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Hypomorphic *CARD11* mutations associated with diverse immunologic phenotypes with or without atopic disease

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Hypomorphic *CARD11* mutations associated with diverse immunologic phenotypes with or without atopic disease.

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Abstract

Background

CARD11 encodes a scaffold protein in lymphocytes that links antigen receptor engagement with downstream signaling to NF-κB, JNK, and mTORC1. Germline *CARD11* mutations cause several distinct primary immune disorders in humans, including SCID (biallelic null mutations), B cell Expansion with NF-κB and T cell Anergy (BENTA; heterozygous, gain-of-function mutations), and severe atopic disease (loss-of-function, heterozygous, dominant interfering mutations), which has focused attention on *CARD11* mutations discovered by whole exome sequencing.

Objectives

To determine the molecular actions of an extended allelic series of *CARD11*, and to characterize the expanding range of clinical phenotypes associated with heterozygous *CARD11* loss-of-function alleles.

Methods

Cell transfections and primary T cell assays were utilized to evaluate signaling and function of *CARD11* variants.

Results

Here we report on an expanded cohort of patients harboring novel heterozygous *CARD11* mutations that extend beyond atopy to include other immunologic phenotypes not previously associated with *CARD11* mutations. In addition to (and sometimes excluding) severe atopy, heterozygous missense and indel mutations in *CARD11* presented with immunologic phenotypes similar to those observed in STAT3-LOF, DOCK8 deficiency, common variable immune deficiency (CVID), neutropenia, and immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX)-like syndrome. Pathogenic variants exhibited dominant negative activity, and were largely confined to the CARD or coiled-coil domains of the CARD11 protein.

Conclusion

These results illuminate a broader phenotypic spectrum associated with *CARD11* mutations in humans, and underscore the need for functional studies to demonstrate that rare gene variants encountered in expected and unexpected phenotypes must nonetheless be validated for pathogenic activity.

Keywords: CARD11; atopy; atopic dermatitis; dominant negative, primary immunodeficiency, immune dysregulation

Abbreviations:

AHA: autoimmune hemolytic anemia
BENTA: B cell Expansion with NF-κB and T cell Anergy
CARD: caspase activation and recruitment domain
CC: coiled-coil
CVID: common variable immunodeficiency
DN: dominant negative
EoE: eosinophilic espophagitis
EBV: Epstein-Barr virus
FTT: failure to thrive
GOF: gain-of-function
HSV-1: herpes simplex virus 1
IPEX: immune dysregulation, polyendocrinopathy, enteropathy, X-linked
ITP: idiopathic thrombocytopenic purpura
JNK: c-Jun N-terminal kinase
LGL: large granular lymphocytic leukemia
LOF: loss-of-function
MAGUK: membrane-associated guanylate kinase domain
MAS: macrophage activation syndrome
mTORC1: mechanistic target of rapamycin complex 1
PMA: Phorbol 12-myristate 13-acetate
NF-κB: nuclear factor kappa B
SCID: severe combined immune deficiency
sJIA: systematic juvenile idiopathic arthritis
T1D/T2D: type 1 / type 2 diabetes
TCR: T cell receptor
WES: whole exome sequencing
WGS: whole genome sequencing

Key Messages:

- *CARD11* DN mutations are associated with a broader spectrum of human disease phenotypes than previously appreciated, extending beyond atopy to include cutaneous viral and respiratory infections, hypogammaglobulinemia, autoimmunity, neutropenia, and lymphoma.

- Pathogenic DN mutations are most likely located in the N-terminal CARD and CC domains of CARD11, compromising TCR-induced NF-κB activation

- Clinicians should test for causative *CARD11* DN mutations in patients that present with an autosomal dominant pattern of atopy, viral skin infections, and/or respiratory infections and exhibit defective TCR-induced NF-κB activation in vitro, with or without impaired TCR-induced S6 phosphorylation.

Capsule Summary

This description of a large cohort of patients broadens the spectrum of clinical disease phenotypes linked to *CARD11* DN mutations, including atopy, cutaneous viral and respiratory infections, hypogammaglobulinemia, autoimmunity, neutropenia, and lymphoma.

Introduction

Hypomorphic mutations in genes whose protein products are critical for immune function provide an opportunity to assess the protein's roles in the context of a functioning, albeit impaired, immune response. Unlike the unresponsive or absent effector compartments often associated with null mutations that manifest as severe combined immune deficiency (SCID), these mutations preserve sufficient function to allow for development of the particular cell(s) or organ(s) with which it is involved. Studies of such allelic variants in mice and other model organisms have taught us much about immune system development and function. With the wider availability and application of next-generation sequencing technologies, we now have more opportunities to explore the phenomenon of allelic variance and phenotypic heterogeneity in humans. One example can be found in the case of *RAG1* – null mutants lead to the absence of T-cells and grossly abnormal thymic development. Hypomorphic mutations lead to functioning T-cells but disruptions of repertoire and thymic development that shed light on immune tolerance, RAG1 protein domain function, and other immune processes (1).

Another example is found in different types of mutations described in *CARD11* leading to different phenotypes. CARD11 is a critical 1154 amino acid protein scaffold best known for linking antigen recognition with downstream NF-κB activation in lymphocytes (2, 3). The protein CARD11 (also known as CARMA1, represented by the sequences NP_115791.3 and NM_032415) includes an N-terminal caspase recruitment domain (CARD, 1-110), LATCH (112-130), coiled-coil (CC, 130-449) domains, and a C-terminal membrane-associated guanylate kinase domain (MAGUK, 667-1140) comprised of PDZ, SH3 and GUK domains. Biallelic null mutations of *CARD11*, in both patients and mouse models, lead to severe T and B-cell immune deficiency (4). Somatic gain-of-function (GOF) *CARD11* mutations are commonly found in non-Hodgkin B cell lymphomas, whereas germline GOF mutations give rise to BENTA (B cell Expansion with NF-κB and T cell Anergy) disease in humans (2, 5, 6). Surprisingly, hypomorphic/dominant negative (DN) mutations in both mice and human patients permit

sufficient effector function to reveal a strong disposition toward atopic phenotypes, in addition to variable immune deficiency (7-9). Because CARD11 oligomerization is essential for downstream signaling, heterozygous variants can either enhance or dominantly interfere with CARD11 function (10, 11).

Greater access to next-generation sequencing virtually guarantees that variants in a particular gene can now be identified in patients with ever-broadening phenotypes, especially for cases of GOF and hypomorphic loss of function (LOF) mutations (12). Although algorithms can assist in predicting which variants are likely to be benign or deleterious, previously undescribed rare or novel variants in such genes must always be validated for pathogenicity using relevant biological assays. Moreover, in silico prediction methods do not distinguish between variants seen in the heterozygous state and homozygous state, which is critical for CARD11. Furthermore, collection and testing of variants from as many centers as possible can more accurately determine the breadth of disease associated with a given gene, especially given the role that referral bias can play in any given center.

We therefore report our experience with numerous heterozygous mutations in *CARD11* in the context of severe familial atopic disease and other immunologic phenotypes not previously associated with *CARD11* mutations. The atopy described here is a genetic tendency to develop symptoms of immediate hypersensitivity (e.g. food allergy, allergic rhinitis) or allergic inflammation (e.g. eczema, eosinophilic esophagitis), irrespective of specific allergen sensitization (13). In many cases, rare or novel mutations were uncovered in whole exome/genome sequences (WES/WGS) performed at major referral centers for multiple reasons, particularly in patients without a clear putative genetic diagnosis. Herein we attribute several new DN *CARD11* mutations to an expanded list of disease manifestations, and describe assays designed to help differentiate pathogenic vs. non-pathogenic variants in *CARD11*. Importantly, results of these assays are interpreted within the context of specific genotypic/phenotypic criteria that help to define a differential diagnosis for patients harboring *CARD11* DN mutations. In addition to severe atopy, heterozygous missense mutations in *CARD11* with DN activity can

present with common variable immune deficiency (CVID), neutropenia, cutaneous viral infections, and immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX)-like syndrome. Collectively, our findings define a broader spectrum of immune disease associated with detrimental *CARD11* mutations, which are most often confined to specific domains of the CARD11 protein. Nevertheless, our evaluations underscore the idea that rare gene variants found by WES/WGS can be pathogenic even when not matching with reported phenotypes. At the same time, *CARD11* mutations associated with an expected phenotype must nonetheless be validated for pathogenic activity using functional studies.

Methods

Patients

Informed consent was obtained from all participating patients and their family members according to protocols approved by Institutional Review Boards and ethics boards at their respective institutions.

Whole exome sequencing (WES) and genetic analysis

WES was performed on the majority of patients described here according to established protocols. For example, Kindreds 6, 19 and 29 were analyzed as follows. Genomic DNA was extracted from peripheral blood cells and Illumina paired-end genomic DNA sample preparation kit (PE-102-1001, Illumina) was used for preparing the libraries followed by exome-enriched library using the Illumina TruSeq exome kit (FC-121-1008, Illumina). Samples were sequenced on an Illumina HiSeq as 100-bp paired-end reads. DNA reads were mapped to the GRCh38 human genome reference using the default parameters of the Burrows-Wheeler Aligner (bio-bwa.sourceforge.net). Single nucleotide substitutions and small insertion deletions were identified and filtered based on quality with SAMtools software package (samtools.sourceforge.net) and annotated with Annovar tool (www.openbioinformatics.org). Filtering of variants for novelty was performed according to minor allele frequency, mouse mutant phenotype in the homologous mouse gene, Mendelian disease associations (OMIM), pathway analysis (gene ontology [GO]), immune system expression (Immgen), CADD, SIFT, and PolyPhen-2 scores (14-16). After filtering and ranking variants, heterozygous *CARD11* variants were investigated further.

Kindred 14 (R187P) was initially evaluated separately from the other families. Eight samples were subjected to whole exome sequencing (WES) as described previously (17) (Supplementary Materials and Methods). We selected putative causative variants that had an average read depth ≥20, a quality score of ≥200, and were heterozygous in at least one individual. Genetic analysis showed that seven of the samples were related and one sample was not related. The remaining seven samples were from one

unaffected individual and six affected individuals. We did a combinatorial search for variants present in the heterozygous state in the six affected individuals and absent in the unaffected individual. We performed multipoint genetic linkage analysis with Superlink-online-SNP (18, 19) assuming a rare disease allele and dominant inheritance. Two variants satisfied the combinatorial condition of being heterozygous in six sampled affected individuals and absent in the one sampled unaffected individual: MICALL2 (p.Q202*) and CARD11 (p.R187P), and were at the same time located in a region (chr7:0.0-4.3 Mbp) consistent with genetic linkage.

PBMC signaling analysis

PBMC were isolated by Ficoll gradient centrifugation. Phosphorylation of p65 and/or S6, and degradation of IkB was assessed by intracellular flow cytometry after short ex vivo stimulations with PMA as previously described (9). To assess S6 phosphorylation under Th0 conditions, total PBMC (10^6/ml) were seeded in 1 mL RPMI (Gibco) supplemented with 10% FBS, penicillin/streptomycin and L-glutamine in an anti-CD3 antibody (OKT3, 1 µg/mL) coated 48-well plate. Anti-CD28 antibody (L293, 0.2 µg/mL,) and IL-2 (10 ng/mL, Peprotech) were then added to the culture (Th0), and cells were incubated in a 37℃ CO_2 chamber. After 24 hours, cells were pelleted by centrifugation, stained with Live/Dead dye (Thermo Fisher), fixed with 1.6% paraformaldehyde in PBS, then centrifuged and permeabilized with absolute methanol. Cells were then stained for flow cytometry with fluorochrome-conjugated antibodies after washes with FACS buffer (PBS with 0.5% BSA). The antibodies (Abs) used for flow cytometry were: CD3 (UCHT1), CD4 (RPA-T4, L200), CD45RA (HI100), phospho-S6 (N7-548) (BD Biosciences). Percentages of phospho-$S6^+$ live $CD3^+$ $CD4^+$ cells, gated on the lymphocyte SSC and CD45RA compartments, are shown in Figure 6.

CARD11 mutant expression plasmids

Modified wild type (WT) and mutant pUNO-CARD11-FLAG plasmids were constructed and purified as previously described (9). Briefly, site-directed mutagenesis was utilized to introduce single nucleotide variants that were putative point mutations into the WT CARD11 construct (Invivogen) using primer-directed linear amplification with Pwo or

PfuI polymerase (Roche), followed by *DpnI* digestion of methylated template DNA (ThermoFisher). All inserted variants were confirmed by Sanger sequencing. All resulting plasmids were purified from DH5α E. Coli (New England Biolabs) using a GenElute HP Plasmid Maxi-Prep Kit (Sigma).

Cell transfection assays

Both WT (clone E6.1, ATCC) and CARD11-deficient Jurkat T cells (referred to as JPM50.6) were cultured and transfected as previously described (9). JPM50.6 cells containing an integrated canonical NF-κB-driven GFP reporter were originally provided by Dr. Xin Lin (MD Anderson Cancer Center). Briefly, $5x10^6$ Jurkat or JPM50.6 T cells were resuspended in 0.4 mL RPMI/10% FBS, placed in 0.4cm cuvettes (Bio-Rad) and electroporated (260 V, 950 μF) with 5-10 μg plasmid DNA (BTX Harvard Apparatus). For JPM50.6, cells were stimulated 24 hours post-transfection with 1 μg/ml anti-CD3/CD28 antibodies and incubated overnight (BD Biosciences). Relative canonical NF-κB activation in JPM50.6 was quantified based on mean fluorescence intensity (MFI) of an integrated κB-GFP reporter using an Accuri C6 flow cytometer (BD Biosciences). To assess S6 phosphorylation in Jurkat transfectants, cells were washed 24 hrs post-transfection in PBS and incubated in PBS/1%BSA for 1 hr at 37°C. Following an additional PBS wash, cells were stimulated with 1 μg/ml anti-CD3/CD28 antibodies (BD Biosciences) for 20 min at 37°C. Activation was stopped by adding ice cold PBS, and cells were pelleted and lysed in 1% NP-40 lysis buffer as previously described (9). Lysates (5-10 μg) were separated on 4-20% Tris-Glycine SDS gels and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in 5% milk/TBS/0.1%Tween20 and immunoblotted with the following Abs: anti-phospho-S6 (Ser235/236), anti-S6 (5G10), anti-CARD11 (1D12, Cell Signaling Technology; LS-C368868, LifeSpan Biosciences; OASG00985, Aviva Systems Biology); anti-HA (2-2.2.14, Thermo Fisher), anti-FLAG (M2) and anti-β-actin (AC-15, Sigma). Blots were washed 3x in TBS/0.1%Tween20), incubated in HRP-conjugated secondary Abs (Southern Biotech), and washed again. Bands were visualized by enhanced chemiluminescence (Thermo Fisher) and imaged on a ChemiDoc system (Bio-Rad).

Spot densitometric quantification of phosphor-S6 vs. total S6 was performed using ImageLab software (Bio-Rad).

Hierarchical clustering analysis

Hierarchical Clustering with a complete linkage algorithm and an asymmetric binary distance measure (for binary variables) was used to explore the data contained in Online Repository Table 1. Due primarily to a significant proportion of missing data across many of the variables, only a subset of variables was included in the clustering algorithm. Moreover, only subjects with complete data on the chosen subset were included in the hierarchical clustering. Models were also run using different variable sets, distance measures, and clustering algorithms, and led to different sets of clusters. Hence presented results should be considered exploratory and descriptive.

Unsupervised patient clustering

To depict patients with their phenotypic attributes in 2-dimensional space, we used Gower distance transformation (20) (as implemented in the daisy function in the R cluster v2.0.7-1 package (21, 22), which handles datasets with missing data points, followed by tSNE (23) as implemented in the Rtsne v0.13 library (using settings theta=0 and perplexity=10). Next, we divided the patient cohort into three clusters using K-means clustering in R (K=3). The process of using tSNE and K-means to cluster patients was repeated over 10 trials and consensus assignment was used to assign each patient to a final cluster id. To identify phenotypes important for each cluster, we performed attribute selection and logistic regression in Weka v3.8.2 (24). For attribute selection, we used the Fast Correlation-based Feature Search method (25) to select nonredundant attributes. Using the selected attributes, we performed multinomial logistic regression using the SimpleLogistic function (26) with 5-fold cross-validation to build a classifier for assigning patients to one of the three clusters. The features selected along with their predicted coefficients allowed us to assess the importance of phenotypes for each class separately. Based on 10 randomized trials, the classifier demonstrated an overall accuracy of 88.1% with standard deviation (SD) ±11.7%.

Sensitivity and specificity for the trials were 98.33% (SD: ±8.42%) and 93.90% (SD: ±11.03%), respectively.

Statistics

For JPM50.6 transfections, two-way ANOVAs with Sidak correction were used to compare GFP MFI between WT and mutant CARD11. pS6/total S6 protein densitometric ratios were normalized to WT values and compared by a Wilcoxon signed-rank test. For primary cell assays, experimental and technical replicates were limited by small numbers of patient samples available. For patients in Kindreds 6, 19 and 29, ratios of pS6 gMFI (stimulated/unstimulated) were compared to healthy controls (matched per experiment) using a Kruskal-Wallis test.

Results

Novel dominant negative *CARD11* mutations detected in a broad spectrum of immune disorders

Rare or novel *CARD11* mutations were identified by allergy and primary immunodeficiency referral centers in patients with immune-deficient or dysregulatory phenotypes (Table 1). A total of 48 new patients in 27 families, with 26 different heterozygous germline *CARD11* variants were referred. Salient patient phenotypes, combined with those already reported (7, 9), are summarized in Tables 1-2. These alleles were then evaluated at centers specialized in CARD11 biology and associated diseases and pathways. First, we investigated whether each variant altered T cell receptor (TCR) signaling. Each *CARD11* variant was cloned into an expression construct and transfected in WT Jurkat or CARD11-deficient Jurkat (JPM50.6) T cell lines. As a control, we also tested a variant (p.C150L) identified as a somatic reversion mutant that restored NF-κB signaling in T cells from a CARD11-deficient patient that developed Omenn's syndrome (27). Upon CD3/CD28 stimulation of transfected JPM50.6 cells, 14/26 mutants demonstrated significantly reduced NF-κB activation, indicating loss of function (LOF) (Figure 1A). Two variants (p.P495S, p.R848C) displayed enhanced NF-κB activation only after stimulation; unlike BENTA-associated mutations, these variants did not induce constitutive NF-κB activity. As all variants were heterozygous, we next tested whether each LOF mutant could dominantly interfere with WT CARD11 signaling when co-expressed at 50:50 ratios. Among 14 LOF mutations, dominant negative (DN) activity (defined as significantly reduced NF-κB activation relative to 100% WT expression) was observed for 10 (Figure 1B). Most of these new DN variants were confined to the CARD domain and proximal coiled-coil (CC) domains, which are critical for both CARD11 oligomerization and BCL10-MALT1 interactions (28, 29). All variants were comparably expressed in transfected cells, suggesting none of the mutations tested affected CARD11 protein translation and stability (Figure 1C and data not shown). Although a faint 15 kD band was noted upon prolonged blot exposure, we could not definitively confirm the presence of a truncated K143X protein by immunoblot using available N-terminus specific CARD11 Abs, which cross-reacted with many non-

specific proteins (data not shown). However, the fact that expression of this construct, and not the Q945X truncation mutant (9), exhibits DN activity strongly suggests that it is expressed. Moreover, a K143X CARD11 expression construct engineered to include a C-terminal HA-tag was clearly expressed in JPM50.6 transfectants, and exhibited comparable DN activity compared to the untagged form (Supplemental Figure 1).

CARD11 also affects TCR-induced mTORC1 activation (30, 31). We therefore further tested whether selected *CARD11* variants could disrupt TCR-induced mTORC1 activation by measuring ribosomal S6 protein phosphorylation by immunoblotting. For most mutations within the CARD domain, we observed a trend toward decreased phospho-S6 (Figure 1C-D), similar to previously characterized DN mutants (9). These differences did not reach statistical significance, however, due to inherent variation in our assays. mTORC1 signaling is exquisitely sensitive to subtle perturbations in cell viability, culture media contents and stimulation conditions, particularly in Jurkat T cells (K. Hamilton, personal communication). Interestingly, some mutants (e.g. p.R72G, p.R187P) had no appreciable effect on TCR-induced S6 phosphorylation despite impaired NF-κB activation in Jurkat transfectants (Figure 1C-D). Collectively, these transfection results suggest *bona fide* DN mutations in *CARD11*, often located in the CARD domain, attenuate TCR-induced NF-κB activation with variable effects on mTORC1 signaling (Figure 1E).

Patient phenotypes with dominant negative *CARD11* mutations

We compared a total of 60 patients from 32 kindreds carrying heterozygous *CARD11* variants (Table 1). Including those previously published (7, 9), we identified 28 distinct *CARD11* alleles, of which 14 are DN as determined by the functional assays described above. All kindred pedigrees with confirmed *CARD11* DN variants are shown in Figure 2. All patients were referred with immunological phenotypes for investigation, typically presenting in childhood. We therefore compared the manifestations in those with functional *CARD11* DN defects to those with no obvious DN functional defect, even if hypomorphic (Tables 1-2). Severe atopic dermatitis (AD) was present in most patients with DN mutations (32/44; 73%), compared with non-DN variants (5/16, 31%). Other

atopic symptoms were noted in patients with DN variants, including asthma (55%) and food allergies (32%), and less frequently, rhinitis and eosinophilic esophagitis (Table 1). Cutaneous viral infections were also more common in patients with DN alleles, including molluscum contagiosum (52%), cutaneous herpes simplex virus type 1 (HSV-1) infection (30%) and warts (27%) (Table 1). We also observed cases of neutropenia, presentations similar to other syndromes associated with elevated IgE and infection (Kindred 14), including IPEX-like syndrome (Kindred 4) in patients with functional DN alleles, but not in the others. Of note, the patient with IPEX-like presentation (including failure to thrive, bloody diarrhea, and severe eczema) carried the identical mutation (p.R30W) to that described recently by Roifman and colleagues (Kindred 5) in a family with severe atopy, infection and mild (late onset) autoimmunity (7). Prominent phenotypes in the collective CARD11 DN patient cohort are summarized in Table 2. In addition to atopic disease (89%), significant viral skin infections (68%) and lung disease (e.g. infections, pneumonia, bronchiectasis) (68%) were the most prominent symptoms shared by patients harboring DN *CARD11* mutations (Table 2). Additional phenotypes included autoimmunity (20%)—most commonly alopecia, but also including ITP and bullous pemphigoid. Neutropenia was also observed in five patients (14%, including four with no demonstrable atopic disease), although an autoimmune etiology could not be confirmed. Oral ulcers were also observed (14%), and may be linked to neutropenia. Four patients had lymphoproliferative disease (large granular lymphocytic leukemia (LGL), peripheral T-cell lymphoma, and/or mycosis fungoides). Notably, three patients from two families had little to no atopic disease (one had a mild IgE elevation without clinical manifestations), but did have neutropenia with humoral defects including progressive B-cell lymphopenia, poor class switched B-cell memory, and antibody deficiency. Indeed, low IgM and impaired humoral responses to certain vaccines (e.g. pneumococcal) were observed in several patients. Relevant immunological phenotypes, including total and specific antibody defects and lymphocyte subsets are summarized in Table 3 and Online Repository Table 1.

Hierarchical clustering with a complete linkage algorithm and an asymmetric binary distance measure was used to explore the cohort for specific phenotypic patterns for

patients (n=36) and phenotypes (n=7) for which phenotypic data were more complete. While the small size of the cohort precludes any assessment of statistical significance, descriptively we saw (a) a cluster of neutropenic patients having low IgM and lower IgE, (b) patients with AD and asthma who had low IgM, (c) patients with AD without asthma who had low IgG and low IgA, and (d) patients with high IgA, normal IgM, lack of neutropenia and IgE levels that tracked with the presence of AD (Supplemental Figure 2A). These clusters did not appear to correlate with specific mutations. To be more inclusive of phenotypes with some missing data, we performed an additional analysis using all phenotypes (n=31) and patients (n=44). Data was transformed into a Gower distance matrix and reduced to 2-dimensions using t-Distributed Stochastic Neighbor Embedding (tSNE) before applying K-means clustering to partition the patients into three clusters. Supplemental Figure 2B summarizes phenotypes characteristic of each cluster. Interestingly, cluster 2 was characterized by lack of AD, skin bacterial infections, pneumonia, and allergic rhinitis, but included patients with neutropenia and abnormal IgM and IgA levels. This cluster included all patients with R30G and R974C, most patients with R47H, and the single patient with R75Q. Again, all phenotypic correlations predicted by these clusters are merely descriptive for this limited patient cohort.

The largest family analyzed included ten patients from the United Kingdom (Kindred 14) who presented with autosomal dominant inheritance of symptoms typically associated with DOCK8 deficiency, including elevated serum-IgE levels with significant atopic disease, recurrent respiratory tract infections, skin abscesses, and recurrent or persisting viral skin infections such as molluscum contagiosum, herpes infections or warts (Table 1, Figure 3). Two patients additionally had mycobacterial complications. Surprisingly, mild skeletal/connective tissue abnormalities characteristic for the STAT3-DN autosomal dominant HIES were also observed in six out of ten affected family members. Patient III.4 died from disseminated mycosis fungoides. Immune phenotyping of patient PBMCs from this family revealed low CD8+ T cell numbers and high CD4+ T cell numbers, with a shift towards the naïve compartment and reduction of memory CD4+ T cells. The B cell compartment was normal.

Flow cytometric analyses of other patients, when available, often showed B-cell defects including low total/memory B cells in those with specific antibody deficiency, and low IgM in several patients (Table 3). Other assays performed showed diminished mitogen-induced T-cell proliferation in the majority of patients tested, and relatively normal frequencies of regulatory T-cells, as previously reported (Table 3). Detailed clinical and laboratory findings for individual patients, where available, are included in Online Repository Table 1.

Primary cell signaling defects in CARD11 DN patient lymphocytes

In seven of the patients with confirmed DN mutations, primary cells were available for evaluation of NF-κB signaling using short Phorbol 12-myristate 13-acetate (PMA) activation. Hallmarks of TCR-induced canonical NF-κB activation, including p65 phosphorylation and IκBα degradation, were impaired in all samples tested (Figure 4). Impaired upregulation of activation markers at 24 hours and skewed cytokine secretion was also noted in multiple $CD4^+$ T cell patient samples, consistent with previous findings (Supplemental Figures 3-4). Of note, NF-κB activation in lymphocytes from the patient with the V195L substitution—which was LOF but did not show DN activity in our transfection system—was also abnormal. Because haploinsufficiency does not likely lead to direct DN activity (9), the result suggests either that other lesions explain the cellular and clinical phenotype, or that the effects of this mutation on the pathway are not revealed using our transfection system. The variability in pS6 activation in the primary cell assay precludes making a definitive conclusion regarding its utility, though small but reproducible defects were observed in 4 patients tested from Kindreds 6 and 19 (Figure 5A-B). Moreover, we noted a more reproducible diminution of pS6 could be observed in CARD11 DN patient samples activated for 24 hours (Figure 5C).

Discussion

In this report, we describe multiple new DN *CARD11* mutations associated with additional immune-deficient and dysregulatory conditions in human patients. Recent reports identified DN *CARD11* mutations in a handful of patients presenting with severe atopic dermatitis and other allergic conditions, with or without additional infections (7, 9). The substantially larger cohort assembled here illuminates a broader phenotypic spectrum of disease tied to *CARD11* DN mutations, including frequent sinopulmonary infections, cutaneous viral infections, neutropenia, hypogammaglobulinemia, and lymphoma (Table 2). Atopic disease was the cardinal feature noted in most patients (89%), frequently presenting in childhood as atopic dermatitis, but also including asthma, allergic rhinitis, food allergies, and even eosinophilic esophagitis (Table 2). However, atopy was mild or absent in a number of patients examined in this report, with no measured differences in TCR-driven signaling responses. There were no obvious clinical similarities between non-atopic CARD11 DN patients beyond neutropenia (present in four). It is possible that atopic symptoms improved over time for some older adults for whom detailed clinical history was lacking, similar to previously described patients (9). Furthermore, unrelated patients with the same mutation (e.g. R30Q, R72G) and even family members harboring identical mutations (e.g. p.R47H, p.R187P) demonstrated differences in both the variety and severity of disease symptoms. From our characterization of this expanded patient cohort, we conclude that *CARD11* DN mutations exhibit high penetrance and variable expressivity for several phenotypes that can extend beyond atopy. Other genetic variants or polymorphisms could certainly be influencing the heterogeneity and severity of phenotypes observed in certain patients.

Lack of atopic disease in patients with biallelic *CARD11* null mutations, associated with SCID, is likely due to the relative lack of lymphocyte effector function (32, 33). However, the absence of atopy observed in certain patients described here with *CARD11* DN mutations, all of whom had lived into adulthood without major intervention, is surprising. Interestingly, unsupervised clustering analyses identified a subset of these patients from four kindreds that presented with neutropenia, abnormal Ig levels, and fewer

609 skin/respiratory infections, although the significance of these associations cannot be
610 formally assessed within this limited cohort. We otherwise noted no particular
611 genotype/phenotype correlations, nor were there patterns of other comorbid conditions
612 that suggested substantially different phenotypic manifestations. Phenotypic variation
613 *within* families suggests that other genetic variants, which may not be pathogenic by
614 themselves, or differences in environmental exposures could influence the phenotype.
615 As more *CARD11* variant patients are found, demographic patterns may be possible to
616 establish.
617
618 Non-atopic phenotypes associated with *bona fide* DN mutations shed new light on
619 important biological functions governed by CARD11 signaling in lymphocytes. For
620 example, insufficient (e.g. low IgM) or misdirected humoral responses appear to be a
621 common outcome of attenuated CARD11 signaling, with or without elevated IgE. A
622 number of families presented with more severe humoral defects resembling CVID,
623 which may reflect both intrinsic defects in B cell differentiation and/or poor T cell help.
624 Future studies aimed at elucidating specific abnormalities in class switch recombination
625 and plasma cell differentiation in patient B cells would be helpful. The neutropenia noted
626 in several patients was not associated with obvious bone marrow defects, which could
627 suggest an autoimmune etiology; indeed, CARD11 is primarily expressed in mature
628 lymphocytes. However, given that this is a diagnosis of exclusion, further investigation is
629 warranted.
630
631 Cutaneous viral infections (e.g. molluscum, HSV-1, etc.) were also common to several
632 patients. Impaired CD8+ T cell immunosurveillance could be a factor, and may also help
633 to explain tumor development in certain patients. Interestingly, BENTA patients carrying
634 GOF *CARD11* mutations often present with molluscum and other viral infections (e.g.
635 EBV), and are at greater risk of lymphoma/leukemia development (5). Unlike many
636 CARD11 DN patients, BENTA patient T cells are only mildly “anergic” and proliferate
637 relatively well in response to robust stimuli, even though IL-2 production is often
638 reduced (6, 34). There is also no evidence of Th2-skewing or atopy in BENTA patients
639 described to date, although this has not been investigated thoroughly. Further studies

are needed to elucidate how dysregulated CARD11 variant signaling leads to abnormal CD4+ and CD8+ T cell responses that might predict expressivity of associated phenotypes (e.g. atopy, viral skin infections). Based on our work and the work of others, we suspect alterations in TCR signal strength, metabolic reprogramming, and actin-dependent adhesion and motility could all be contributing factors (13, 35-38).

Our workup of 48 new patients encompassing 25 novel/rare variants further clarifies whether mutations in specific regions of CARD11 are more or less likely to be pathogenic (Figure 6). Notably, missense mutations in the N-terminal CARD domain (aa1-110) are most likely to disrupt NF-κB (and less reliably, mTORC1) signaling, probably by compromising interactions with BCL10 (and by association, MALT1). The N-terminal CC domain (aa130-200) is also a hotspot for pathogenic mutations, although it is still difficult to predict which variants in the CC domain will be LOF or DN; GOF mutations have been identified in the CARD, LATCH, and CC domains (28, 39). In contrast, we have not found DN mutations residing between residues ~200-970, encompassing the C-terminal portion of the CC domain, the flexible linker, and the PDZ and SH3 domains. In fact, two mutations within this region (p.P495S, p.R848C) significantly boosted TCR-induced NF-κB activity in Jurkat cells; however, more work is required to determine if and how this effect contributes to autoimmune manifestations in these patients. Unlike CARD/CC-associated GOF mutations found in BENTA patients, these mutations did not drive constitutive NF-κB activation in the absence of antigen receptor stimulation. The linker itself (aa449-667) contains an array of redundant repressive and activating elements that govern the complex intramolecular regulation of CARD11 (40, 41), making it unlikely to find single point mutations in the linker that affect CARD11 signaling. Finally, one new (p.R974C) and one previously confirmed DN mutation (p.R975W) were located in the guanylate kinase (GUK) domain), although nearby variants in this region showed no effect on TCR-induced NF-kB (p.V983M, p.E1028K, p.D1152N). In the end, LOF and/or DN activity was not detected in all rare variants from patients referred for a variety of phenotypes, including severe atopy. These results highlight the utility of our simple cell transfection assay in addition to the workup of primary patient cells whenever possible—indeed, as highlighted by the

patient with the p.V195L mutation, the observed NF-κB signaling defect detected in primary cells cannot always be ascribed to DN activity. Although defects in mTORC1 signaling are also important, even small perturbations in cell culture conditions make quantification of differences in S6 phosphorylation extremely challenging for both Jurkat and primary T cells. Therefore, we cannot currently recommend pS6 quantification after short term stimulation as a diagnostic assay for CARD11 DN mutations. Our comprehensive allelic series suggests that for CARD11, protein domain architecture might predict functional consequences of specific mutations. However, this too will require broader, more detailed structure-function analyses of the CARD11 protein before predictions could be used for clinical diagnoses.

For clinicians, this study also provides an improved differential diagnosis for immune deficiency and dysregulation linked to *CARD11* mutations. An algorithm detailing our strategy for identifying and diagnosing these patients is depicted in Figure 6. Heterozygous *CARD11* DN mutations may therefore be suspected and sought - either *via* analysis of existing exomes or included in other gene sequencing panels - in patients with histories of atopy (especially atopic dermatitis), often in combination with sinopulmonary bacterial infections or cutaneous viral infections (Figure 6), especially when a dominant family history of any of those phenotypes is present. Failure to thrive, neutropenia, autoimmunity (e.g. alopecia), specific antibody deficiency and/or CVID, and even lymphoma may also be noted. Even in patients without atopy, but with a family history of dominant inheritance of any of the other phenotypes above, it may be worth testing for a heterozygous *CARD11* DN mutation. A simple lab diagnostic test aimed at uncovering a TCR-induced NF-κB signaling defect in primary T cells is strongly recommended, even before sequencing is performed. Although identification of such mutations may not alter patient care currently, continued evaluation and reporting of new *CARD11* variants (perhaps through a registry) will advance our understanding of specific phenotypic associations and hopefully inform future clinical management. Certainly, those with any such phenotypes in whom a rare heterozygous *CARD11* mutation is found even by chance should lead to strong suspicion that it is causal, especially if it resides in the CARD domain. Nevertheless, functional tests such as those described here are imperative for definitive diagnosis.

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840 **Figure Legends**

841

842 **Figure 1. Jurkat T cell transfection screens for LOF/DN activity of new CARD11**
843 **variants. (A)** Quantification of NF-κB-induced GFP reporter activity in CARD11-
844 deficient JPM50.6 T cells transfected with empty vector (EV), wild-type (WT), or mutant
845 CARD11 constructs and subsequently stimulated with anti-CD3/CD28 Abs. Data are
846 mean -/+ SEM for ≥3 separate transfections each. **(B)** Quantification of NF-κB-induced
847 GFP in JPM50.6 cells transfected with equal ratios of WT and mutant CARD11
848 constructs. Data are mean -/+ SEM for ≥3 separate transfections of each LOF variant;
849 dashed boxes in (A-B) indicate SEM for numerous WT transfections. Asterisks denote
850 statistically significant reductions in GFP MFI versus WT (A) or WT+WT CARD11 (B)
851 after stimulation, indicating DN activity (p<0.05). **(C)** Immunoblot for phospho-S6, total
852 S6, and FLAG-CARD11 expression in Jurkat T cells transfected with CARD11
853 constructs -/+ 20 min stimulation with anti-CD3/CD28 Abs. Data are representative of
854 several independent experiments. **(D)** Spot densitometric quantification of phospho-
855 S6/S6 ratio for each mutant, normalized to WT (dashed line = 1). Data are mean -/+ SD
856 for 2-3 experiments for each variant. **(E)** Schematic diagram of CARD11 protein
857 including new DN, LOF, hypermorphic and benign (i.e. no effect) variants. Stars indicate
858 mutations with reduced ph-S6 in (D).

859

860 **Figure 2. Pedigrees for new CARD11 DN variants.** Key indicates healthy (white),
861 affected (symptomatic with confirmed heterozygous DN mutation), and possibly affected
862 (symptomatic, no genotype available). Asterisks denote patients that were definitively
863 genotyped.

864

865 **Figure 3. Clinical presentation of patients with DN CARD11 mutations (A) R30Q**
866 **(Kindred 2) and (B-F) R187P (Kindred 14). (A)** Patient II.1 displaying HSV-1 skin
867 disease. **(B)** Patient IV.6 displaying severe eczema. **(C)** Cutaneous vasculitis (both) and
868 alopecia (left) in patient IV.3. **(D)** Chest radiograph of patient III.5 from 2008 depicting
869 bronchiectasis. **(E)** Abnormal dentition (left) and brachial hypermelanosis (right) in
870 patient IV.8. **(F)** Histology findings in patient III.4 with cutaneous T cell lymphoma

(mycosis fungoides). Left: viable pleomorphic blasts x40; middle: necrotic lymph node replaced by lymphoma low power; right: CD2 staining viable and semiviable cells.

Figure 4. Defective NF-κB activation in primary T cells from CARD11 DN/LOF patients. (A) Primary CD4+ and CD8+ T cells from 3 Kindred 14 patients (III.1, III.2, IV.4) and 2 controls were left unstimulated (gray filled histograms) or stimulated for 15 min with PMA + ionomycin (black histograms). IκB degradation and p65 phosphorylation was detected by intracellular flow cytometry. **(B)** Total PBMC from affected patients, unaffected family members and controls (labeled at right, + standing for WT) were stimulated for 20 min with PMA or left unstimulated (basal: gray histograms). IκB degradation and p65 phosphorylation was detected in gated CD4+ T cells by intracellular flow cytometry. Geometric mean fluorescence intensity (gMFI) values are listed within each histogram.

Figure 5. Defective S6 phosphorylation in primary T cells from affected CARD11 DN patients after acute and prolonged stimulation. (A) Primary CD4+ T cells from affected patients in Kindreds 6, 19 and 29, healthy relatives and controls were not stimulated (NS) or stimulated with PMA for 20 min. S6 phosphorylation was measured by intracellular flow cytometry. **(B)** The ratio of pS6 gMFI for stimulated versus unstimulated cells is plotted for each of 3 experiments represented in (A). Asterisks denote statistical significance relative to controls (Kruskal-Wallis test, $p<0.05$). **(C)** Total PBMC from affected CARD11 DN patients, controls (C) and travel controls (TC) were cultured under Th0 conditions for 24 hours. S6 phosphorylation was measured in CD4+CD45RA- or RA+ cells gated for high vs. low side scatter (SSC) by intracellular flow cytometry; % pS6+ cells are labeled within each histogram.

Figure 6. Clinical diagnostic algorithm for recognizing CARD11 DN patients. Schematic diagram of an algorithm to be used in identifying and diagnosing potential *CARD11* DN patients. Suspected patients typically present with atopy with or without cutaneous viral infections and respiratory infections. Simple lab diagnostic tests to pinpoint TCR-induced NF-κB activation defects in primary patient T cells (e.g. phospho-

p65, IκB degradation) are recommended prior to genomic sequencing analysis, or after a *CARD11* mutation is uncovered (e.g. via WES). Within these selected patients, a mutation in the CARD domain (or N-terminal CC domain) is highly likely to be DN, whereas mutations in the rest of the protein are more likely benign. Functional testing (e.g. Jurkat transfection assays for NF-κB activation) is recommended for all novel CARD11 variants, especially for those outside the CARD domain.

Table 1. Clinical phenotypes for all patients with *CARD11* variants. Kindreds are listed in the order each variant appears within the CARD11 protein.

Table 2. Clinical summary of *CARD11* DN patients. Summary table of mean age, gender ratio, and relevant phenotypes shared by *CARD11* DN patients, with the % of affected patients shown for each category.

Table 3. Immunological phenotypes for patients with *CARD11* DN variants. Descriptors are based on institution-specific reference ranges for each parameter (see Online Repository Table 1). Absent = no cells detected, Low = >10% below reference range minimum, Borderline low = within 10% of reference minimum, High = >10% above reference range maximum. Numbers in parentheses = number of patients with the observed defect / number of patients tested within each kindred.

Kindred	Mutation	Genotype	Functional defect	# patients	Atopic dermatitis	Asthma	Food allergy	Pneumonia	Bronchiectasis	Molluscum contagiosum	Cutaneous HSV	Warts	Other Phenotypes
1	R30G	c.88C>G	DN	6	1	3	0	1	0	2	0	0	bacterial sinusitis/meningitis, visceral leishmaniasis, severe vernal keratoconjunctivitis, florid EoE, severe oral ulcers
2	R30Q	c.89G>A	DN	1	0	0	1	0	0	0	1	0	bullous pemphigoid
3	R30Q	c.89G>A	DN	2	2	1	2	2	0	2	0	2	viral pneumonia, S. aureus skin infection, flu, constipation
4	R30W	c.88C>T	DN	1	1	0	0	0	0	0	0	0	IPEX-like, FTT
5	R30W	c.88C>T	DN	4	3	4	4	3	1	0	0	0	recurrent respiratory infections, oral candidiasis, lichen sclerosis of vulva, psoriasis, impetigo
6	R47H	c.140G>A	DN	3	1	1	0	0	0	3	3	3	neutropenia, LGL
7	R47H	c.140G>A	DN	1	1	1	0	1	0	1	0	1	progressive hypogammaglobulinemia
8	E57D	c.171A>C	DN	2	2	2	1	2	0	1	0	0	ulcerative colitis, stroke, peripheral T lymphoma
9	R72G	c.214C>G	DN	2	2	2	0	1	1	0	0	1	alopecia, joint pain, oral ulcers, pulm TB
10	R72G	c.214C>G	DN	1	1	1	0	1	0	1	0	0	persistent skin infections (VZV, HPV), EBV viremia, progressive B cell lymphopenia, frequent OM
11	R75Q	c.224G>A	DN	1	0	0	0	0	0	1	0	0	neutropenia
12	L92W	c.275T>G	DN	1	1	0	0	0	0	0	1	0	
13	K143X	c.427A>T	Weak DN	1	1	0	0	0	0	0	0	0	ITP, alopecia, EoE
14	R187P	c.560G>C	DN	10	10	5	4	4	2	8	6	5	alopecia, cutaneous vasculitis, mycosis fungoides, broad nose, retained teeth, shingles
15	dup183-196	c.701_713insT	DN	3	3	1	0	3	1	2	1	0	prom forehead, broad nose, poor dentition, pulm TB, eosinophilic coloproctitis
16	L194P	c.581T>C	DN	1	1	1	1	0	0	1	0	0	
17	V195L	c.583G>C	LOF	1	1	1	0	0	0	0	0	0	diverticulitis, T2D
18	K362E	c.1084A>G	LOF	1	0	0	0	0	0	0	0	0	healthy
19	R408H	c.1223G>A	LOF	1	0	0	0	1	0	0	0	0	Evan's syndrome, anhidrosis
20	P495S	c.C>T	Hypermorphic w/ TCR stim	1	0	0	0	1	0	0	0	0	IPEX-like, FTT, T1D, alopecia, skin tags
21	I544L	c.1630A>C	Nil	1	1	0	0	0	0	0	0	0	atopic dermatitis
22	R608H	c.1823G>A	Nil	1	1	0	0	0	0	0	0	0	CMV myocarditis, adenoviral hepatitis
23	V659M	c.1975G>A	Nil	1	0	0	0	1	0	0	0	0	recurrent sinopulmonary/skin infections
24	T670M	c.2009C>T	Nil	1	0	0	0	0	0	0	0	0	Seizures, mental retardation, TB, candidiasis, facial dysmorphism
25	E766D	c2298G>T	Nil	1	1	0	0	0	0	0	0	0	severe atopic dermatitis
26	R848C	c.2542C>T	Hypermorphic w/ TCR stim	2	1	1	0	1	0	0	0	0	AHA, ITP, refractory cytopenias, drug-induced lupus, Crohn's disease
27	R912Q/D1152N	c.2735G>A, c.3454G>A	Nil	1	0	0	0	1	0	0	0	0	late onset recurrent sinopulmonary infections
28	S923L	c.2768C>T	LOF	1	0	0	0	1	0	0	0	0	agammaglobulinemia, giardiasis
29	R974C	c.2920C>T	Weak DN	2	0	1	0	1	0	1	0	0	neutropenia, mycobacterial disease
30	R975W	c.2923C>T	DN	2	2	1	1	0	0	0	1	0	
31	V983M	c.2947G>A	Nil	2	1	1	1	1	0	0	1	0	
32	E1028K	c.3082G>A	Nil	1	0	0	0	0	0	0	0	0	sJIA, MAS, pheochromocytoma, migraines
		DN	**13**	**44**	**32**	**24**	**14**	**19**	**5**	**23**	**13**	**12**	
		%			**73%**	**55%**	**32%**	**43%**	**11%**	**52%**	**30%**	**27%**	
		Non-DN	**15**	**16**	**6**	**3**	**1**	**7**	**0**	**0**	**1**	**0**	
		%			**38%**	**19%**	**6%**	**44%**	**0%**	**0%**	**6%**	**0%**	
		TOTAL	**28**	**60**	**38**	**27**	**15**	**26**	**5**	**23**	**14**	**12**	

Table 1. Clinical phenotypes for all patients with *CARD11* variants.

CARD11 DN Patients	
Age (mean -/+ SD)	23.3 -/+ 19.5
Age of disease onset (mean -/+ SD)	5.2 -/+ 6.7
% Female	50%

Clinical Phenotype	% Patients Affected
Atopic disease	89%
Atopic dermatitis	73%
Asthma	55%
Food allergies	32%
Eosinophilic esophagitis	7%
Cutaneous viral infections	68%
Respiratory infections	68%
Autoimmunity	20%
Neutropenia	14%
Oral ulcers	14%
Hypogammaglobulinemia	11%
Lymphoma	7%

Table 2. Clinical summary of *CARD11* DN patients.

Kindred	Mutation	# patients	T cell prolif defect	Spec Ab response defect	Total Ab defect	Total CD4	Memory CD4	Total CD8	Memory CD8	Total B cells	Low CS/Mem B cells	NK cells	Tregs	IgE	Eosinophils
1	R30G	6	no	yes (2/6)	no	normal	ND	normal	ND	normal	normal	normal	ND	high (3/6)	high (2/6)
2	R30Q	1	yes	yes	yes (low IgM)	normal	normal	normal	normal	borderline low	ND	borderline low	ND	high	high
3	R30Q	2	yes (2/2)	yes (2/2)	yes (2/2)	normal	normal	normal	normal	normal	low	normal	ND	high (2/2)	high (1/2)
4	R30W	1	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown	low	high	ND
5	R30W	4	yes (4/4)	yes (2/4)	yes (2/4)	normal	ND	normal	ND	low (1/4)	ND	normal	ND	high	high (3/4)
6	R47H	3	yes (3/3)	yes (2/2)	yes (1/3)	normal	low (3/3)	normal	low (3/3)	normal	low (3/3)	normal	normal	high 1/3	normal
7	R47H	1	yes	yes	yes	normal	low	normal	ND	low	low	normal	normal	high	normal
8	E57D	2	yes (1/2)	unknown	yes (1/1)	normal	ND	normal	ND	normal	normal	low (1/2)	normal	high	high
9	R72G	2	yes (1/2)	yes (2/2)	no	normal	ND	normal	ND	low (1/2)	absent	normal	ND	ND	ND
10	R72G	1	no	yes	no	high	normal	high	normal	low	normal	normal	normal	high	high
11	R75Q	1	yes	yes	no	normal	normal	normal	normal (high TEMRA)	low	low	low	low	normal	normal
12	L92W	1	ND	no	yes	normal	normal	normal	normal	normal	normal	normal	normal	high	ND
13	K143X	1	ND	yes	yes	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
14	R187P	10	ND	yes (2/10)	yes (1/10)	high (3/3)	low (3/3)	low (1/3)	ND	low (1/3)	low (1/3)	low (3/4)	normal (0/3)	high (8/10)	high (8/10)
15	dup183-196	3	yes (3/3)	no	no	normal	ND	normal	ND	low (1/3)	ND	normal	normal	high	high
16	L194P	1	yes	yes	no	normal	borderline low	normal	borderline low	normal	normal	normal	normal	high	high
29	R974C	2	no	yes (2/2)	IgA (1/2)	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
30	R975W	2	yes (1/2)	no	no	normal	borderline low (1/2)	normal	normal	normal	normal	borderline low	normal	high	high
	TOTAL	44	61% (19/31)	49% (20/41)	29% (12/42)										

Table 3. Immunological phenotypes for patients with *CARD11* DN variants.

CARD11 Variants: Clinical Information

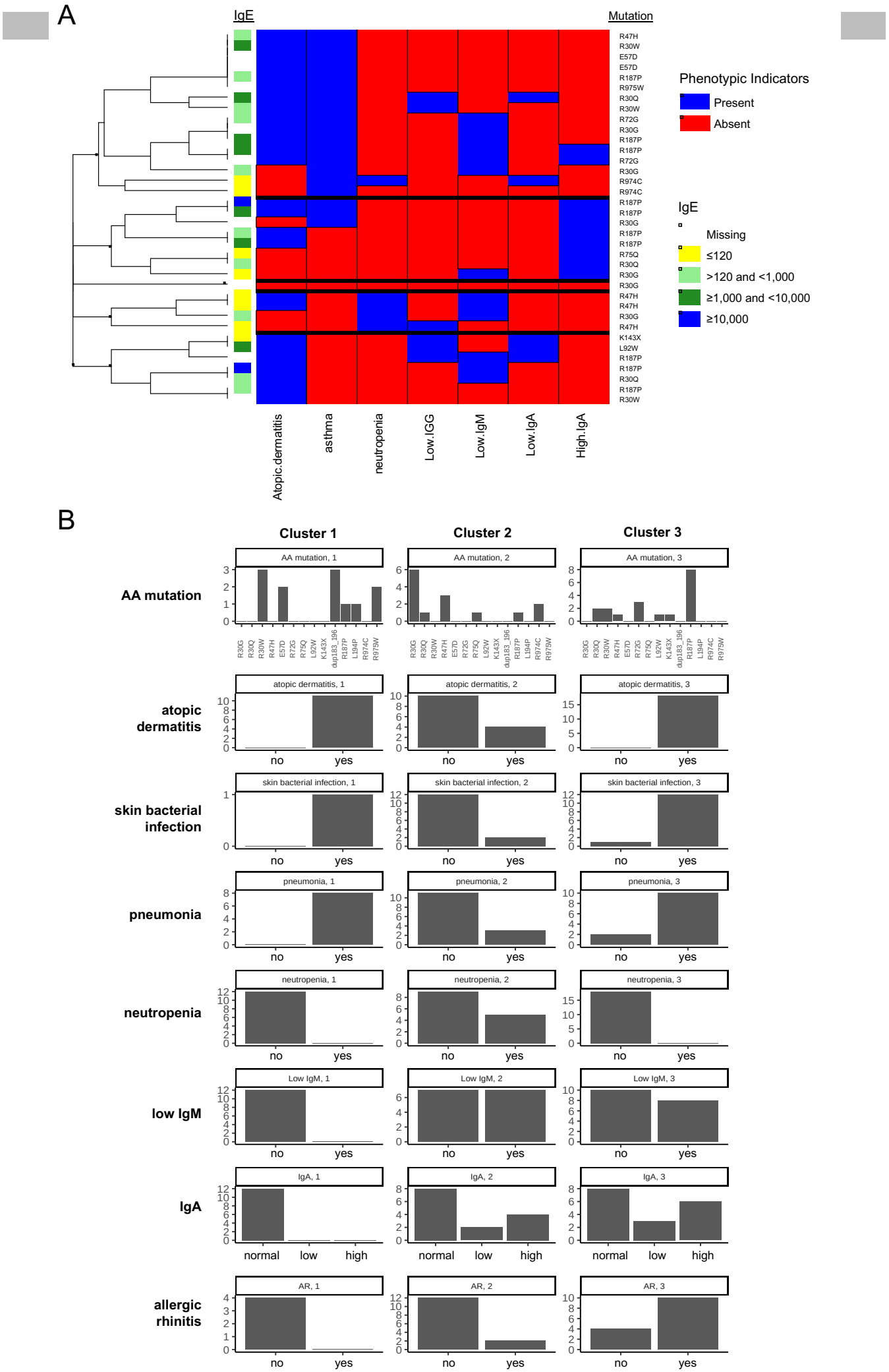
AA Mutation	Nucleotide Mutation	ExAC MAF	Sequencing Method	Age	Sex	Patient Phenotype	Age of Onset	atopic dermatitis	erythroderma	asthma	AR	food allergy	other
Confirmed LOF/DN Mutations													
R30G (II.1)	c.88C>G	0	WES	49	M	sinusitis, sibling died at age 9 with eczema/asthma	14 yo	no	no	no	no	no	
R30G (II.2)	c.88C>G	0	WES	45	F	mild asthma	7 yo	no	no	yes	yes	no	
R30G (III.1)	c.88C>G	0	WES	14	F	Staph meningitis, sepsis, intermittent neutropenia, visceral leishmaniasis	3 mo	no	no	no	no	no	
R30G (III.2)	c.88C>G	0	WES	10	F	Severe atopic dermatitis	10 mo	yes	no	yes	yes	no	
R30G (III.3)	c.88C>G	0	WES	15	M	Hay fever	10 yo	no	no	yes	no	no	
R30G (III.4)	c.88C>G	0	WES	11	M	Pn meningitis, recurrent tonsillitis, florid EoE, diarrhea	4 mo	no	no	no	no	no	EoE, severe persistent lip swelling and mouth ulcers
R30Q	c.89G>A	0	Invitae panel	5	M	5 yo Hispanic male, severe recurrent HSV	6 mo	no		no	no	yes (multiple IgE titers to food, no history of reactions)	biopsy-proven EoE
R30Q (I.1)	c.89G>A	0	WES	34	M	severe AD in childhood (now mild), atopic (asthma, rhinitis and food allergies), pneumonia x3, recurrent chest infections from childhood	in childhood	yes	no	yes	yes	yes	
R30Q (II.1)	c.89G>A	0	WES	2	F	severe AD, hypogam, viral pneumonia, disseminated molluscum	<6 mo	yes	no	no	no	yes	
R30W	chr7:2,987,341	0		1	M	Infant with bloody diarrhea, severe eczema, FTT, and elevated IgE levels		yes	no	no	no		
R30W	c.88C>T	0	WES	31	F	see Dadi et al (2018) *JACI* 141: 181-30.		yes	no	yes	yes		
R30W	c.88C>T	0	WES	4	M	see Dadi et al (2018) *JACI* 141: 181-30.		yes	no	yes			
R30W	c.88C>T	0	WES	4	F	see Dadi et al (2018) *JACI* 141: 181-30.		yes	no			yes	
R30W	c.88C>T	0	WES	37	F	see Dadi et al (2018) *JACI* 141: 181-30.		?	no	yes			
R47H (II.2)	c.140G>A	0.00000823	WES	38	F	Hypogammaglobulinemia, neutropenia, (indolent) LGL	20	no	no	no	no	no	
R47H (III.2)	c.140G>A	0.00000823	WES	7	M	Cutaneous viral infection, neutropenia	2	yes	no	no	no	no	
R47H (III.1)	c.140G>A	0.00000823	WES	3	F	Cutaneous viral infection, neutropenia	1 year	yes	no	no	no	no	
R47H	c.140G>A	0.00000823	Invitae panel	7	F	Recurrent sinopulmonary infections, severe diffuse molluscum, progressive hypogammaglobulinemia, absent vaccine responses, absent lymphocyte proliferation to antigen stim	1 yo (recurrent sinopulmonary infections, severe molluscum)	yes	no	yes	yes	not clinically allergic but low + to egg	
E57D (C-II.1)	CHR7:2987258 T>G		WES, Sanger	51	M	severe AD, pneumonia	childhood	yes	no	yes	no		
E57D (C-I)	CHR7:2987258 T>G	0	WES, Sanger	16	M	Severe AD, asthma, food allergy, molluscum, resp distress	childhood	yes	no	yes	no	yes	
R72G (I.1)	c.C214G	0	WES	53	M	CVID, atopic dermatitis	1 yo (eczema)	yes	no	yes	yes	no	
R72G (II.1)	c.C214G	0	WES	18	F	CVID, atopic dermatitis		yes	no	yes			
R72G	c.C214G	0	WGS (100,000 Genome Project)	20	F	Undefined CID presented to clinic at 15 yo. Early onset eczema/asthma, recurrent bacterial sinopulmonary infections, severe recurrent molluscum, persistent cutaneous HPV, EBV viremia	~6 mo (otitis media, atopic dermatitis)	yes	no	yes	yes	no	
R75Q	c.224G>A	0	WES	18	M	severe neutropenia	14 yo	no	no	no	no	no	no
L92W	c.T275G	0	WES	10	M	severe atopic dermatitis, elevated IgE, hypogam, recurrent infections	3 mo (severe eczema), 11 mo (hypogammaglobulinemia)	yes (severe)	no	no		yes	
K143X	c.427A>T	0	WES	4	F	ITP, hypogam, allopecia, and eosinophilic esophagitis	early childhood	yes	no	no	no	no	EoE
dup183_196 (D-II.2)	c.588_589ins42bp	0	WES	15	M	severe AD, pneumonia, molluscum, prom forehead, broad nose, poor dentition		yes	yes				
dup183_196 (D-II.1)	c.588_589ins42bp	0	WES	21	M	severe AD, pneumonia, molluscum, prom forehead, broad nose, poor dentition		yes	yes				
dup183_196 (D-I)	c.588_589ins42bp	0	WES	1.5	M	severe AD, pneumonia, molluscum, prom forehead, broad nose, poor dentition		yes (severe)	yes				
R187P (II.3)	g.2983970C>G	0	Sanger	77	F	no definitive diagnosis	early childhood	severe	yes	yes, mild	no	yes, fish	
R187P (III.1)	g.2983970C>G	0	WES	63	F	bronchiectasis, recurrent cellulitis, eczema	childhood	severe	yes	yes	yes	yes, oranges	allergic rhinoconj
R187P (III.2)	g.2983970C>G	0	WES	57	F	no definitive diagnosis	18 yo	yes	yes	yes	yes	unknown	allergic rhinitis
R187P (III.4)	g.2983970C>G	0	WES	46	M	no definitive diagnosis	13 yo	severe	no	no	no	no	
R187P (III.5)	g.2983970C>G	0	WES	50	M	no definitive diagnosis	childhood	severe	no	no	yes	no	allergic rhinitis
R187P (IV.3)	g.2983970C>G	0	WES	33	F	no definitive diagnosis	childhood	yes	yes	yes	yes	yes	allergic rhinitis
R187P (IV.4)	g.2983970C>G	0	WES	20	M	eczema, food allergies, recurrent molluscum	<3 yo (eczema)	yes	no	yes	yes	yes	allergic rhinitis
R187P (IV.6)	g.2983970C>G	0	WES	31	M	no definitive diagnosis	3 yo	severe	no	no	no	no	
R187P (IV.8)	g.2983970C>G	0	Sanger	20	F	no definitive diagnosis	birth	severe	no	no	no	no	
R187P (V.2)	g.2983970C>G	0	Sanger	2	F	no definitive diagnosis	childhood	mild	no	no	no	no	
L194P		0		16	M	severe AD, recurrent respiratory distress, diffuse molluscum		severe		yes			
R974C (II.2)	g.130560C>T	0.000376	WES	30	F	Cervical lymph node mycobacterial infection (childhood)	childhood	no	no	yes	no	no	
R974C (III.1) daughter	g.130560C>T	0.000376	WES	1	F	Neutropenia, viral skin infections, impaired vaccine responses, IgAD	childhood	no	no	yes	no	no	
R975W (B-II.2)	c.2923C>T	0	WES	28	F	grew out of mild eczema	childhood	mild eczema					
R975W (B-I)	c.2923C>T	0	WES	6	M	Severe AD, asthma, food allergy, eczema herpeticum	childhood	severe atopic dermatitis.		yes			
Additional Mutations		**BOLD = LOF only**											
C150L	c.449G>T	0	Sanger	1	F	Omenn's - reversion from C150X	3 mo						
V195L	c.583G>C	0.0002142	ion torrent	52	M	atopy, diverticulitis complicated by perforation, with hemicolectomy complicated by wound dehisence	infancy (eczema)	yes	no	yes	yes	yes, shellfish	
K362E	chr7:2977600 T>C	0	WES	30	F	healthy		no					
R408H (II.4)	g.106791G>A	0.00000823	WES	35		CVID, Evan's syndrome, anhidrosis, hypogammaglobulinemia	childhood	no					
P495S	chr7:2974122	0.000008488		1	M	Infant with watery diarrhea, villous atrophy, mild eczema, T1 Diabetes, FTT, alopecia arreata, skin tags, club foot.	childhood						
I544L	HGVS.C c.1630A>C	.00219 (dbSNP)				Severe atopic dermatitis		severe AD					
R608H	c.G1823A	0	WES	8	M	CMV myocarditis, adenoviral hepatitis, carried by unaffected mother		mild eczema	no	no	no	no	no
V659M	c.1975 G>A	.0002417 (dbSNP)				Late-onset (40's) with recurrent sinopulmonary infections, life-long severe dermatitis, recurrent skin infections.		yes	no	no	no	no	no
T670M	NM_032415:exon16:c.C2009T	0.00008539 (dbSNP)	WES	11	M	Seizures, mental retardation, tuberculosis, candidiasis, and facial dysmorphism		no	no	no	no	no	no
E766D	c.2298G>T	0.0004454	Ion Torrent			Severe atopic dermatitis							
R848C	c.2542 C>T	0.00005133	WES	16	M	AHA, ITP, refractory cytopenias, psoriasis/skin lesions/eczema, eosinophilia (mother carries same mutation - drug-induced lupus, Crohn's)	11 yo	yes	no	no	no	no	
R912Q	ENST00000396946.4:c.27	0.00003319	Ion Torrent	29	F	Compund het: 29 y AA Female, Healthy until her mid 20s with onset of recurrent bacterial sinopulmonary infections	early 20s	Medication allergy to amoxicillin (urticaria) and		no		no	antibiotic allergies
D1152N	ENST00000396946.4:c.34	0.0007528 (dbSNP)											
S923L	c.C2768T	0.00004143	WES	15	F	Agammaglobulinemia or CVID, no switched memory B cells		no	no	no	no	no	no
V983M	HGVS.C c.2947G>A	0.0001172			M	atopic son, unaffected father		eczema, airborne and food allergy					
E1028K	c.G3082A	0.00002488	WES	9	M	Systemic JIA/macrophage activation syndrome		no	no	no	no	no	no

AA Mutation	infection - respiratory	pneumonia	bronchiectasis	skin abscess	skin bacterial superinfection/cellulitis	Molluscum contagiosum	Herpesvirus	Warts	infection-other	autoimmunity	connective tissue	dentition abnormalities	scoliosis	oral ulcers	lymphoma	other
Confirmed LOF/DN Mutations																
R30G (II.1)	ND	no	no	no	no	no	no	no	no	no	no	no	no	no	no	no
R30G (II.2)	ND	no	no	no	no	no	no	no	no	no	no	no	no	no	no	no
R30G (III.1)	no	no	no	no	no	yes (dissemintated)	no	no	Staph meningitis, bacterial sepsis after chicken pox, visceral leishmaniasis	no	no	no	no	no	no	intermittent neutropenia
R30G (III.2)	no	no	no	no	no	no	no	no	no	no	no	no	no	no	no	chronic green nasal discharge
R30G (III.3)	no	no	no	no	no	no	no	no	no	no	no	no	no	no	no	hay fever
R30G (III.4)	yes	yes age 2	no	no	no	yes (extensive)	no	no	Pn meningitis, recurrent tonsillitis, Camph diarrhea	no	no	no	no	yes	no	florid EoE, chronic inflammatory mouth ulcers & lip swelling
R30Q	Surface cultures pos for MRSA at least twice; Blood cultures have never been positive.	no	no	no	no	no	yes (multiple recurrences of cutaneous HSV)	no	no	Bullous pemphigoid (vs severe cutaneous HSV?)	no	no	no	yes (HSV-1)	no	no
R30Q (I.1)	yes	yes (x3, hospitalized)				unknown	yes (primary VZV)			no	no	no	no	no	no	
R30Q (II.1)	yes	yes (viral			yes (Staph aureus)	yes (dissemintated)			eczema herpeticum, influenza A	no	no	no	no	no	no	constipation
R30W										bloody diarrhea, FTT						
R30W	yes	yes	yes						oral candidiasis	granulomatous skin inflammation, lichen sclerosis, psoriasis						
R30W	recurrent otitis	yes														
R30W										lichen sclerosis						
R30W	yes	yes												?		
R47H (II.2)	Pneumonia	yes	no	no	no	no	yes (cutaneous HSV)	yes		no	no	no	no	no	yes	(indolent) LGL, neutropenia
R47H (III.2)	URTI	no	no	no	no	yes	yes (cutaneous HSV)	Yes		no	no	no	no	no	no	neutropenia
R47H (III.1)	URTI	no	no	no	no	yes	yes (cutaneous HSV)	Yes		no	no	no	no	no	no	neutropenia
R47H	recurrent OM and sinusitis	yes with strep pneumo bacteremia		yes	yes, staph	yes, severe in early childhood, now resolved		yes		no	no	no	no	no	no	
E57D (C-II.1)	pneumonia	yes													yes	T-cell lymphoma
E57D (C-I)	pneumonia	yes								ulcerative colitis						
R72G (I.1)	Recurrent upper respiratory tract infections , OM	yes	yes	no	yes (S. aureus, cellulitis)	no	no	no	pulmonary TB	alopecia	no	no	no	yes	no	recurrent oral ulcers, joint pain
R72G (II.1)	recurrent respiratory tract infection, sinusitis.		no					yes		alopecia	no			yes		recurrent oral ulcers, migraine, joint pain
R72G	yes (recurrent upper/lower resp tract infections - Strep pneumoniae,Hib)	yes		no	no	yes (severe)	yes (prolonged VZV infection at age 6)	yes	persistent cutaneous HPV infection affecting right upper labia)	no	no	no	no	no	no	EBV viremia, fracture of 5th metartarsal after minor trauma- patient has been on multiple steroid courses
R75Q	no	no	no	no	yes (MRSA & Staph aureus infection)	yes	no	no	no	no	no	no	no	yes	no	
L92W	yes				bacterial skin infection (Staph MS).	no	Eczema herpeticum X3, recurrent herpes keratitis			no						
K143X	sinusitis, strep throat	no	no	no	no	no	no	no	no	alopecia, ITP	no	no	no	no	no	no
dup183_196 (D-II.2)	yes	yes		yes		yes	kaposi varicelliform			chronic diarrhea	prominent forehead, broad nose					
dup183_196 (D-II.1)	yes	yes	yes	yes		yes					broad nose	very poor dentition				
dup183_196 (D-I)	yes	yes							CMV pneumonitis		prominent forehead, broad nose	very poor dentition				
R187P (II.3)	no since 2010	2-3 /year, moderate (Haemophilus	atelectasis, mild	no	yes (MRSA & Staph aureus infection)	yes, severe, persistent since	Yes, HSV -1, shingles	yes	no	alopecia, hypothyroid	mild broad nasal bridge	no	no			
R187P (III.1)	yes	recurrent pseudomonas/ H. infl. Cultured	bronchiectasis with infective exacerbations	occasional superficia	yes	no	yes, shingels	yes	pulmonary tuberculosis	alopecia	no	no	yes			
R187P (III.2)	unknown	unknown	bronchiectasis	unknown	yes	yes	unknown	unknown	fingernail CMC	alopecia	unknown	unknown	unknown			
R187P (III.4)	no	yes	no	no	yes	yes, severe	yes (head, neck, limbs)	no	nodule thigh with atypical		no	yes, retained primary teeth	no		yes	(mycosis fungoides), disseminated to
R187P (III.5)	no	yes	no	no	yes	yes, severe	yes, shingles	yes	no		no	yes, retained primary teeth	no			
R187P (IV.3)	yes (sinusiti,otitis)	unknown	unknown	unknown	yes	yes	eczema herpeticum	yes	unknown	alopecia, cutaneous vasculitis	unknown	unknown	unknown			
R187P (IV.4)	no	no	no	no	yes	yes, persistent	no	no	no		no	no	no			
R187P (IV.6)	Otitis, chronic sinusitis	yes	no	one- two	yes	yes, severe, for 12 months when aged	yes, shingles	yes	no		mild (2013)	yes, retained primary teeth	no			
R187P (IV.8)	otitis >4/year	no	no	1 (staph. aureus)	yes	yes, severe in early childhood,	no	no	no		mild (2007), broad nasal bridge, slightly prominent	yes, brittle, notched incisors	no			
R187P (V.2)	yes	no	no	no	yes	no	no	no	no		no	unknown	no			
L194P						yes										
R974C (II.2)	no	no	no	no	no	no	no	no	mycobacterial disease in	no	no	no	no	no	no	no
R974C (III.1) daughter	Pneumonia	yes	no	no	no	yes	no	no	no	no	no	no	no	no	no	neutropenia
R975W (B-II.2)																
R975W (B-I)							eczema herpeticum									
Additional Mutations																
C150L																
V195L	sinusitis and bronchitis Episodes of sepsis complicating hemicolectomy for colon perforation Recurrent C diff	no	no	no	yes	Not increased	no	no	no	No	Poor wound healing after surgery		no		no	Type II Diabetes Colon Diverticuli Mother with history of Ehler Danlos
K362E	no					no				no	no					no
R408H (II.4)	Pneumonia		yes			no				ITP+AIHA (Evan's syndrome)						Anhidrosis
P495S																
I544L	none					none				none	none					none
R608H	no	no	no	no	no	CMV myocarditis, adenoviral hepatitis	no	no	no	no	no	no	no	no	no	no
V659M																
T670M	no	no	no	no	no	no	no	no	no	no	no	no	no	no	no	no
E766D																
R848C	Pneumonia, Cellulitisx1 (MRSA), abcessx1	yes (1x)	no	yes	yes	HSV stomatitis on CellCept	HSV stomatitis on CellCept	no	sinusitis	Evan's Syndrome, immune neutropenia	psoriasis	no	no	no	no	Behavioral problems, headaches and leptomeningeal enhacement
R912Q / D1152N	Recurrrent sinopulmolnary; *Streptococcus pneumoniae*	yes	no	no	yes	no	no	no		no	no		no		no	
S923L	pneumococcal pneumonia					CMV pneumonia				none	none					giardiasis
V983M	recurrent pneumonia, staph , strep, infection						recurrent HSV infection			none	none					
E1028K	MRSA bullous impetigo					none				systemic JIA with episode of MAS	none					history of pheochromocytoma, migraines

AA Mutation	laboratory abnormalities	CD4 memory	CD4 naïve	CD8 memory	CD8 naïve	Total CD19+	CD19+ 27+ IgM+ (% B cells)	CD19+ 27+ IgM- (% B cells)	CD19 27- (% B cells)	NK	tetanus response	pneumococcal response	IgG/A/M/E	G	M	A
Confirmed LOF/DN Mutations																
R30G (II.1)		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		ND	ND	ND
R30G (II.2)		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	elevated IgA	12.0 g/L (5.4-16.1)	2.0 g/L (0.5-2)	5.0 g/L (0.8-2.8)
R30G (III.1)	reverse CD4/CD8 ratio	normal	normal	normal	normal	normal	87%	2%	6%	normal	normal	poor	low IgM	11.8 g/L (5.4-16.1)	0.33 g/L (0.5-2)	1.46 g/L (0.8-2.8)
R30G (III.2)	subsets normal	ND	ND	ND	ND	normal	100%	0%	0%	normal	poor	poor	low IgM	10.1 g/L (5.4-16.1)	0.17 g/L (0.5-2)	2.3 g/L (0.8-2.8)
R30G (III.3)	subsets normal	ND	ND	ND	ND	normal	ND	ND	ND	normal	ND	ND	low IgM	5.64 g/L (5.4-16.1)	0.27 g/L (0.5-2)	2.11 g/L (0.8-2.8)
R30G (III.4)	subsets normal	ND	ND	ND	ND	normal	83%	2%	4%	normal	normal	poor	low IgM, high IgA	7.74 g/L (5.4-16.1)	0.10 g/L (0.5-2)	3.74 g/L (0.8-2.8)
R30Q	Elevated IgA and IgE, IgM now trending low, normal IgG, absent responses to pneumovax x 2	Normal at Cincinnati Childrens March 2016	Normal at Cincinnati Childrens March 2016	Normal at Cincinnati Childrens March 2016	Normal at Cincinnati Childrens March 2016	borderline low	ND	ND	ND	borderline low	low positive response (0.33)	virtually absent x 2	Normal IgG, Normal to elevated IgA, Normal trending low IgM, High IgE	5.30 (3.91-10.47) (performed at 29 mo prior to IVIG)	0.31 (0.49-2.02) (performed at 29 mo)	1.00 (0.15-0.95) (performed at 29 mo)
R30Q (I.1)	low IgM, low class switched memory B cells, increased CD4 naive, reduced PHA, but normal anti-CD3, high IgE	178 (normal)	712 (normal)	83 (normal)	165 (normal)	125 (100-500)	4% (low)	4%(low)	90% (High)	100 (90-600)	pre-scIg, present, 0.166 IU/ml(Ref <0.01 inadequate, 0.01-0.15 suboptimal, >0.15 adequate)	<3.3 micrg/ml (Ref <10 inadequat, 10-30 suboptimal, >30 adequate	Pre-scIg results (aged 18m): IgG 2.2 g/L, IgA 0.35, IgM 0.09, IgE 408 (IgE rising to > 2327 IU/L over past 6m; IgM remains low)	2.2 g/L (6-16)	0.09 g/L (0.5-2)	0.35 g/L (0.8-4)
R30Q (II.1)	Profound hypogammaglobulinaemia, low class switched memory B cells, persistent eosinophilia	1475 (normal)	1312 (normal)	296 (normal)	1049 (normal)	1500 (600-3100)	6% (low)	<1% (low)	92% (previous measurements 98,99%)	521 (100-1400)	0.091 IU/ml (Ref <0.01 inadequate, 0.01-0.15 suboptimal, >0.15 adequate)	9.5 micrg/ml (Ref <10 inadequat, 10-30 suboptimal, >30 adequate)	IgG 9.12 g/l (ref 6-16), IgA 3.22 g/l (ref 0.8-4), IgM 0.29 g/l (ref 0.5-2), IgE 554 iu/l (ref	9.12 g/L (6-16)	0.29 (0.5-2)	3.22 (0.8-4)
R30W													high IgE			
R30W	low PHA, anti-CD3										.01	absent		4.7	1.5	3.9
R30W	low PHA, anti-CD3					LOW					.37	absent		10.7	.5	3.5
R30W	low PHA, anti-CD3										.22	nd		5.6	.3	2
R30W	low PHA, anti-CD3										.27	nd		6.3	.9	1.7
R47H (II.2)	reduced memory B cells / plasmablasts	normal	normal	normal	normal	normal	3%	2%	94%	normal	0.04	0		5.2 (7-16)	1.4 (0.4-2.3)	1.7 (0.7-4.0)
R47H (III.2)	reduced memory B cells / plasmablasts	normal	normal	normal	normal	normal	2%	1%	95%	normal	0.16	poor		6.35 (5-13)	0.2 (0.4-1.8)	1.4 (0.48-3.45)
R47H (III.1)	reduced memory B cells / plasmablasts	normal	normal	normal	normal	normal	2%	1%	94%	normal	0	0		3.9 (3.5-10)	0.1 (0.4-1.4)	0.45 (0.19-2.2)
R47H	low memory B cells	ND	ND (total CD4 = 1.59x10^9//L (1.17-3.52)	ND	ND (total CD8=0.79x10^9/L (0.92-2.76)	0.26x10^9/L (0.76-2.28)	3 (>2.9%)	1 (>5.9%)	ND	0.06x10^9/L (0.13-1.01)	absent	absent despite multiple pneumovax	2016 G 1080, A 108, M 17, E 165 on IVIG	1080 mg/dL (468-1233)	17.6 mg/dL (26.8-147.4)	108 mg/dL (23.2-116.2)
E57D (C-II.1)																
E57D (C-I)	high IgE, AEC, decreased T prolif															
R72G (I.1)	Father: CD4 927 (53%), CD8 619 (35%), CD19 29 (2%), CD16/56 78 (5%)		403		14	29	2.2 (7.4%)	0 (0%)	26.5 (91.3%)	78	Father: pre - 0.05, post - 0.43	Father: pre serotypes >1.35 = 1/12, post serotypes >1.35 = 1/12	Father: IgG 4.92, IgA 3.22, IgM 0.33, IgA	4.92 g/L	0.33 g/L	3.22 g/L
R72G (II.1)	B cell lymphopenia, low IgM/IgG1, poor vaccine responses		386		185	105	3% of B	6% of B	85% of B	126	Daughter: 0.35 IU/ml	Daughter: 0/12 serotypes protective pre booster, 3/12 serotypes protective post booster	Daughter: IgG 8.9 g/L IgA 5.4 g/L IgM 0.3 g/L **(pre-IVIg)**			
R72G	dygammaglobulinaemia (normal IgG with poor vaccine responses), raised IgE, eosinophillia, Normal PHA T cell proliferation	20.7 (% total CD4)	70.1 (% total CD4); total CD4 = 1.5x10^9/L (0.3-1.4)	13.3 (% total CD8)	53.6 (% total CD8); total CD8 = 0.67 x10^9/L (0.2-0.9)	<0.03 x10^9/L (0.1-0.5)	4.9	20.8	71.5	0.16 x10^9/L (0.09-0.6)	0.05IU/ML to 0.01IU/ML	poor to Preevnar (S4=0.03; S6B=0.01; S9V=0.01; S14=0.14; S18C=0.01; S19F=0.12; S23F=0.01; S5=0.3; S7F=0.29; S19A=0.33) (ug/ml)	At presentation IgG 6.62g/l, IgA 3.44g/l and IgM	6.62 g/L (6-16)	0.29 g/L (0.4-2.5)	3.44 g/L (0.8-3)
R75Q	high % CD3 T cells, high CD8 (TEMRA), low B/NK	33% (26.3-66.3)	66.8% (33-73.5)	17.9% (12.9-51.3)	36.3% (36.3-83.5)	2.2% (4.3-23.2)	0.8% (0.8-37.8)	3.0% (5.2-74.2)	77% (61-83%)	3% (3-20)	n/a	9/14	1060/406/49 /63.7	1060 mg/dL (641-1353)	49 mg/dL (40-184)	406 mg/dL (66-295)
L92W	Lymphocyte subpopulation test: WBC 9.200, CD3 1687.8 x mm3, CD4 888.1 x mm3, CD8 675.5 x mm3, CD19 174.7 x mm3, CD56 136.8 x mm3, CD4/CD8 index 1.31. T lymphocyte subpopulation test: CD3+CD45RA+ 84.6% CD3+CD45RO+ 23.7%	CD3+CD45RO 23.7%	CD3+CD45RA 84.6%	N/A	N/A	174 cells/mm3	4% (% B cells)	11%	79%		within normal values	Pneumococcal antibodies 6/10 >1.3	IgA<6, IgG 202 at evaluation then normal values on IVIG therapy, IgM <10, IgE1500-1800.	202 mg/dL (223-1411)	8 mg/dL (19-146)	<6 mg/dL (5-83)
K143X	CD3 ABS: 2673 (74.6%), CD4 ABS: 1490 (57.9%), CD8 ABS: 899 (30.8%), CD19 ABS: 542, CD16/56 ABS: 483	17.7%	82.4%	14%	85.7%	13%	0.5% (low)	7.5%	72%	483	DIPHTH AB: 0.1, TETANUS TITER: 0.1	PNEUM 1 IGG (P13, PNX): 12.35, PNEUM 3 (P13, PNX): 0.50, PNEUM 4 (P7, P13, PNX): 0.43, PNEUM 5 (P13, PNX): 7.86, PNEUM 6B (P7, P13, PNX): 0.45, PNEUM 7F (P13, PNX): 0.88, PNEUM 8 (PNX): 0.23, PNEUM 9N (PNX): 0.34, PNEUM 9V (P7, P13, PNX): 0.77, PNEUM 12F (PNX): 0.09, PNEUM 14 (P7, P13, PNX): 1.18, PNEUM 18C (P7, P13, PNX): 5.19, PNEUM 19F (P7, P13, PNX): 3.78, PNEUM 23F (P7, P13, PNX): 1.80	IgG 581, IgM 18, IgA: 117.0	581 mg/dL (low)	117 mg/dL	18 mg/dL (low)
dup183_196 (D-II.2)	high IgE, AEC, decreased T prolif, low B, high CD8 T															
dup183_196 (D-II.1)	high IgE, AEC, decreased T prolif, low B, high CD8 T															
dup183_196 (D-I)	high IgE, AEC, decreased T prolif, low B, high CD8 T															
R187P (II.3)											yes	yes		10.2 g/L (6-16)	0.55 g/L (0.5-2)	3.39 g/L (0.8-4)
R187P (III.1)		24.1% (35-82)	74.3% (13-56)	ND	total CD8=8.18% (10-39)	13.3% (6-19)	1.14% (7.2-30.8)	1.69% (6.5-29.2)	96.7% (43.2-82.4)	6.73% (7-31)	poor	6/13 protective		12.5 g/L (6-16)	0.25-0.48 g/L (0.5-2)	1.99-2.38 g/L (0.8-4)
R187P (III.2)		18.5% (35-82)	80.1% (13-56)	ND	total CD8=14.33%	2.21% (6-19)	11.3% (7.2-30.8)	21% (6.5-29.2)	47.3% (43.2-82.4)	3.1 (7-31)	poor	4-10/12 protective		8.73-10.4 g/L (6-16)	0.42-0.66 g/L (0.5-2)	3.72- 4.32 g/L (0.8-4)
R187P (III.4)											not done	6/12 protective		12.2 g/L (5.4-	1.3 g/L (0.5-2)	3 g/L (0.8-2.8
R187P (III.5)											not done	4/12 protective		9.43 g/L (5.4-	0.23 g/L (0.5-2)	2.24 g/L (0.8-
R187P (IV.3)										low	unknown	protective		7.22-8.16 g/L (6-16)	0.32- 1.12 g/L (0.5-1.9)	2.13- 3.27 g/L (0.8-2.8)
R187P (IV.4)		10.3% (35-82)	88.4% (13-56)	ND	total CD8=20.57% (10-39)	7.57% (6-19)	2.52% (7.2-30.8)	9.91% (6.5-29.2)	80.2% (43.2-82.4)	1.21% (7-31)	yes	2/13 protective		9.06-9.2 g/L (6-16)	0.18- 0.21 g/L (0.5-1.9)	5.14- 5.57 g/L (0.8-2.8)
R187P (IV.6)											not done	5/12 protective		10.6-11.3 g/L (5.4-	0.5- 0.57 g/L (0.5-2)	3.51- 3.75 g/L (0.8-2.8)
R187P (IV.8)											poor	9/12 protective		13.3 g/L (5.4-16.1)	1.06 g/L (0.5-2)	1.13- 1.73 g/L (0.8-2.8)
R187P (V.2)	high IgE, AEC										awaited	unknown		2.14 g/L (5.4-16.1)	0.07 g/L (0.5-2)	0.22 g/L (0.8-2.8)
L194P	high IgE, AEC, decreased T prolif															
R974C (II.2)						Normal	Normal	Normal	Normal	Normal	Normal	Normal		10.3 (6-16)	0.9 (0.4-2.3)	2.83 (0.7-4.0)
R974C (III.1) daughter						Normal	Normal	Normal	Normal	Normal	0.15	Low		6.7 (3.5-10)	0.78 (0.4-1.4)	0.08 (0.19-2.20)
R975W (B-II.2)																
R975W (B-I)	high IgE, AEC															
Additional Mutations																
C150L																
V195L	High IgE	461	163	138	81	119	10	8	99	125	Positive >2.24	21/23 >1.3	IgG 664 IgA 206 IgM 56 IgE 2108	664 (700-1600)	56 (40-230)	206 (70-400)
K362E	no	ND														
R408H (II.4)							10%	50%	40%	Normal				0	0	0
P495S																
I544L																
R608H		9% (16-42%), 652 (301-919)	22% (3-33%), 1593 (134-969)	N/A	N/A	17% (11-28%), 1231 (249-865)	10.3% (no ref), 76 (no ref)	16.6% (0-30%), 123 (0-94)	52.1% (31-91%), 387 (12-336)	7% (6-23), 507 (137-478)	N/A	6/14 >1.3		1020 (635-1284)	126 (44-190)	177 (32-191)
V659M																
T670M	no AgR prolif, normal PHA, reduced B, high memB	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
E766D																
R848C	hypogamma on steroids and s/p ritux. IgG/A/M (mg/dl) 598/52/39. IgE <4 (IU/ml) (2012). IgM would later increase to 330 (2014)	200 cells/ul (25.5 % of CD4s) (2012)nl range 152-583	393 cells/ul (13 % of CD4s) (2012)nl range 41-1121	total CD8 was 549 (2012)nl range 200-1200	N/A	237 cells/ul (2012 prior to ritux) nl range 200-600	we have this only after ritux	we have this only after ritux	we have this only after ritux	171 cells/ul nl range 70-1200	nonresponsive on ritux	nonresponsive on ritux	hypogamma on steroids and s/p ritux which started in 2013. Prior	598 mg/dL	39 mg/dL	52 mg/dL
R912Q / D1152N	CD3=945, hypogammaglobulinemia	355	103	177	248	134	7	1	126	40	0.01 (very low)	0/23	IgG/A/M/E0 all below level of detections	<40 (635-1284)	<5 (44-190)	<5 (32-191)
S923L		22% (16-42%), 310 (301-919)	19% (3-33%), 268 (134-969)	N/A	N/A	18% (3-25%), 254 (59-457)	2.8% (no ref), 8 (no ref)	0.1% (0-30%), 0 (0-94)	84.5% (31-91%), 264 (12-336)	15% (6-23%), 212 (137-478)	N/A	N/A		<7 (641-1353)	15.6 (40-180)	<6 (66-295)
V983M	low IgM															
E1028K	total CD3CD45RA 56.2%, CD3CD45RO 31.7%	N/A	N/A	N/A	N/A	2.9% of WBCs (no ref), 0.25 (0.2-3.3)	N/A	N/A	N/A	3.4% of WBCs (no ref), 0.29 (0.1-1.0)	N/A	N/A		1500 (641-1353)	72.9 (40-180)	136 (66-295)

E	Ag-specific IgE	Other Labs (including Th1/Th2/Th17)	Referred From
ND	ND	ND	Peter Arkwright (Manchester, UK)
ND	ND	ND	Peter Arkwright (Manchester, UK)
180 KU/ml (0-114)	specific IgE negative for HDM and milk	normal mitogen stim to PHA and PWM	Peter Arkwright (Manchester, UK)
18,000 KU/ml (0-114)	specific IgE to HDM >100, milk	ND	Peter Arkwright (Manchester, UK)
164 KU/ml (0-114)	specific IgE to mixed grass 50	ND	Peter Arkwright (Manchester, UK)
13.7 KU/ml (0-114)	ND	ND	Peter Arkwright (Manchester, UK)
687 KU/L (0-60) (Age 29 mo)	Positive RAST for egg white (5.47 KU/ml), milk (0.68 KU/ml), wheat (0.4 KU/ml), soybean (1.08 KU/ml), peanut (6.08 KU/ml)	Absent T cell proliferative responses	C. Prescott Atkinson, Jeffrey Lebensburger (UAB)
>2327 IU/L (0-120)	Positive RASTs to egg white, wheat, animal dander, soya	reduced PHA response, normal anti-C	Clinics Sivic (Leeds)
914 (0-120)	Positive RASTs to house dust mite, egg, cow/dog dander, milk	reduced PHA response, normal anti-CD3, normal Tregs	Sophie Hambleton (Newcastle)
			Troy Torgerson (Seattle)
707			Todd et al (2018) JACI 141: 183-93.
4904			Todd et al (2018) JACI 141: 183-93.
707			Todd et al (2018) JACI 141: 183-93.
707			Todd et al (2018) JACI 141: 183-93.
3		reduced effector T cells (IFN-g, IL-17), normal Tregs	Matthew Cook (ANU)
7		reduced effector T cells (IFN-g, IL-17), normal Tregs	Matthew Cook (ANU)
6		reduced effector T cells (IFN-g, IL-17), normal Tregs	Matthew Cook (ANU)
466 KU/ml (0-161)	ImmunoCAP (3 yo): positive to egg (0.4 KU/ml) and dog (0.43 KU/ml)	Normal Mitogen Stim, Absent Prolif to Candida and Tetanus, low Tregs (3% total CD4)	Kenneth Paris (LSUHSC)
		high IgE, AEC, decreased T prolif	Ma et al (2017) Nat Genet 49: 1192-1201.
648 KU/ml	Negative RAST for HDM, D.Pt, Aspergillus >3.35	HDM IgE not detected, Reduced T cell proliferation	Ronan Leahy, Niall Conlon (Dublin)
		HDM IgE not detected, Aspergillus IgE not detected	Ronan Leahy, Niall Conlon (Dublin)
338 KU/ml (0-120)	Positive RAST for grass mix (gx5)	normal PHA response, eosinophilia	Smita Patel (Oxford)
63 IU/ml (<114 KU/ml)	N/A	92%CD3, high CD8 (TEMRA), low IL/mL, Th17 0.71% - normal study, Antigen proliferation: normal recall response to antigens candida and tetanus, decreased response to mitogen PHA, lymphocyte activation: decreased CD69 expression of CD25 on CD4+ T-cells.	Vinay Chandrakasan (Emory)
1555 mg/dL (0-99)	Immulite Panel (11 mo) Der P >100 kU/L Cat E 1.12 kU/L Egg White >100 kU/L Chocolate 0.12 kU/L Egg yolk 76.9 kU/L Tomato 0.62 kU/L Shrimp 1.46 kU/L	Normal endocrinology workup (Thyroid, prolactin, IGI, IGFBP3)	Ivan Chinn (Baylor)
7 IU/ml	N/A	Abs Foxp3+ Tregs = 86/uL (normal >20)	Michael Jordan (CCHMC)
		high IgE, AEC, decreased T prolif, low	Ma et al (2017) Nat Genet 49: 1192-1201.
		high IgE, AEC, decreased T prolif, low	Ma et al (2017) Nat Genet 49: 1192-1201.
		high IgE, AEC, decreased T prolif, low	Ma et al (2017) Nat Genet 49: 1192-1201.
19-203 IU/ml (0-114)	Negative RAST to cat/dog/fox, feces,	elevated AEC	Ilana Alachkar (Manchester, UK), Bodo Grimbacher (Freiburg)
1216-6209 IU/ml (0-107)	Positive RAST: house dust, mite, cat, dog, Asp, fumigatus	elevated AEC, normal Treg %	Claire Bethune, Andrew Whyte (Plymouth UK), Bodo Grimbacher (Freiburg)
3907-6962 IU/ml (0-107)	Positive RAST: Asp, fumigatus	normal AEC, normal Treg %	Claire Bethune, Andrew Whyte (Plymouth UK), Bodo Grimbacher (Freiburg)
1464 IU/ml (0-114)	unknown	elevated AEC	Peter Arkwright (Manchester, UK), Bodo Grimbacher (Freiburg)
15000 IU/ml (0-114)	Positive RAST: house dust, mite, grass, tree, dog, cat	elevated AEC	Peter Arkwright (Manchester, UK), Bodo Grimbacher (Freiburg)
5100-54120 IU/ml (0-107)	unknown	normal AEC	Claire Bethune, Andrew Whyte (Plymouth UK), Bodo Grimbacher (Freiburg)
1464 IU/ml (0-107)	unknown	elevated AEC, normal Treg %	Claire Bethune, Andrew Whyte (Plymouth UK), Bodo Grimbacher (Freiburg)
1464 IU/ml (0-114)	none	elevated AEC	Peter Arkwright (Manchester, UK), Bodo Grimbacher (Freiburg)
290 IU/ml (0-114)	none	elevated AEC	Peter Arkwright (Manchester, UK), Bodo Grimbacher (Freiburg)
unknown	unknown	elevated AEC	Peter Arkwright (Manchester, UK), Bodo Grimbacher (Freiburg)
		high IgE, AEC, decreased T prolif	Ma et al (2017) Nat Genet 49: 1192-1201.
70			Matthew Cook (ANU)
9		Normal plasmablasts; Normal T cells	Matthew Cook (ANU)
			Ma et al (2017) Nat Genet 49: 1192-1201.
		high IgE, AEC	Ma et al (2017) Nat Genet 49: 1192-1201.

			Stephan Ehl newsmare (Mosaic 2015)
3108 (<90)	unknown		Alex Freeman (NIH)
			Josh Milner, Jeffrey Cohen (NIH)
0		Normal plasmablasts; Normal T cells	Matthew Cook (ANU)
			Troy Torgerson (Seattle)
			Josh Milner
33.1 (0 - 90)		N/A	Ivan Chinn (Baylor)
			Eric Allenspach (Seattle)
N/A		no AgR prolif, normal PHA, reduced IL, high memB	Ivan Chinn (Baylor)
			Josh Milner (NIH)
>1 IU/ml	>1mg/L = 9% of total CD4 T cells (s/p rituximab and bortezomib on steroids MMF, rapamycin)in 2017. Their frequency was 1.8% of the CD4 gate. Also has a TACI mutation (C104R) that may act as a disease modifier	We have >1mg/L sig also and bortezomib on steroids MMF, rapamycin in 2017. Their frequency was 1.8% of the CD4 gate. Also has a TACI mutation (C104R) that may act as a disease modifier	Kate Sullivan / Neil Romberg (CHOP)
<1 (0-90)		CD19/4S, Very low Abs	Alex Freeman (NIH)
<4 (0-200)		N/A	Ivan Chinn (Baylor)
			Josh Milner (NIH)
		N/A	Ivan Chinn (Baylor)


A
IgE
Mutation
R47H
R30W
E57D
E57D
R187P
R975W
R30Q
R30W
R72G
R30G
R187P
R187P
R72G
R30G
R974C
R974C
R187P
R187P
R30G
R187P
R187P
R75Q
R30Q
R30G
R30G
R47H
R47H
R30G
R47H
K143X
L92W
R187P
R187P
R30Q
R187P
R30W
Phenotypic Indicators
Present
Absent
IgE
Missing
≤120
>120 and <1,000
≥1,000 and <10,000
≥10,000
Atopic.dermatitis
asthma
neutropenia
Low.IGG
Low.IgM
Low.IgA
High.IgA
B
Cluster 1
Cluster 2
Cluster 3
AA mutation
atopic dermatitis
skin bacterial infection
pneumonia
neutropenia
low IgM
IgA
allergic rhinitis
AA mutation, 1
AA mutation, 2
AA mutation, 3
atopic dermatitis, 1
atopic dermatitis, 2
atopic dermatitis, 3
skin bacterial infection, 1
skin bacterial infection, 2
skin bacterial infection, 3
pneumonia, 1
pneumonia, 2
pneumonia, 3
neutropenia, 1
neutropenia, 2
neutropenia, 3
Low IgM, 1
Low IgM, 2
Low IgM, 3
IgA, 1
IgA, 2
IgA, 3
AR, 1
AR, 2
AR, 3
R30G R30Q R30W R47H E57D R72G R75Q L92W K143X dup183_196 R187P L194P R974C R975W
no
yes
normal
low
high