma\) and IL-17 are supposedly involved in the defense against \textit{Pneumocystis} infection\textsuperscript{25}. Regarding NK-cell differentiation, their number seems to depend on the genetic background of the mouse model\textsuperscript{17,21,26}. In accordance with the report by Malarkannan et al, that the CARD11/MALT1/bcl10 complex is involved in NF-\(\kappa\)B-dependent cytokine production in NK cells, but does not affect their differentiation\textsuperscript{27}, we observed normal NK-cell numbers, a regular distribution of subsets, but markedly decreased production of IFN-\(\gamma\) by patient’s NK cells.

Most striking are the changes in the B-cell compartment. We observed a severe developmental block at the IgD\textsuperscript{low}/IgM\textsuperscript{high} late transitional B-cell stage affecting all mature B-cell subpopulations in CARD11 deficiency. In accordance, all mouse models demonstrated a reduction of mature B cells\textsuperscript{10,17}. This finding was corroborated by the defective B-cell differentiation in mice with targeted deletion of the other members of the CARD11/MALT1/bcl10 complex\textsuperscript{28, 29}. The exact reason of this developmental block remains unknown. Beside the BCR signal, B cells depend on BAFF-R expression and signaling at this stage. Thus, also BAFF-R-deficient mice and humans have a block at the transitional stage and mature B-cell numbers are severely reduced\textsuperscript{13,30,31}. Since the alternative NF-\(\kappa\)B pathway downstream of BAFF-R is independent of CARD11 and BAFF-R itself is a potential NF-\(\kappa\)B target gene\textsuperscript{32}, we speculated about a reduced BAFF-R expression on CARD11-deficient B cells. Indeed, CARD11-deficient B cells failed to express normal amounts of BAFF-R on the surface. Whole exome sequencing excluded any mutations in BAFF-R itself and the analysis of CARD11-deficient murine B cells confirmed this finding. Therefore, beside the disturbed BCR signal, low BAFF-R expression may contribute to the disturbed B-cell differentiation beyond the late transitional stage in human CARD11 deficiency. This seems to differ from mice where the developmental block at the transitional stage is less...
prominent\textsuperscript{10} despite reduced BAFF-R expression. This aspect needs to be revisited more carefully in the different murine models of CARD11 deficiency.

The crucial defect in late B-cell differentiation leading to reduced serum immunoglobulin levels, defective T-dependent and T-independent immune responses was in common to all mouse models\textsuperscript{10,17-19}. Jun et al additionally described poor germinal center (GC) formation\textsuperscript{10}.

Similarly, MALT1- and bcl10-deficient mice show insufficient formation of GC B cells after immunization with T-dependent antigens\textsuperscript{33}. Again it remains unknown, which mechanisms contribute to the failure of GC formation. Interestingly, in addition to the reduced induction of OX40 and CD25, we found a severe defect in the up-regulation of ICOS after TCR activation and confirmed it in our mouse model. In contrast to the global affected upregulation of activation markers in CARD11 deficient murine T cells CD40L induction on patient’s T cells was still within the normal range. Given the essential role of ICOS in GC formation in mice and men\textsuperscript{34-38} it is very tempting to speculate that impaired ICOS up-regulation plays an important role in poor B cell memory formation in human CARD11 deficiency.

While hypogammaglobulinemia is a common finding in all reports of CARD11 deficiency, our data reveal that differentiation of plasmablasts is not completely dependent on CARD11 expression, since CD40L/IL-21 stimulation allowed efficient generation of plasmablast differentiation and immunoglobulin production in vitro. In this context it is noteworthy, that CD40 activation of the canonical NF-κB pathway was normal and therefore CD40-induced B-cell activation is independent of CARD11 in contrast to early reports\textsuperscript{18}.

In the case of our patient, because of the severe clinical presentation it was decided to proceed to allogeneic bone marrow transplantation from her fully matched, heterozygous brother. She was successfully transplanted and 5 months later, has full donor chimerism, normal immunoglobulin levels, without evidence of graft versus host disease.

In summary, we have discovered a new form of profound combined immunodeficiency due to a genetic deletion in \textit{CARD11}. The described genetic recombination between homologous regions up- and downstream of exon 21 suggests a potential hot-spot mutation in this gene worthwhile screening for in a larger cohort of pCID patients. In the absence of lymphopenia,
the diagnostic clues in CARD11 deficiency derive from abnormal B-cell differentiation and
absent T-cell proliferation after TCR/CD28 stimulation. Signaling studies reveal defective
canonical NF-κB activation after antigen receptor but not CD40 stimulation. CARD11 is a
crucial scaffold of the adaptive immune system controlling peripheral B-cell differentiation, a
variety of critical T-cell effector functions and the GC response. The restricted expression of
CARD11 in the hematopoietic system makes patients with CARD11 deficiency excellent
candidates for stem cell transplantation.
Acknowledgements

We would like to thank the patient and her parents for their support, Mehmet Yabas for FACS measurement and J. Hodges and B. Roller for critical reading of the manuscript.
Stepensky 21

References


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<td>γδ T cells*</td>
<td></td>
<td></td>
<td></td>
<td>10.1</td>
</tr>
<tr>
<td>DN T cells**</td>
<td></td>
<td></td>
<td></td>
<td>1.45</td>
</tr>
<tr>
<td>NK T cells*</td>
<td></td>
<td></td>
<td></td>
<td>0.044</td>
</tr>
<tr>
<td><strong>B cells</strong></td>
<td>990</td>
<td>871-1553</td>
<td>20.2</td>
<td>16.3-26.8</td>
</tr>
<tr>
<td>Transitional</td>
<td>576</td>
<td>109-278</td>
<td>58.2</td>
<td>9.7-17.9</td>
</tr>
<tr>
<td>Naïve</td>
<td>363</td>
<td>586-955</td>
<td>36.7</td>
<td>61.5-68.7</td>
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<tr>
<td>IgG switched</td>
<td>8</td>
<td>13-55</td>
<td>0.83</td>
<td>1.5-4.2</td>
</tr>
<tr>
<td>IgA switched</td>
<td>0</td>
<td>8-20</td>
<td>0.01</td>
<td>0.8-1.7</td>
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<td>IgM Memory</td>
<td>34</td>
<td>40-177</td>
<td>3.41</td>
<td>4.6-15.0</td>
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<tr>
<td><strong>NK cells</strong></td>
<td>217</td>
<td>55-4000</td>
<td>4.42</td>
<td>1-96</td>
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</table>

**Table 1 Peripheral lymphocyte subpopulations**

Patient’s absolute and relative counts of lymphocyte subpopulations are indicated. Percentage refers to the respective parental population. Age matched reference values are taken from a van Gent et al14, b Shatorje et al15 or are c internal reference values.

* % of CD3pos T cells, ** of CD3pos αβ T cells
Figure legends

Fig1 Pedigree of the CARD11-deficient family
Circles represent females, squares males; filled symbols, homozygous individuals; half-filled symbols, heterozygous individuals; crossed-out symbols, deceased individuals. The pedigree shows five generations of the family. The arrow indicates the index patient.

Fig2 Abnormal lymphocyte homeostasis in CARD11 deficiency
CD127neg/CD25hi regulatory T cells gated on CD4pos/CD3pos lymphocytes (A), CD38high/CD10pos transitional B cells (top), CD27/IgD expression gated on CD19pos lymphocytes (bottom) (B) of the patient (Pat) compared to an age-matched control (HD). (C) BAFF-R expression on the indicated subpopulations.

Fig3 Molecular analysis of CARD11 deficiency
(A) RT-PCR covering cDNA of exons 16-22 of CARD11 (left) and schematic representation of the deletion in CARD11, depicted by the grey shaded box (right). (B) PCR products amplified from gDNA of a HD, the patient’s father, mother, the patient and her brother flanking exon 21 of CARD11 (left). Sanger sequencing of the gDNA including exon 21 of CARD11 (right). (C) Immunoblot for CARD11 protein expression in EBV lines of the patient and three controls. β-actin was used as loading control.

Fig4 Signaling in CARD11-deficient lymphocytes
(A) Degradation of IκBα (upper panel) and phosphorylation of p65 (pp65) (lower panel) gated on CD19pos B cells upon stimulation as indicated. (B) Degradation of IκBα (upper figures) and phosphorylation of p65 (lower figures) upon stimulation of PBMCs with PMA (dashed line) and un-stimulated gated on CD3pos T cells. (C) Phosphorylation of ERK1/2 (pERK) in CD19pos B cells after stimulation. (D) Panel shows phosphorylation of ERK in CD3pos T cells after stimulation with PMA and un-stimulated. (E) Calcium mobilization in
CD19^{pos}/CD27^{pos}/IgG^{neg}/IgA^{neg} B cells upon anti-IgM stimulation. Depicted is the ratio of Indo-1 bound/unbound. Arrows indicate time points of the addition of anti-IgM and ionomycin.

Fig5 B-cell function in CARD11 deficiency
(A) ICAM-1, CD25, CD69 and CD86 expression after stimulation of PBMCs as indicated gated on CD19^{pos} cells. (B) Differentiation of plasmablasts, defined as CD38^{hi}/CD27^{hi} B cells, after in-vitro stimulation of PBMCs with IL-21 and CD40L (upper panel). IgG^{pos} and IgA^{pos} plasmablasts are depicted after gating on CD38^{hi}/CD27^{hi} B cells (lower panel). (D) IgM, IgG and IgA levels from cell culture supernatants of the patient’s or cord blood PBMCs after in-vitro stimulation with IL-21 and CD40L.

Fig6 T-cell and NK-cell function in CARD11 deficiency
(A) PBMCs were stimulated with PMA/ionomycin in order to determine IL-2 (upper left), IL-4 and IFN-γ production (upper right) and IL-17 and IFN-γ (lower right) on CD4^{pos} T cells. (B) Expression of CD69, CD40L, OX40, ICOS and CD25 after stimulation of CD4^{pos} T cells as indicated (top). Percent positive cells for indicated marker of CD4^{pos} T cells of the patient (open circle) compared to healthy controls (closed circle) (bottom). (C) Proliferation of CD4^{pos} T cells upon stimulation with anti-CD3/anti-CD28 (left) and PHA (right). (D) IFN-γ production in CD3^{neg}/CD56^{pos} NK cells after stimulation with PMA/ionomycin compared to un-stimulated samples.

Fig7 Expression of BAFF-R and T cell activation markers in CARD11-mutant mice
(A) Expression of BAFF-R on CD93^{pos} immature and CD93^{neg} mature splenic B cells from Card11um/unm mice and age-matched controls (top panel). By staining for IgM and CD23 the CD93^{pos} immature B cells were further divided into T1 (IgM^{pos}/CD23^{neg}), T2 (IgM^{pos}/CD23^{pos}) and T3 (IgM^{low}/CD23^{pos}) stages (bottom panel). (B) Expression of CD69, CD25, CD40L, OX40 and ICOS on 7AAD^{neg}/CD4^{pos} T cells after stimulation as indicated. Triangles represent mice from a C57BL/6 background, circles mice from a B10.BR
background. Statistical analysis was done using ANOVA followed by Bonferroni post-Test. *

p<0.05; ** p<0.005; *** p<0.0005.
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Fig 2 Abnormal lymphocyte homeostasis in CARD11 deficiency
Fig3 Molecular analysis of CARD11 deficiency
Fig 4 Signaling in CARD11-deficient lymphocytes
Anti-IgM
CD40L
unstimulated

Fig 5 B-cell function in CARD11 deficiency
Fig 6 T-cell and NK-cell function in CARD11 deficiency
Fig 7 Expression of BAFF-R and T cell activation markers in CARD11-mutant mice