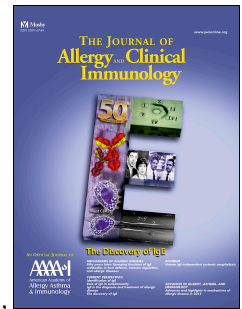


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**Combined immunodeficiency and atopy caused by
a dominant negative mutation in CARD11**

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ABSTRACT

Background: Combined immunodeficiency is a T cell defect frequently presenting with recurrent infections as well as associated immune dysregulation manifesting as autoimmunity or allergic inflammation.

Objective: We sought to identify the genetic aberration in four related patients with combined immunodeficiency, early onset asthma, eczema and food allergies, as well as autoimmunity.

Methods: Whole exome sequencing (WES) followed by Sanger confirmation, assessment of the genetic variant impact on cell signaling and evaluation of the resultant immune function.

Results: A heterozygous novel c.C88T one base pair substitution resulting in the amino acid change R30W in CARD11 was identified by WES and segregated perfectly to family members with severe atopy only, but was not found in healthy individuals. We demonstrate that the R30W mutation results in a loss of function while also exerting a dominant negative effect on wild-type CARD11. The CARD11 defect altered the classical Nuclear Factor- κ B (NF- κ B) pathway, resulting in poor in vitro T cell responses to mitogens and antigens caused by reduced secretion of IFN γ and IL-2.

Conclusion: Unlike patients with biallelic mutations in CARD11 causing severe combined immunodeficiency, the R30W defect results in a less profound yet prominent susceptibility to infections as well as multi-organ atopy and autoimmunity.

CLINICAL IMPLICATIONS

A single dominant negative gene defect in CARD11 can cause combined immunodeficiency, autoimmunity and multi-organ atopy.

KEY WORDS

CARD11, combined immunodeficiency, hypogammaglobulinemia, NF- κ B, cytokine secretion, T-cell repertoire, T cell mitogen and antigen responses, autoimmunity, asthma, eczema and food allergies, atopy.

CAPSULE SUMMARY

We show here for the first time that a novel R30W dominant negative mutation in CARD11 can cause a familial autosomal dominant disorder encompassing combined immunodeficiency, severe multi-system atopy and autoimmunity.

ABBREVIATIONS USED

BENTA:	B cell Expansion with Nuclear Factor κ B and T cell Anergy
BCL10:	B cell lymphoma/leukemia 10
BCR:	B cell receptor
CARD:	Caspase activation and recruitment domain
CARD11:	Caspase activation and recruitment domain family, member 11
CARMA1:	CARD-containing MAGUK protein 1
CBM:	CARD11-BCL10-MALT1

82	CMV:	Cytomegalovirus
83	GWAS:	Genome wide association studies
84	ID:	Inhibitory domain
85	Ig:	Immunoglobulin
86	IL:	Interleukin
87	LPS:	lipopolysaccharide
88	MALT1:	mucosa-associated lymphoid tissue lymphoma-translocation gene 1
89	NF- κ B:	nuclear factor κ B
90	PBL:	Peripheral blood lymphocyte
91	PHA:	Phytohemagglutinin
92	TCR:	T cell receptor
93	TH:	T helper
94	TREC:	T cell receptor excision circle
95	Treg:	Regulatory T
96	TSLP:	Thymic stromal lymphopoietin
97	WES:	Whole exome sequencing
98	WGS:	Whole genome sequencing

99

INTRODUCTION

Combined immunodeficiency (CID) is a term used to describe a diverse group of inherited T cell immunodeficiencies^{1, 2}. CID patients, unlike patients with severe combined immunodeficiency (SCID), typically have a substantial body of circulating lymphocytes, sometimes comparable to control, but are characteristically dysfunctional³. The severity of T cell dysfunction may vary according to the genotype or even specifically, be mutation-dependent.

Frequently, CID is caused by hypomorphic mutations in SCID-associated genes like IL-2R γ (R222C variant)⁴, JAK3⁵, RAG 1, 2⁶ and others⁷⁻¹¹.

Recently, profound T cell deficiency was linked to mutations in the CARD11 gene^{12, 13}. CARD11 (CARMA1 or BIMP3) is a scaffold protein that belongs to a family of membrane-associated guanylate kinases, and is required for B cell receptor (BCR) and T cell receptor (TCR) signaling to the NF- κ B transcription factor. This pathway controls an array of genes that participate in lymphocyte differentiation, proliferation and survival¹⁴. In resting cells, NF- κ B is kept inactive in the cytoplasm by I κ B inhibitory proteins. Upon antigen-receptor engagement, the I κ B kinase (IKK) complex is activated, resulting in phosphorylation and degradation of I κ Bs. As a result, NF- κ B active transcription factors are liberated, enabling translocation to the cell nucleus and regulation of target genes.

Following antigen receptor stimulation, CARD11 undergoes a conformational change from an inactive state to an active scaffold. This change is controlled by the CARD11 inhibitory domain (ID), which is neutralized upon serine phosphorylation in part by protein kinase C θ (T cells) or PKCB (B cells). ID neutralization allows CARD11 to recruit several cofactors, including

Bcl10, MALT1, TRAF6, TAK1, caspase-8, IKK γ , CK1 α , and HOIP¹⁵⁻¹⁷. The critical role of many components of the signaling cascade in human immunity is highlighted by inherited deleterious mutations that result in lymphocyte dysfunction, causing immunodeficiency^{18, 19}. Typically, these patients present in early life with repeated or persistent infections, as well as increased susceptibility to cancer and autoimmunity.

Homozygous null mutations in BCL10 and MALT1 were identified in infants who suffer repeated and severe infections characteristic of combined immunodeficiency^{20, 21}. In these patients, T cell function and B cell maturation were consistently aberrant and NF- κ B signaling abrogated. In full agreement with the concept that the CARD11/Bcl10/MALT1 (CBM) signalosome is essential for downstream signaling pathways required for maturation and function of lymphocytes, null mutations in CARD11 lead to a phenotype similar to MALT1 and BCL10 deficiencies.

Patients with CARD11 deficiency presented with severe chest infections caused by *P. jirovecii* pneumonia infection. Evaluation of the immune system recorded mostly normal numbers of circulating T and B cells, which were predominately of naïve phenotype and unresponsive to mitogens. Immunoglobulin levels were also reduced. CARD11 null mice display similar features²²⁻²⁵.

Recently, monoallelic mutations causing gain-of-function of CARD11 have also been identified in patients with the lymphoproliferative disorder known as B cell Expansion with Nuclear Factor κ B and T cell Anergy (BENTA)²⁶. These patients present shortly after birth with splenomegaly and marked B-cell lymphocytosis of naïve phenotype. They also suffer repeated microbial infections. Similar to somatic mutations commonly found in large B-cell lymphomas,

CARD11 gain-of-function mutations are localized to the CARD, LATCH, and coiled-coil domains²⁶⁻²⁹. These mutations interfere with auto-inhibition by four repressive elements in the CARD11 ID and bypass the need for their antigen receptor-induced neutralization²⁹⁻³².

In the *unmodulated* mouse, a homozygous T to A mutation leading to a Leu to Gln (L298Q) amino acid substitution in the coiled-coil domain results in loss-of-function of the murine CARD11²³. *Unmodulated* mice have combined selective defects in both B and T cell antigen receptor signaling²³. Most interestingly, reminiscent of the patients described here, most *unmodulated* mice gradually develop spontaneous progressive atopy consisting of teary eyes and intensely itchy dermatitis in the ear and neck^{23, 33}. Histological inspections of these lesions revealed skin hyperkeratosis and mixed allergic inflammatory infiltrates consisting of mast cell, lymphocytes and eosinophils³³.

The clinical manifestations observed in CID typically consist of susceptibility to viral, fungal and microbial infections, but frequently include features attributed to immune dysregulation such as autoimmunity. This is believed to develop because of poorly controlled residual immunity, resulting in a spectrum of disorders including endocrinopathies, rheumatologic diseases and hematologic cytopenias.

Severe allergic inflammation was also previously reported in patients with T cell dysfunction. Severe erythroderma and marked eosinophilia are the hallmarks of Omenn Syndrome (OS), which was described in a variety of 'leaky' defects in SCID-associated genes^{6-9, 34}. Food allergies were also described in patients with IPEX syndrome.

Here we describe an autosomal dominant transmission of CID which uniquely consists of multi-organ severe atopy in addition to susceptibility to infections.

MATERIALS AND METHODS

Patients

Patient data was compiled prospectively and retrospectively from medical records and entered into the Canadian Centre for Primary Immunodeficiency Registry and Tissue Bank, which has been approved by the SickKids Research Ethics board (protocol no. 1000005598). Consent and assent from each patient and parents were obtained for genetic testing, including WES and WGS analysis as well as extensive investigations to unravel the functional effect of immune abnormalities. Patients and families have also consented to permit publication of findings.

Exome Sequencing and Variant Calling

DNA from blood of patient 1 was submitted to The Centre for Applied Genomics (TCAG), Toronto, Canada for exome library preparation and sequencing. DNA was quantified by Qubit DNA HS assay (Life Technologies, Carlsbad, CA) and 100 ng of input DNA was used for library preparation using the Ion AmpliSeq Exome Kit (Life Technologies) according to the manufacturer's recommendations. The Ampliseq Exome library was immobilized on Ion PI™ Ion Sphere™ particles using the Ion PI Template OT2 200 Kit v3. Sequencing was performed with the Ion PI Sequencing 200 Kit v3 and Ion PI Chip v2 in the Ion Proton™ semiconductor sequencing system following the manufacturer's recommendation.

Alignment and variant calling were performed using Torrent Suite (v4.0) on the Ion Proton Server, using the Ion Proton ampliseq germline low stringency setting and the hg19 reference genome. The variants were annotated using an in-house annotation pipeline³⁵ based

on Annovar (November 2014 version)³⁶ and RefSeq gene models (downloaded from UCSC 01 August 2015).

Variant Prioritization

High quality variants were prioritized^{37, 38} based on (i) allele frequency based on the public databases 1000 Genomes³⁹, ExAC⁴⁰, Welllderly⁴¹ and in-house Ion Proton™ AmpliSeq platform-matched unaffected controls⁴²; (ii) clinical classification as pathogenic or likely pathogenic, if available in the public databases ClinVar⁴³ and HGMD⁴⁴; (iii) genomic conservation based on the phyloP⁴⁵ and PhastCons⁴⁶ scores, effect and predicted damaging impact on the gene product based on the predictors SIFT⁴⁷, PolyPhen2⁴⁸, MutationAssessor⁴⁹, CADD⁵⁰, Spidex⁵¹; (iv) gene implication in human immune disorder and Mendelian mode of inheritance based on the Clinical Genomics Database (CGD)⁵², the Human Phenotype Ontology (HPO)⁵³, Online Mendelian Inheritance in Man (OMIM; <https://omim.org/>), abnormal immune or hematopoietic phenotype in mouse based on the Mammalian Phenotype Ontology (MPO) annotations⁵⁴ provided by the Mouse Genome Informatics (MGI) database⁵⁵, immune gene function annotations based on Gene Ontology⁵⁶ and pathway databases^{57, 58}, and constraint to truncating or missense mutation for heterozygous variants provided by ExAC⁴⁰. Minimum damaging impact for missense variants was defined as at least 2 / 6 impact predictors and phyloP conservation passing threshold for damaging impact³⁵. OMIM morbidmap annotations were downloaded in Jan 2015; Gene Ontology and pathway annotations were downloaded in March 2013; MPO/MGI and HPO annotations were downloaded in June 2015.

Flow Cytometry

B and T cell phenotypes were determined by flow cytometry analysis using the four laser BD analyzer LSR II CFI BGRV. Antibodies used include: CD4-BV510, CD3-APC, CD127-PerCP-Cy5.5, CD25-BB515, CD194-BV42, CD45RO-PE, CD3-PerCP-Cy5.5, CD4-BV510, CD8-BB515, CD183-AF700, CD19-BV711, CD27-BV421, IgD-PE-Cy7, CD24-BB515, CD38-APC, CD21-PE-CF594, CD45RO-PE, CD197-BV421, CD196-PE-Cy7; all from BD Bioscience. Anti-IL-4-PE for intra-cellular staining was from eBioscience, anti-CD69-PE (eBioscience) and anti-CD25-AF488 (R&D System) were used to assess T cell activation. For activation analyses, PBL were stimulated with soluble anti-CD3 (UCHT1) 2µg/ml plus anti-CD28 (CD28.2) 10µg/ml, or PMA (Sigma Aldrich) (50ng/ml) as indicated for 22-24 hours and harvested for staining.

Cytokine Determinations

Post-Ficoll peripheral blood mononuclear cells were cultured with PHA or anti-CD3 and anti-CD28 for 48 hours or as indicated, and culture supernatants collected for analysis by ELISA. IL-2, IFN-γ, IL-4, IL-13, IL-10, IL-5 and IL-17 analysis kits were from R&D Systems (MN). Control data are represented as mean ± standard deviation. Comparisons among groups were made using the unpaired t test. An associated probability (p value <0.05) was considered significant.

Serum concentration of immunoglobulins and specific antibodies

Serum concentrations of immunoglobulins (IgG, IgA and IgM) were measured by nephelometry and IgE concentrations measured by radioimmunoassay using the IgE PRIST kit (Pharmacia Diagnostics, Dorval, Canada). Levels of serum antibodies to tetanus toxoid and pneumococcus

(pneumococcal capsular polysaccharide) were measured by ELISA according the manufacturer's instructions (Binding Site, Birmingham, UK). Serum antibodies to measles, mumps and rubella were measured by ELISA using kits available from Euroimmune (Gross-Groenau, Germany). Serum isohemagglutinin titres were determined by two-fold serial dilution with erythrocytes, and are reported as antiglobulin phase, the dilution at which macroscopic agglutination is observed.

T cell and B cell proliferative responses

Lymphocyte proliferative responses to mitogens, including phytohemagglutinin (PHA) and the combination of anti-CD3 antibodies and anti-CD28 antibodies, and to a panel of recall antigens (including Candida, Tetanus Toxoid, Herpes Zoster, and Cytomegalovirus) were determined by thymidine incorporation⁵⁹. All assays were performed in triplicate and were compared with simultaneously stimulated random normal controls.

Assessment of CARD11 R30W signaling activity

HEK293T cells and CARD11-deficient Jurkat T cells (CARD11-KO) were cultured as described previously¹⁵. pcDNA3-based expression vectors for myc-tagged wild-type and R30W CARD11 variants were titrated in HEK293T cells in the presence of 1500ng Ig κ ₂-IFN-LUC and 200 ng CSK-LacZ reporter and control vectors in transient transfections using LT-1 (Mirus) and 3 μ g total DNA. β -gal activity in lysates was measured as described³¹ and used to normalize the amounts of lysate for each sample that were resolved on SDS-PAGE gels prior to analysis by western blotting with anti-myc antibody (Santa Cruz Biotechnology sc-40). The western blots bands

were quantitated by ImageJ and linear regression was used to determine the amount of expression vector required to achieve equivalent amounts of wild-type and mutant CARD11 proteins over a 5-fold range of expression (Supplementary Figure 1).

To assess signaling activity, CARD11-KO Jurkat T cells were transiently transfected with 1500ng Ig κ ₂-IFN-LUC, 200 ng CSK-LacZ, and expression vectors for wild-type and R30W myc-tagged CARD11 as previously described⁶⁰. Each sample was supplemented with empty pcDNA3 so as to keep the total amount of pcDNA3-based expression vector constant and the total amount of DNA at 3 μ g. Forty hours later cells were treated with 1 μ g/ml each of mouse anti-human CD3 (BD Pharmingen 555329), mouse anti-human CD28 (BD Pharmingen 555725), and rat anti-mouse IgG1 (BD Pharmingen 553440) for 5 hours. Cells were harvested, luciferase and β -gal activities measured, and fold activation determined as previously described¹⁵.

Cofactor interaction studies

pcDNA3-based expression vectors for myc-tagged wild-type and R30W CARD11 Δ ID variants were cotransfected into HEK293T cells with expression vectors for FLAG-Bcl10, FLAG-MALT1, and untagged Bcl10 as indicated and anti-FLAG immunoprecipitations were performed as previously described¹⁶.

T cell stimulation

Ficoll gradient purified peripheral blood lymphocytes were stimulated for the indicated times at 37°C with either anti-CD3 (UCHT1) 2 μ g/ml plus anti-CD28 (CD28.2), 10 μ g/ml, or PMA (50ng/ml) as indicated.

RESULTS

Patients

A pedigree of the patients described in this study is shown in Figure 1A.

Patient 1: A 31 year old female who has been followed up for combined immunodeficiency and severe atopy since the age of 14 years. She was born at term to non-consanguineous parents of English descent. She began suffering from atopic dermatitis from the first few weeks of her life. Between two and eight years of age, she experienced multiple episodes of bronchitis, recurrent upper respiratory tract infections and repeated episodes of sinusitis which required admission to hospital. During one of these admissions, low serum IgG was detected and she was given replacement IVIG. She was also diagnosed with asthma and environmental allergies. At the age of nine years she began experiencing recurrent episodes of diffuse erythematous papular skin lesions on her extremities. A skin biopsy revealed necrotizing granulomatous inflammation. At the age of 15 years she suffered repeated exacerbations of oral candidiasis with no preceding treatment of antibiotics or steroids.

At the age of 17 years she was diagnosed with lichen sclerosis of the vulva which resolved with topical steroid treatment. Subsequently, at 20 years she had prolonged and persistent symptoms of abdominal pain and diarrhea and was diagnosed with colitis. At 28 years she started developing scaly skin lesions on her scalp and lower extremities and was diagnosed with psoriasis.

A chest CT scan performed at age 18 and 24 confirmed bilateral changes consistent with bronchiectasis.

Patient 2: The daughter of patient 1 was born at term after a normal pregnancy. From one week of age, she was noticed to have eczema and at one month she developed asthma. During the first two years of life, she experienced multiple episodes of otitis media, six episodes of impetigo as well as three bouts of pneumonia. At two years she had cellulitis on her hands and her face. During the first two years of life she was also diagnosed with milk allergy and at the age of four with nut and peanut allergies.

She continues to suffer from severe extensive eczema as well as repeated episodes of upper and lower respiratory infections.

Patient 3: The four year old male was born at 41 weeks gestation after a normal pregnancy and spontaneous delivery. He is the son of patient 1 and half-brother of patient 2. His father has another six year old daughter from a previous relationship which is healthy. He was breast fed for the first year of life. His first episodes of asthma started at four months of age, exacerbated by repeated respiratory infections severe enough to merit hospital admission.

At six months, upon his first exposure to milk product, he developed swelling around his lips, eyes and face and developed hives. A similar episode occurred following exposure to egg. At seven months, he was introduced to kiwi for the first time and developed lip swelling, hives and irritability. He also developed hives upon exposure to shrimp. Since infancy, he has also had eczema most prominently affecting his hands and face.

Patient 4: This is the 37 year old sister of patient 1. She was assessed because of her striking family history and her life-long struggle with recurrent infections, multiple allergies and autoimmune manifestations.

During infancy and childhood she had multiple episodes of upper and lower chest infections which required antibiotic treatment and hospital infections. She was also diagnosed with primary infertility and like her sister, she suffered lichen sclerosis when she was a teenager.

She too began suffering from severe atopic dermatitis at infancy, which was treated throughout her life with topical steroids as well as tacrolimus, with only partial response requiring periodical intervention with systemic steroids. Over the past 5 years, the dermatitis was most severe in the periorbital and perianal areas.

Simultaneously, she developed asthma which was difficult to control, sometime requiring constant therapy with inhaled steroids and long-acting beta agonists.

The mother of patients 1 and 4, and grandmother of patients 2 and 3, also suffered severe atopy and asthma since early age but medical records were unavailable to us. Patients 1 and 4 have 2 male brothers aged 37 and 29 years, who were always healthy with no evidence of atopy. Patient 2 and 3 share a 9 year old sister who was also always healthy with no signs of atopy or susceptibility to infections.

Identification of CARD11 mutation

Analysis of whole exome sequencing showed that the subjects did not have any rare (allele frequency < 1%) homozygous or potential compound heterozygous variant with minimum damaging impact and occurring in genes with immune function or phenotype. The subjects had only one variant with 0 frequency in control databases, minimum damaging impact and occurring in a gene with established dominant Mendelian inheritance and implicated in human

immune disorder, the CARD11 (caspase recruitment domain family, member 11) missense variant hg19:chr7:2987341:G>A NM_032415:exon3:c.C88T:p.R30W (CARD11 R30W; Figure 1A, B). Sanger sequencing analysis demonstrated that the heterozygous R30W mutation segregated with disease within the family.

CARD11 R30W is a novel variant and has not been previously reported in dbSNP⁶¹, ClinVar or HGMD, although it overlaps with a very rare synonymous variant reported in dbSNP (rs145474800). As expected by the dramatic nature of the amino acid change from a positively charged to a bulky aromatic chain, the R30W amino acid change is deemed damaging by SIFT (score: 0.00), PolyPhen2-HDIV (score: 1.00), MutationAssessor (score: 2.76), and CADD (phred score: 27.30). In addition, R30 is perfectly conserved across 100 vertebrate genome sequences available in UCSC, while the genomic position is highly conserved in vertebrates (phyloP score: 7.47), partially conserved in mammals (phyloP score: 1.25) and overlaps a mammalian conserved genome interval (PhastCons score: 550). R30 maps to the CARD (caspase activation and recruitment) domain⁶² in an alpha-helical secondary structure element (PFAM and SwissProt UCSC annotation track) (Figure 1B).

In the monomeric CARD structure, R30 is adjacent to residues H31 and M32, which when mutated disrupt intramolecular interaction with the inhibitory domain (ID)²⁹. The R30-M32 region is distinct from the BCL10 interface (R35, K69, K41, R72; Figure 1C)⁶³.

Functional impact of the R30W mutation

To assess the effect of the R30W mutation on CARD11 signaling function, we expressed wild-type murine CARD11 and the R30W mutant in CARD11-deficient Jurkat T cells¹⁶ and assayed the

cells for T Cell Receptor-induced activation of NF- κ B³¹. First, we titrated expression vectors for wild-type and R30W variants to insure equivalent expression of each protein in this reconstitution assay (Figure 2A). As shown in Figure 2B, CARD11-deficient Jurkat T cells, which lack CARD11 expression as a result of CRISPR/Cas9 genomic editing¹⁶, failed to activate the NF- κ B-responsive luciferase reporter Ig κ ₂-IFN-LUC in response to TCR crosslinking with anti-CD3/anti-CD28 antibodies. While expression of wild-type CARD11 restored TCR-induced NF- κ B activation to a level of 72-fold, the expression of an equivalent level of R30W failed to rescue signaling at all, indicating that the R30W is a severe loss-of-function mutant (Figure 2B). Since the patients described are heterozygous for the R30W mutation we assessed the effect of R30W on wild-type CARD11 when coexpressed. First, we co-expressed equivalent levels of wild-type and R30W CARD11 proteins, each at half of the previously assayed level. Surprisingly, the R30W mutant eliminated any signaling activity of co-expressed wild-type CARD11, even though this level of CARD11 expression alone yielded 41-fold TCR-induced activation of NF- κ B (Figure 2B). The results clearly indicate that the R30W CARD11 mutant has potent dominant negative properties and can severely compromise signaling by a coexpressed wild-type CARD11 allele. Second, to more closely simulate the conditions in heterozygous cells, we co-expressed wild-type and R30W CARD11 proteins using equivalent amounts of DNA expression vector (Figure 2C). Under these conditions, the R30W mutant still displayed potent dominant negative activity, but the inhibition of signaling from the wild-type protein was not complete, leading to residual NF- κ B activation and reporter stimulation (Figure 2C). At these equivalent DNA expression vector levels, the R30W protein was expressed at ~4-fold lower levels than the wild-type protein (Figure 2D), which likely explains its incomplete inhibition of the wild-type allele.

To verify that the R30W mutant interferes with NF- κ B signaling, we have studied p65 NF- κ B phosphorylation in the patient's T cells stimulated with anti-CD3 and anti-CD28. Consistent with the results in Jurkat T cells, there was a marked reduction when compared with control (Figure 2E). Expression of CARD11 was comparable between the patient and control samples (Figure 2F).

Effect of CARD11 R30W on Bcl10 and MALT1 association

To assess the effect of R30W on binding to Bcl10 and MALT1, we introduced the R30W mutation into the CARD11 Δ ID construct¹⁵, which displays constitutive ability to bind a Bcl10/MALT1 complex due to the absence of the inhibitory domain (ID). CARD11 Δ ID simulates the open, active conformation of CARD11 that is induced by antigen receptor signaling¹⁵. Upon coexpression of with FLAG-Bcl10, Δ ID R30W showed a modest, but clearly reduced ability to co-immunoprecipitate with FLAG-Bcl10 as compared to the unmutated Δ ID construct (Figure 3). As previously described, FLAG-MALT1 could only interact with CARD11 Δ ID in the presence of untagged Bcl10, since MALT1 binds CARD11 through Bcl10 (Figure 3). Again, Δ ID R30W showed a modest, but clearly reduced ability to co-immunoprecipitate with FLAG-MALT1 in the presence of co-expressed Bcl10 under these conditions (Figure 3). The results suggest that the R30W mutation may impact CARD11 signaling by preventing optimal association with Bcl10 and MALT1.

Effect of CARD11 R30W on immune function

The numbers of circulating CD3⁺ T cells as well as CD4⁺ and CD8⁺ subpopulations were found to be normal in all patients. In patients 1 and 2, further analysis of naïve and memory (central and effector) T cell proportions as well as the numbers of Treg cells did not reveal any significant differences from normal controls (data not shown). Patient 2 did, however, demonstrate an increased proportion of CD3⁺ CD4⁻ CD8⁻ DN T cells (12.7% of CD3⁺), whereas patient 1 had only a minor elevation (3.9%) compared to controls (1-1.5%).

Despite apparently normal T cell populations, in vitro responses to mitogens such as PHA were diminished at 19.2% ± 17.3 of control in all four patients (Table 1). Similarly, optimal stimulation of T cells with anti-CD3 and anti-CD28 was markedly reduced in 3 of 4 patients, implying the presence of a T cell defect. In vitro responses to a series of antigens including Candida, Tetanus Toxoid, Zoster and CMV viruses were also extremely low in patients 1, 2 and 3 (Figure 4), suggesting that antigen-specific memory T cells are either lacking or exist at low frequencies in these patients

This would raise the possibility of an aberrant T cell expansion repertoire. Indeed, all four patients had underrepresentation of between 3 to 6 TCR Vβ families, while displaying 4 to 7 overrepresented TCR Vβ families (Figure 5). Within the CD8⁺ sub-population, patient 1 had underrepresentation of TCR Vβ 2, 5.3, and 9 and overrepresentation of TCR Vβ 3 and 13.1. Similarly in CD3⁺CD4⁺ cells, TCR Vβ 5.2, 5.3, 11 and 12 families were all underrepresented while TCR Vβ 13.1 and 16 were overrepresented. A similar pattern was recorded in all three other patients (Figure 5). Abnormal TCR Vβ expression is typically found in patients with combined immunodeficiency and is suggestive of immune dysregulation.

Further evidence for T cell dysfunction likely caused by an abnormal NF- κ B signaling pathway was revealed upon in vitro stimulation of patient cells with T cell mitogens, resulting in marked decreases in levels of IFN γ , and IL-2 secretion (Figure 6A, B). In a quest to determine whether TH2 cells play a major role in this phenotype, we measured cytokine secretions after stimulation of lymphocytes with PHA or anti-CD3 and anti-CD28. While levels of IL-5 were significantly increased after maximum stimulation of T cells (Figure 6C), secreted levels of IL-4, IL-10, IL-13 and IL-17 were comparable to controls (not shown).

Numbers of CD19/CD20 circulating B cells were also normal in all cases. Further assessment of B cell development in patients 1 and 2 failed to reveal any abnormalities, revealing the presence of naïve, transitional and un-switched and switched memory B cells in normal proportions. Yet, serum immunoglobulin levels were low in patient 1 and borderline low in patient 4, while the younger subjects (patients 2, 3) had normal concentrations (Table 1). IgA and IgM levels were normal. Antibody formation in response to vaccination was compromised in patient 1 for both the T cell dependent and independent antibodies. Antibody levels against Tetanus Toxoid were normal in the three other patients and antibody levels to mumps were detectable in all patients. Variable antibody levels to measles and rubella were also recorded. The response to a challenge with the Pneumovax polysaccharide antigens was markedly low in the patients tested (1 and 2). Similarly, antibody levels to isohemagglutinin were depressed in patient 2 but unfortunately could not be tested in patients 1 and 4 because of their AB+ blood group.

Assessment of Atopy

Serum IgE levels were increased in all four patients and eosinophils were above normal range in three of four patients (Table 1). Skin testing for various allergens was performed in all four patients. A standard panel of allergens including ragweed, trees, cat, dog, cockroach, feathers, house dust, egg, milk peanuts and mixed nuts were used. Patient 1 was assessed at 17 years of age and found positive for ragweed with a wheal of 10 mm, cat with a wheal of 8 mm, eggs with a wheal of 4 mm, peanuts resulted in a 7 mm wheal and mixed nuts induced a 3 mm wheal. At the age of 5 years, patient 2 tested positive to peanuts with a wheal of 12 mm, mixed nuts induced a wheal of 22 mm and milk was positive with a wheal of 6 mm. Patient 3 was studied at 3.5 years and tests were positive to milk with a wheal of 12 mm, egg with a wheal of 15 mm, mixed nuts with a wheal of 10 mm and kiwi with a wheal of 6 mm. Patient 4 had skin tests positive to dust mites, feathers, cat, dog, trees and ragweed. In all patients, the histamine positive control had a wheal of 2 mm and the negative was 0 mm.

Patient 3 ImmunoCAP tests of specific IgE levels showed 4.65 kUA/l for casein, 8.07 kUA/l for milk, 27.2 kUA/l for egg and 23.6 kUA/l for egg white. These results correlated well with the clinical history as well as skin testing, confirming multiple allergies.

Evaluation of NF κ B pathway function in patients

Previous reports have shown defects in T cell activation in the absence of CARD11 expression, including a specific inhibition of IL-2 high affinity receptor chain (CD25) induction. To assess this possibility, control and patient T cells were stimulated with a combination of soluble anti-CD3 plus anti-CD28 or the PKC-activating phorbol ester PMA, which activates CARD11 to initiate NF- κ B pathway activation, and CD25 induction examined after 24 hours (Figure 7). A sizeable

decrease in CD25 expression was noted with either stimulus in patient T cells compared to controls. The patient responses suggested that there was a significant blockage in NF- κ B activation. In contrast, PMA-induced up-regulation of CD69 expression, which has previously been shown to be unaffected by the loss of CARD11 expression, was essentially normal within the patient group.

These findings all strongly supported our studies in transfected Jurkat T cells that CARD11 R30W acts as a dominant negative, blocking the activity of the wild-type CARD11 protein.

DISCUSSION

We have described here an extended multi-generational family consisting of five members who suffered recurrent viral, fungal and bacterial infections since infancy, as well as manifesting severe atopy and autoimmune disorders. The index case (patient 1) had two affected children conceived from two different husbands who were unaffected, one of whom had one healthy offspring with another partner, suggesting autosomal dominant inheritance. This combination of features prompted the use of whole exome studies in order to identify a causal defect. The sole credible variant identified was a novel mutation in the CARD domain of CARD11. Sanger sequencing verified the variant in patient 1 and validated perfect segregation in the other family members. These findings could not explain the phenotype based on previous immunodeficiencies described with mutations in CARD11.

Heterozygous mutations in CARD11 were previously reported in patients with BENTA who, unlike our patients, typically present in infancy with B-cell lymphoproliferation. In contrast, monoallelic loss-of-function mutations found in parents of patients with autosomal recessive CARD11 deficiency were asymptomatic¹³. As the clinical manifestations of the patients were consistent with CID and atopy, we hypothesized that R30W CARD11 was a heterozygous mutant with a dominant negative effect. This was supported by the observation that a homozygous loss-of-function mutation in CARD11 leads to severe atopy in the immunodeficient *unmodulated* mouse^{23, 33}. Indeed, transfection of the CARD11 R30W mutant into CARD11-deficient Jurkat T cells demonstrated markedly reduced NF- κ B induction compared to the wild-type. Moreover, co-transfection of R30W CARD11 along with wild-type

CARD11 resulted in dramatic suppression of wild-type-mediated NF- κ B activation, indicating a strong dominant negative effect. This observation was further confirmed in primary lymphocytes of the patients.

It appears from our cofactor interaction studies that the R30W mutation modestly impacts the ability of CARD11 to bind to Bcl10 and MALT1 through Bcl10. The effect on Bcl10 binding could explain the signaling defect of the R30W mutant if a certain threshold of cofactor interaction not met by the R30W variant is required for signaling to NF- κ B. Alternatively, it is possible that a distinct step in CARD11 signaling is also affected by the R30W mutation and that R30 participates in an interaction with an undetermined positive signaling cofactor, or promotes a conformation of CARD11 required for signaling. Since CARD11 functions as an oligomer of undetermined stoichiometry, the R30W mutation could interfere with CARD11-CARD11 interactions within the same molecule or between distinct subunits in an oligomer.

The ablative effect of R30W on NF- κ B signaling was consistent with the markedly reduced TCR-triggered secretion of IL-2 and IFN γ , as well as proliferative responses to mitogens and antigens. Together, these findings can explain the patient manifestations of repeated and severe infections. Despite these functional T cell defects, the number of circulating T cells and the proportion of naïve and memory, as well as Treg cells, remained largely intact. However, analysis of TCR V β families revealed a significant skewing of the T cell repertoire. Of note is the over-representation of several TCR V β families suggesting clonal expansion, which is frequently seen in CID^{64, 65}, and is reminiscent of observations in autoimmune disorders⁶⁶. These results are consistent with the multiple autoimmune disorders observed in R30W^{-/+} patients.

The number of circulating B cells in our cohort was consistently normal but IgG levels as well as the ability to produce specific antibodies in response to vaccinations were variable. Patients 1 and 4 had low serum IgG levels, but only patient 1 had a universal inability to produce specific antibodies.

The phenotypic difference between autosomal recessive CARD11 deficiency and the heterozygous R30W carriers is striking, suggesting that signaling downstream of CARD11 in our patients is not as universally and completely ablated, despite the strong dominant negative effect of the mutant CARD11 in the Jurkat T cell assay. Induction of CD25 expression in primary T cell activation assays showed a significant reduction in R30W T cells compared to normal (typically < 50%); however, while blunted, responses were not absent. PMA-induced responses were found to be enhanced by co-stimulation with anti-CD28 in all patients (not shown), demonstrating that maximal stimulation is capable of inducing some significant activation. The residual signaling activity observed in the patients' cells was mimicked in our NF- κ B reporter assay in Jurkat T cells when we used equivalent levels of DNA expression vectors for wild-type and R30W CARD11 (Figure 2C). Under these conditions, wild-type CARD11 is expressed at ~4-fold higher levels than the R30W mutant, likely leading to a fraction of wild-type CARD11 proteins that is resistant to the dominant negative effect of R30W.

The precise mechanism underlying the severe atopy afflicting R30W defect patients remains largely unclear and requires further extensive investigation. We explored the possibility that an increase in TH2 secreted cytokines occurred in these patients. TH2 as well as some TH1 and CD8⁺ cells were implicated in orchestrating allergic inflammation through release of the cytokines IL-4, IL-5, IL-9 and IL-13. While IL-5 secretion from circulating T cells was

elevated, secretion of IL-4 and IL-13 were indistinguishable from controls. The increased secretion of IL-5 is consistent with the rise in eosinophil numbers in these patients, but the concomitant increase in total IgE as well as specific antibodies could not be explained by these results. It is still possible that the increase in IgE levels may be triggered by increased IL-4 and IL-13 stemming from tissue infiltrating TH2 cells. Alternatively, it is possible that the expanded CD8⁺ T cells found in the patients' infiltrated tissues also secrete TH2 cytokines (Tc2).

In conclusion, we have described here for the first time an autosomal dominant transmission of CARD11 deficiency caused by a dominant negative mutation. The clinical spectrum in this disorder is unique, encompassing recurrent infections, autoimmunity and severe atopy. The novel R30W mutations described abrogates the NF- κ B pathway and leads to decreased IL-2 and IFN γ secretion and lymphocyte proliferation. The signaling defect results in a unique form of CID with prominent features of autoimmunity and atopy.

560 Table 1: Immune characteristics of four patients with the R30W CARD11 mutation

	Patient 1		Patient 2		Patient 3		Patient 4		Normal Values
		Normal Range/Control		Normal Range / Control		Normal Range / Control		Normal Range / Control	
Eosinophil count ($\times 10^9/L$)	0.66		2.33		0.87		0.49		0.02-0.5
Lymphocyte immunophenotyping (cells/ μL)									
CD3	2017	700-2100	1178	800-3500	2963	900-4500	2019	700-2100	
CD4	1148	300-1400	671	420-2100	1428	500-2400	1066	300-1400	
CD8	868	200-900	338	200-1200	1305	300-1600	845	200-900	
CD56	189	90-600	94	70-1200	230	100-1000	216	90-600	
CD19/20	153	100-500	56	200-600	375	200-2100	154	100-500	
Responses to mitogens (Stimulation Index; P/C)									
PHA	162	410	99	1451	171.5	646	97	753	Stim. Index >400 Patient control >50
CD3+CD28	79	111	3.1	111	304	383			
Immunoglobulins									
IgG (g/L)	4.7	6.6-15.3	10.7	6.6-15.3	5.6	5.4-13.6	6.3	6.6-15.3	
IgA (g/L)	3.9	0.54-4.17	3.5	0.5-2.2	2.0	0.3-1.5	1.7	0.54-4.17	
IgM (g/L)	1.5	0.3-2.3	0.5	0.5-1.9	0.3	0.4-1.5	0.9	0.3-2.3	
IgE (IU/mL)	707		9404		423		480		<200
Specific antibodies									
Tetanus	0.01		0.37		0.22		0.27		>0.1
Measles/ Mumps / Rubella	-ve / -ve / -ve		-ve / -ve / +ve		-ve / -ve / +ve		+ve / -ve / +ve		+ve / +ve / +ve
α -Pneumococcus	No response		No response		Not done		Not done		>5% >4-fold rise
isohemagglutinin	NA		$\alpha A - 1:2$		Not done		NA		>1:32

561 NA – Not applicable due to AB blood type

562

FIGURE LEGENDS

Figure 1. Pedigree and structural analysis of CARD11 R30W mutant. (A) Pedigree of multi-generational family with mutation in CARD11. The pedigree shows the heterozygous CARD11 R30W (c.88C>T) mutation in four genotyped cases. [=] indicates no variant detected. Circles represent female subjects while squares denote male subjects. Half-solid symbols represent heterozygous subjects. The pedigree shows 3 generations of the family with 5 affected individuals. **(B)** Structure of CARD11 protein with domains required for signaling activity. The R30W loss-of-function variant targeting the CARD domain is shown in green, whereas gain-of-function variants are shown in blue. The CARD sequence and secondary structure (helices $\alpha 1$ to $\alpha 6$) are shown in the inset **(C)** Representation of the CARD11 CARD domain monomer structure. R30 is indicated in yellow. Residues at the CARD11:Bcl10 interface in violet and residues required for inhibitory domain interaction in green.

Figure 2. CARD11 R30W is a loss-of-function mutant with dominant negative activity. (A) The indicated amounts in ng of expression vectors for wild-type or R30W mutant murine myc-CARD11 were expressed in 293T cells with the Ig κ ₂-IFN-LUC reporter and the CSK-LacZ control and levels of protein expression were assayed by western blot with anti-myc antibodies. **(B)** Jurkat-KO T cells were transfected with Ig κ ₂-IFN-LUC, CSK-LacZ, and expression vectors for wild-type or R30W CARD11 as indicated. To achieve a relative level of expression referred to as 1.0, 148 ng and 348 ng of expression vector were used, respectively, for the wild-type and R30W variants. To achieve 50% of that level of expression (referred to as 0.5), 107 ng and 200 ng of

expression vector were used, respectively, for the wild-type and R30W variants. Forty hours after transfection, cells were stimulated for 5 hours with anti-CD3/anti-CD28 treatment, and then harvested for luciferase and β -galactosidase assays. **(C)** Jurkat-KO T cells were transfected with Ig κ ₂-IFN-LUC, CSK-LacZ, and expression vectors for wild-type or R30W CARD11 as indicated. Forty hours after transfection, cells were stimulated for 5 hours with anti-CD3/anti-CD28 treatment, and then harvested for luciferase and β -galactosidase assays. **(D)** Equivalent amounts (107 ng) of expression vectors for wild-type or R30W mutant murine myc-CARD11 were expressed in 293T cells with the Ig κ ₂-IFN-LUC reporter and the CSK-LacZ control and levels of protein expression were assayed by western blot with anti-myc antibodies. **(E)** Peripheral blood lymphocytes of patients 1 and 3 were stimulated with anti-CD3 plus anti-CD28 for the indicated period of time (min). Protein samples were blotted with anti-phospho-p65 NF- κ B demonstrating a markedly diminished phosphorylation of p65 in patients as compared to controls. **(F)** Western blots show comparable expression of CARD11 in EBV transformed B cell lines obtained from patients and control as indicated.

Figure 3. The R30W mutation affects binding to Bcl10 and MALT1. HEK293T cells were transfected with expression vectors for wild-type and R30W variants of CARD11 Δ ID, FLAG-Bcl10, FLAG-MALT1, and untagged Bcl10 as indicated. Forty hours after transfection, anti-FLAG immunoprecipitations were performed and the contents of the lysate input and immunoprecipitate were evaluated by western blotting with the indicated antibodies.

Figure 4. T cell responses to antigens. Patient and control PBL were incubated with antigens (Candida, Tetanus Toxoid, Zoster, CMV) as indicated for 6 days, labelled with ^3H Thymidine, harvested and radioactivity counted. Three of four patients had no responses to cell antigens. Normal response is considered >40 stimulation index. The figure displays a group of 44 control responses (mean $\pm$ SD).

Figure 5. Clonal expansion in affected patients. The CD4^+ and CD8^+ T cell repertoire of patients 1 (A), 2 (B), 3 (C) and 4 (D) were assessed by flow cytometry. All 4 patients had underrepresentation of 3-6 TCR $\text{V}\beta$ families and 4-7 overrepresented TCR $\text{V}\beta$ families. The mean $\pm$ SD was determined by assessing a large number of controls (>75 normal individuals; mostly children).

Figure 6. Cytokine secretion in response to PHA or anti-CD3 and anti-CD28. Patient ($n=2-3$) or control ($n=7-17$) PBL were stimulated with PHA or anti-CD3 and anti-CD28 for a period of 48 hr, and subsequently assessed for IFN- γ (A), IL-2 (B) and IL-5 (C) secretion by ELISA. Control data are represented as mean $\pm$ SD. Comparisons were made using unpaired t-test.

Figure 7. Defective T cell activation in CARD11 R30W patients. (A) Control and patient T cells were stimulated with anti-CD3 and anti-CD28 or PMA as indicated for 24 hours and induction of CD25 assessed by flow cytometry. (B) Comparison of CD25 and CD69 induction in control and patient T cells. The results are representative of two separate experiments with similar results.

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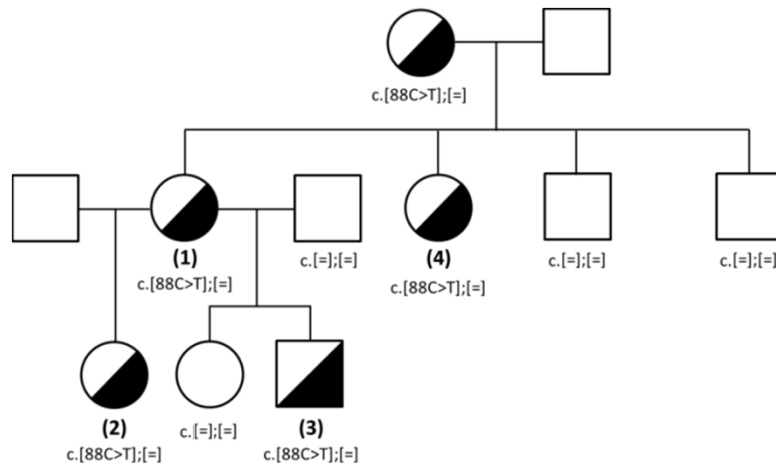
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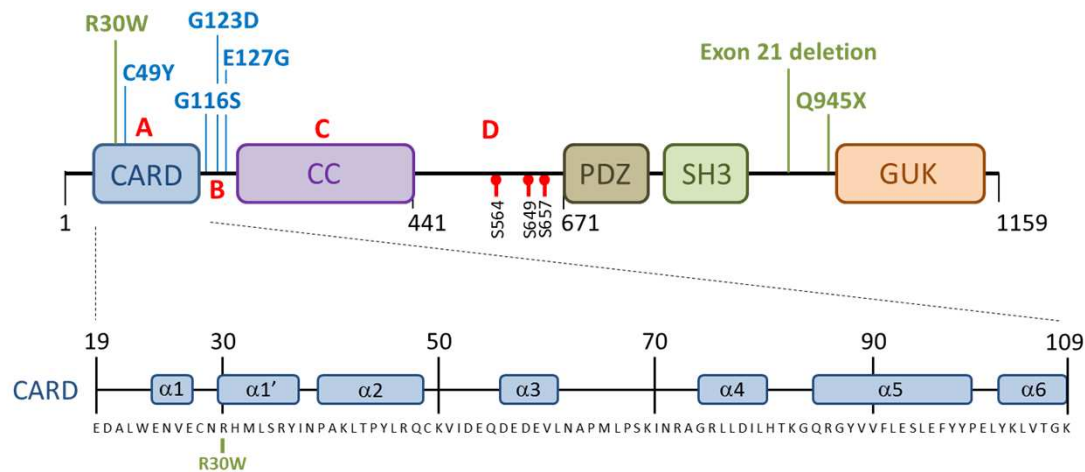
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A



B



C

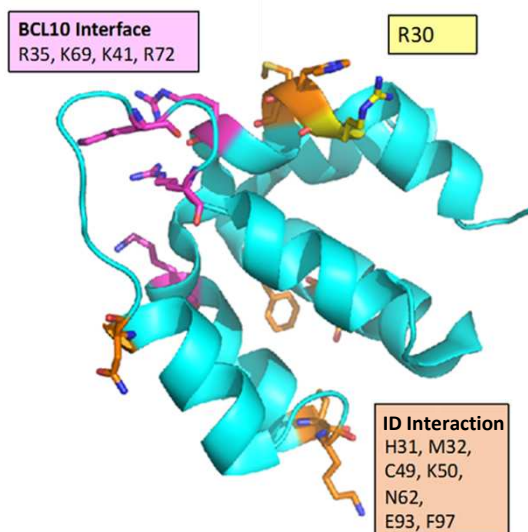
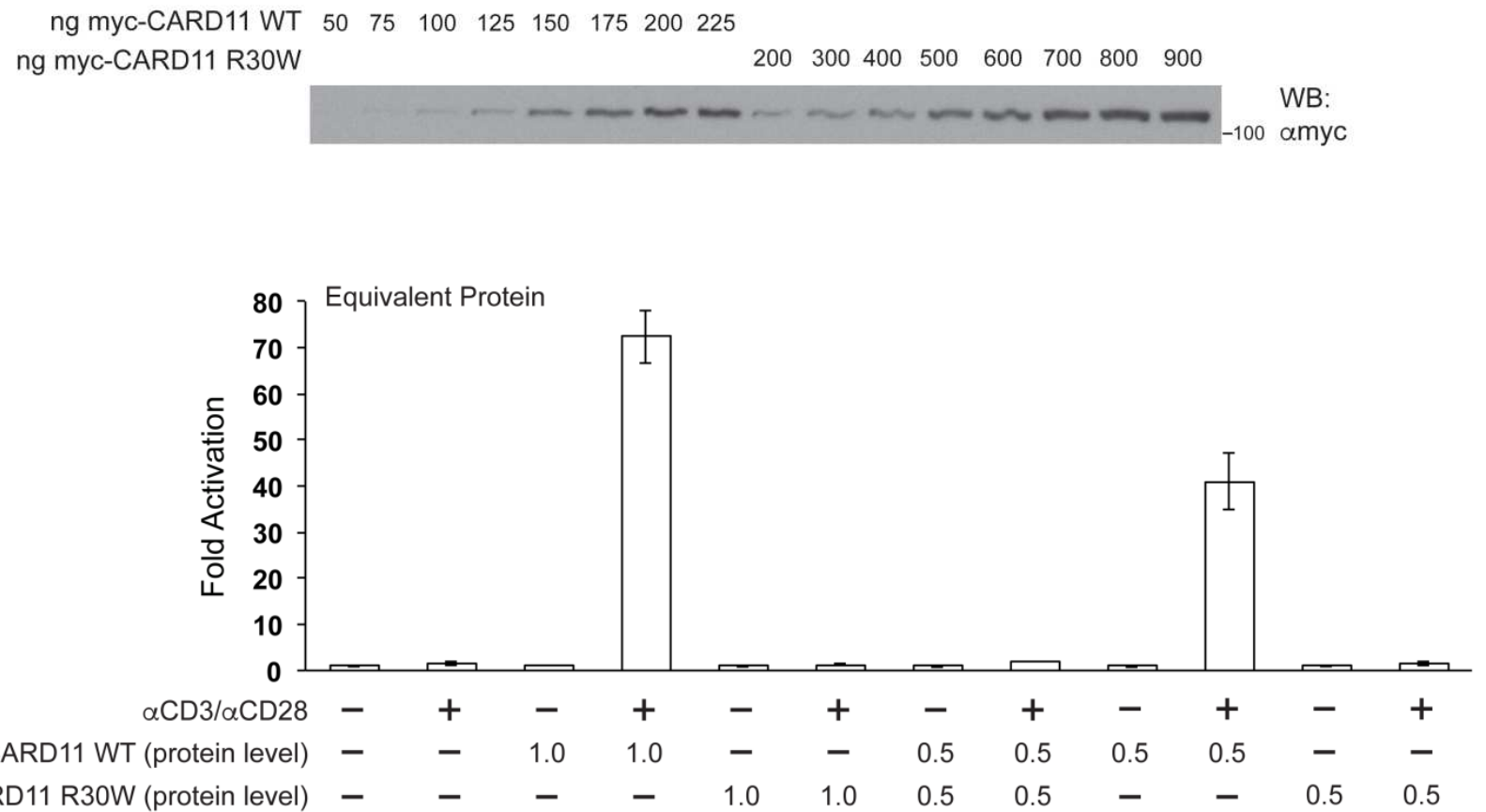
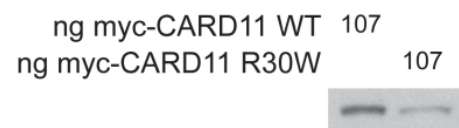
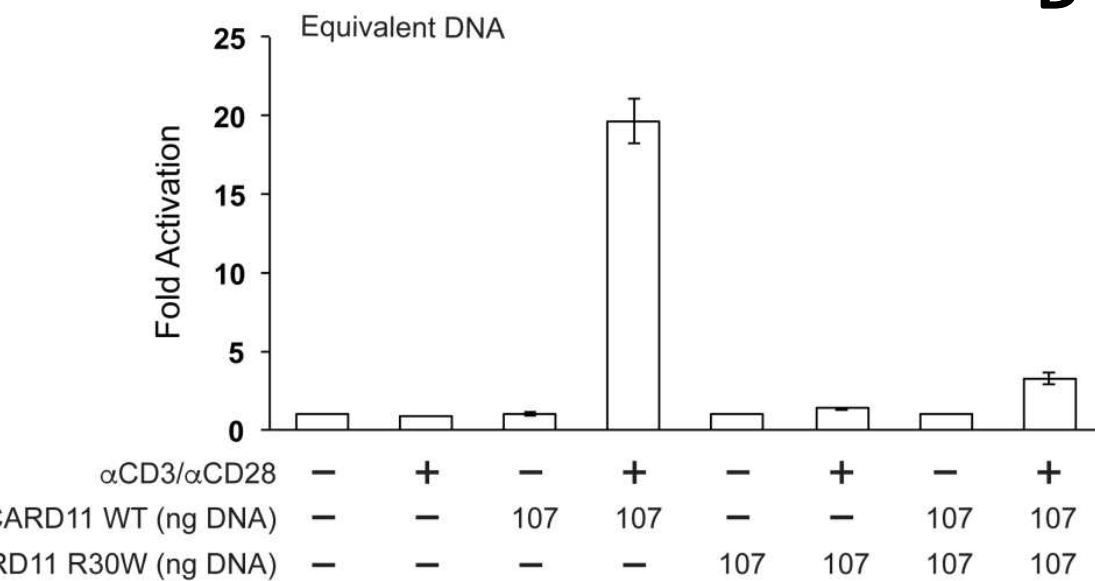


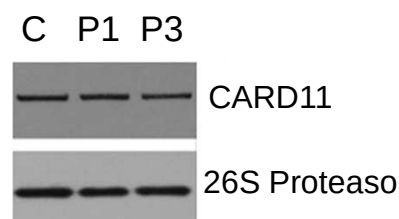
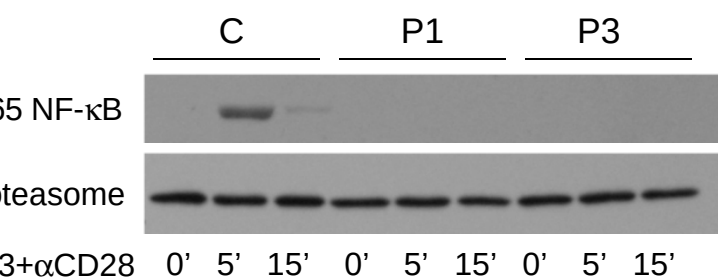
Figure 1



D



F



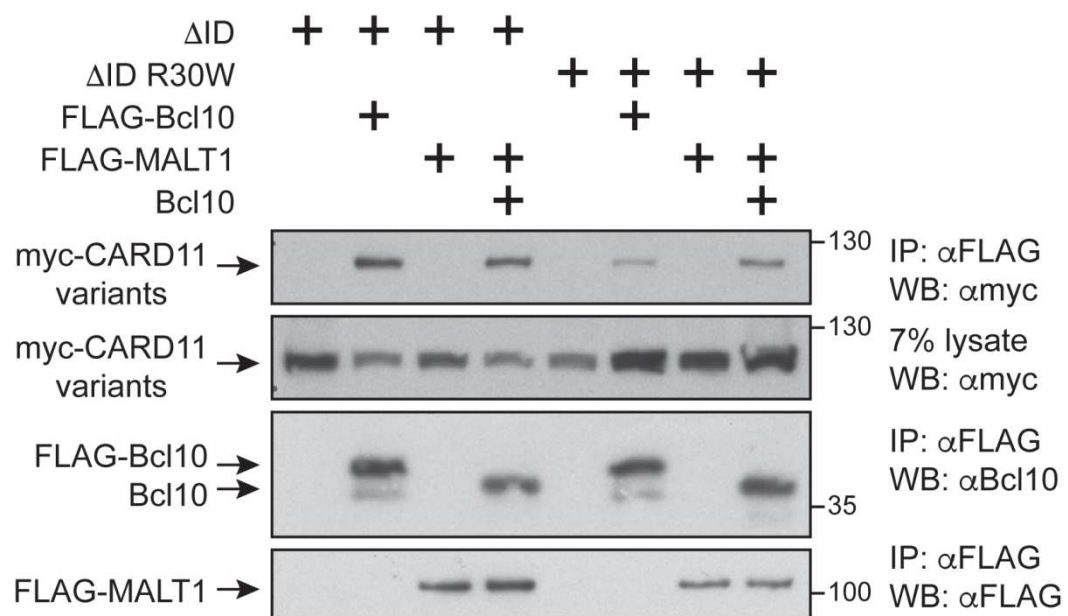


Figure 3

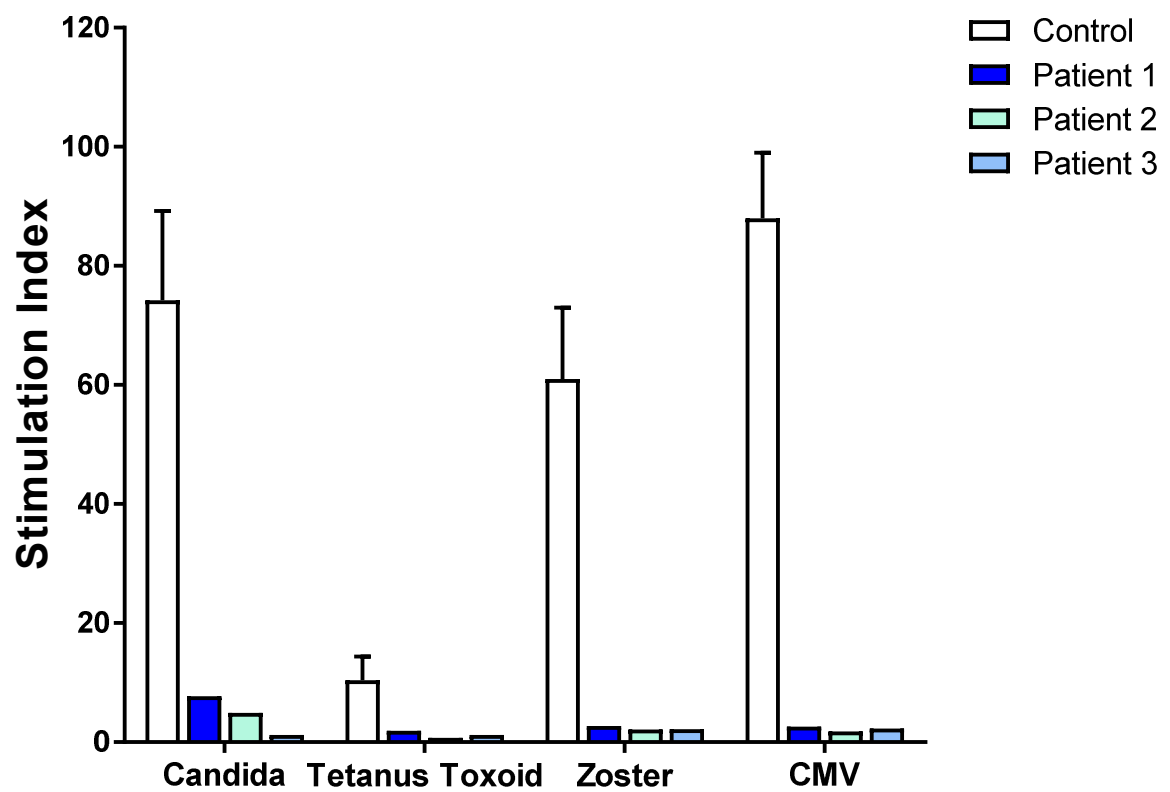


Figure 4

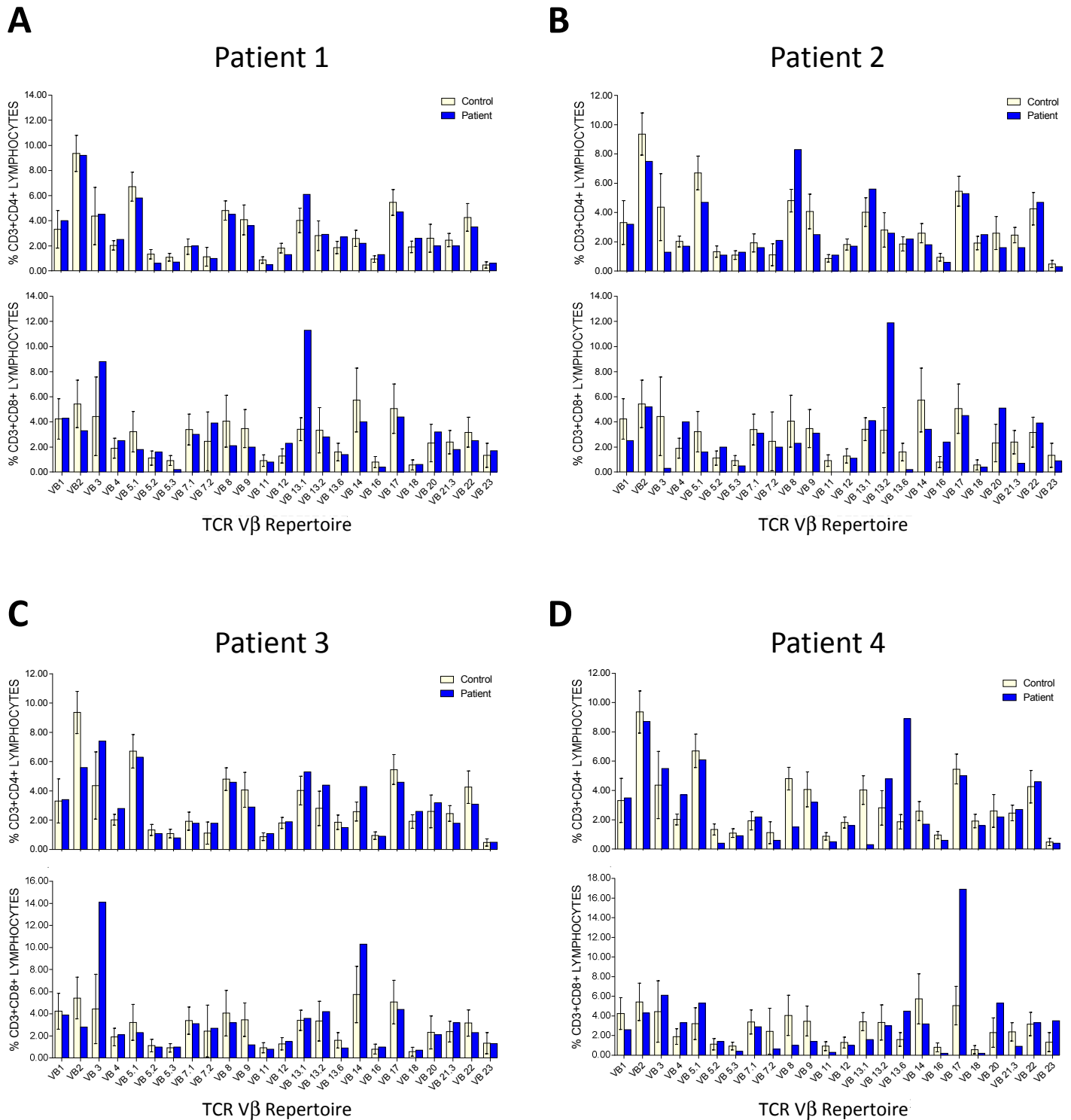


Figure 5

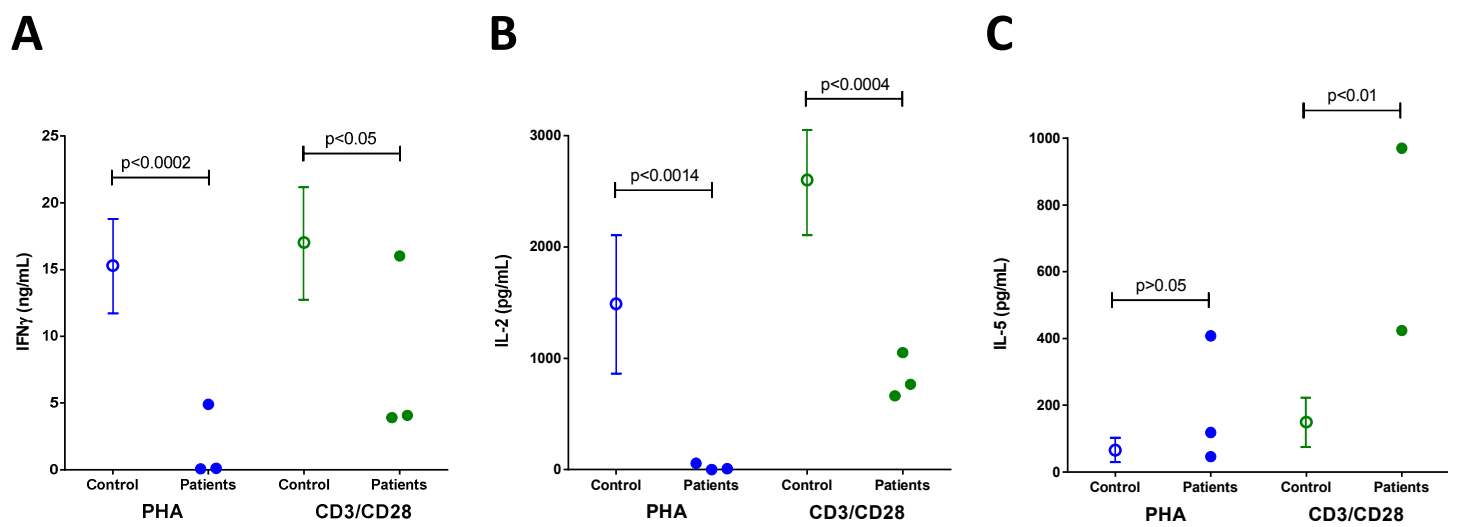
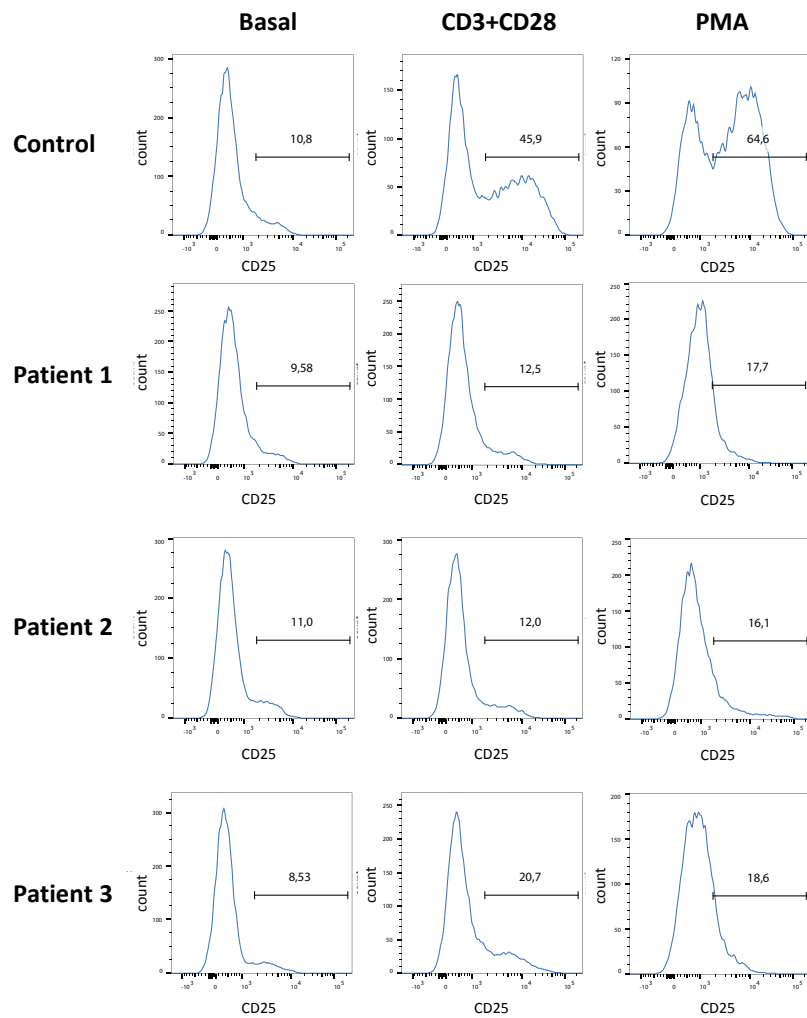


Figure 6

A



B

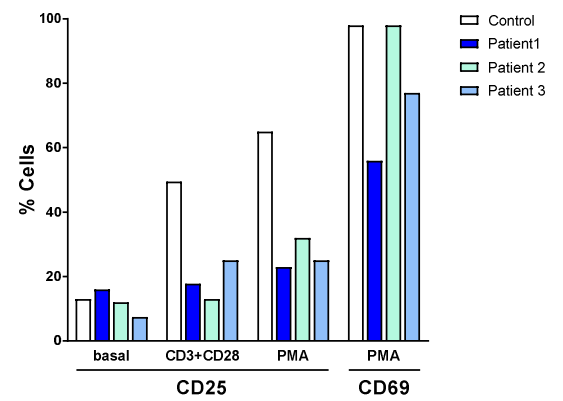
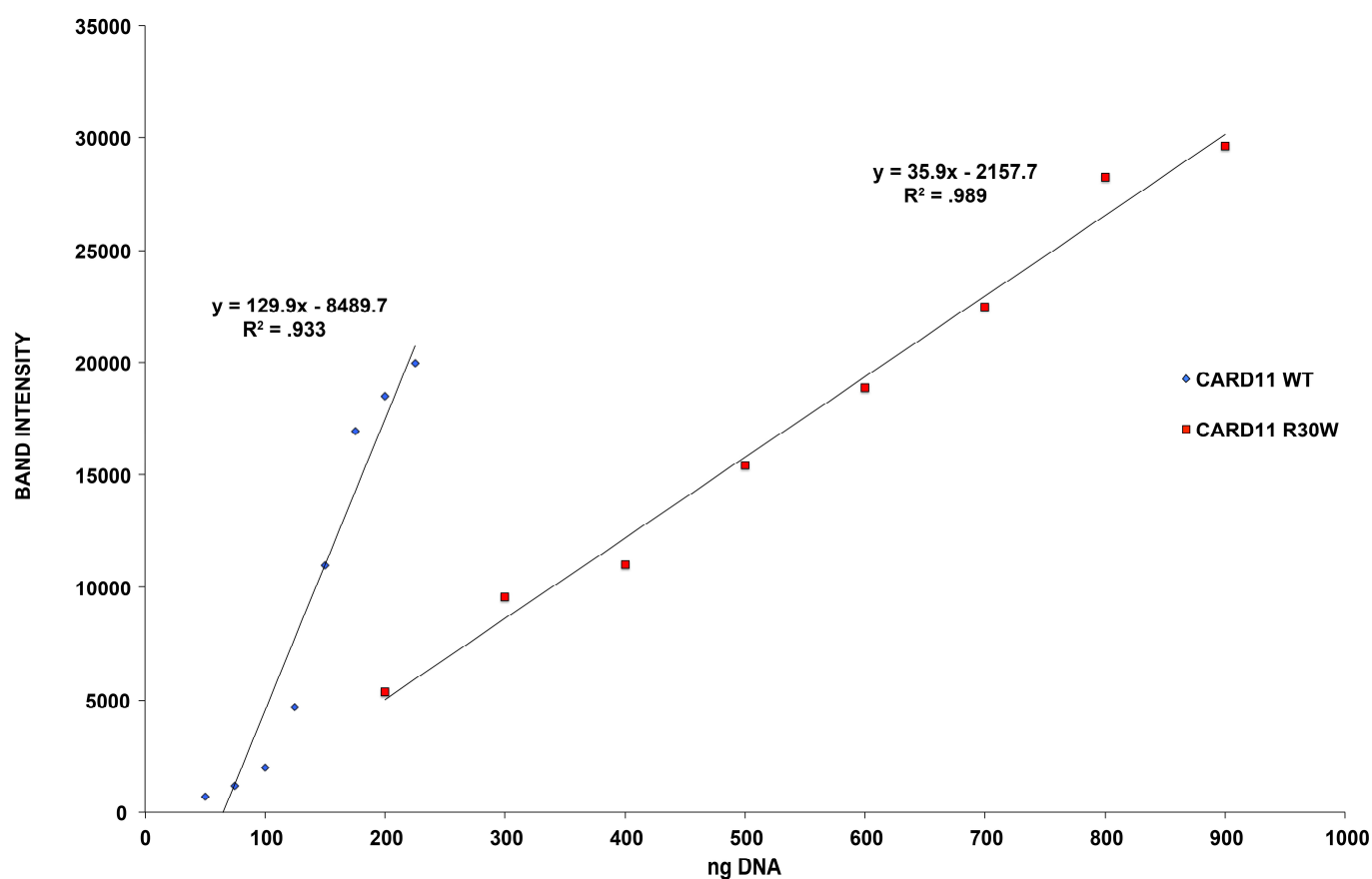


Figure 7

Supplementary Table 1: Clinical features of patients

	Patient 1	Patient 2	Patient 3	Patient 4
Infections	Recurrent sinusitis, chronic bronchitis (bronchiectasis), recurrent upper respiratory infection	Recurrent pneumonia, cellulitis	Repeated upper respiratory infections	Upper respiratory infections, pneumonia
Atopy	Asthma, eczema, environmental allergies	Asthma, eczema, food allergies	Asthma, eczema, food allergies	Asthma, eczema, environmental allergies
Autoimmune Features	Colitis, lichen sclerosis, necrotizing granulomatous inflammation			Lichen sclerosis, ovarian failure



Supplementary Figure S1. Linear regression curves used to determine relative amounts of CARD11 WT and R30W expressed from a given DNA amount of expression vector. Band intensities over background from Figure 2A were quantitated using ImageJ and plotted. The best-fit linear regression equations and associated R^2 values are shown.