Germline hypomorphic CARD11 mutations in severe atopic disease

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Few monogenic causes for severe manifestations of common allergic diseases have been identified. Through next-generation sequencing on a cohort of patients with severe atopic dermatitis with and without comorbid infections, we found eight individuals, from four families, with novel heterozygous mutations in CARD11, which encodes a scaffolding protein involved in lymphocyte receptor signaling. Disease improved over time in most patients. Transfection of mutant CARD11 expression constructs into T cell lines demonstrated both loss-of-function and dominant-interfering activity upon antigen receptor–induced activation of nuclear factor-κB and mammalian target of rapamycin complex 1 (mTORC1). Patient T cells had similar defects, as well as low production of the cytokine interferon-γ (IFN-γ). The mTORC1 and IFN-γ production defects were partially rescued by supplementation with glutamine, which requires CARD11 for import into T cells. Our findings indicate that a single hypomorphic mutation in CARD11 can cause potentially correctable cellular defects that lead to atopic dermatitis.

Monogenic causes for immune disorders have provided critical insight into the role of specific immune pathways in the pathogenesis of common diseases, including atopy and allergic diseases. In the case of some allergic disorders involving atopic dermatitis and elevated IgE coupled with infection, it may be difficult to distinguish the contribution of impaired host defense from that of the defined genetic lesion in allergic disease. Severe atopic disease and elevated serum IgE levels have now been linked to mutations in immune-mediated host defense pathway genes, including DOCK8, STAT3 and PGM3 (refs. 1–3). In some patients with severe atopic dermatitis, elevated IgE and eosinophilia, infections beyond the skin are less common and they lack comorbidities seen with the genetically defined disorders, so there is little reason to suspect an immune deficiency. Reports of single gene mutations in common allergic disease without overt syndromic features have been rare4, but examples are increasingly described. One such example is a single gain-of-function (GOF) mutation in IL4RA, identified initially in a few severely atopic patients5, that is a common risk allele in allergic disease6. Another is loss-of-function (LOF) mutations in IFNGRI leading to atopic dermatitis with eczema herpeticum without additional immune phenotypes2. LOF mutations in cytokine signaling components such as STAT3 and STAT5B can also contribute to atopic phenotypes with substantial multisystem comorbidity, seen also in putative antigen receptor signaling gene mutations such as WASP, DOCK8 (ref. 9) and MALT1 (ref. 10). Diminished antigen-driven TCR signaling can predispose to type 2 helper T cell (Th2) phenotypes in a variety of in vivo and in vitro settings9, and mouse models have shown that hypomorphic mutations in ZAP70 (ref. 11) and CARD11 (ref. 12) can also lead to severe atopy.

CARD11 (also known as CARMA1) encodes a membrane-associated guanylate kinase (MAGUK) family protein that partners with BCL10 and MALT1 to form the CBM complex, a scaffold classically required for T cell receptor signaling. Mutations in CARD11 can lead to potentially correctable defects in immune cell signaling and T cell receptor signaling in the skin, as well as reduced IFN-γ production. Our findings suggest that CARD11 mutations can contribute to atopic dermatitis through impaired T cell receptor signaling.

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for activation of IκB kinase (IKK) and nuclear factor-κB (NF-κB) in response to lymphocyte receptor ligation. While homozygous null mutations in CARD11 lead to severe combined immune deficiency (SCID) in humans and mice, heterozygous GOF mutations give rise to a selective B cell lymphoproliferative disease known as BENTA. CARD11 has also been identified as a risk locus for atopic dermatitis.

Here we describe rare hypomorphic dominant-negative mutations in CARD11, in four unrelated families, that lead to dominantly inherited severe atopy with variable infection beyond the skin.

RESULTS

CARD11 mutations in patients with severe atopic dermatitis

We performed next-generation sequencing on a series of patients with recalcitrant, severe atopic disease (Fig. 1). In four of these patients (A-I, B-I, C-I and D-I), we detected novel heterozygous CARD11 mutations. These included three distinct missense mutations (encoding p.Glu57Asp (E57D), p.Leu194Pro (L194P)) and p.Arg975Trp (R975W)) and one in-frame mutation encoding a 14-aa insertion (p.Met183_Lys196; herein referred to as dup183_196) (Table 1). The PolyPhen values are 1 for all three missense mutations, and the CADD scores are 21.2, 19.5 and 17.8, respectively. The mutations are predicted to be deleterious. A parent and/or other relative in two of the four patients had a history of atopic dermatitis that waned in severity over time; these relatives were confirmed as carriers of the mutation (Fig. 1a). Subject B-II.2, the mother of patient B-I, was unaffected but reported having had eczema when younger. Subjects A-I and C-I had histories of pneumonia early in life, and C-II.1 had transient hypogammaglobulinemia of childhood. Subjects A-I, C-I, D-I, D-II.1 and D-II.2 had multiple episodes of respiratory distress in early childhood that were treated as pneumonia. Patients A-I and C-I were also diagnosed with asthma. Oral steroids were required to resolve all asthmatic episodes for patient A-I, whose computerized tomography imaging showed normal lungs, raising the likelihood that his respiratory symptoms were not of infectious etiology. The possibility that the patients’ presentation was not of infectious etiology, the infectious history of family D (dup183_196) includes abscesses, bacteremia and pulmonary infections. Affected members of family D had some unique features that may or may not have been related to their primary genetic diagnosis, including a prominent forehead and broad nasal base from birth. Unlike the subjects with STAT3 LOF mutations, there was no characteristic facial asymmetry or coarseness, and the birth onset differed as well (Table 1). Patient C-I had history of stroke and ulcerative colitis. C-II.1, his father, who also carried the E57D variant, had a history of lymphoma. Cancer and stroke were not otherwise reported. Two of the seven subjects had B cell lymphopenia with no decrease in other lymphocyte lineages; three of seven patients showed low IgM with no decrease in IgG and normal or elevated IgA. Elevated IgE (5/7) and eosinophilia (6/7) were prominent, and the subjects without these features had more substantial atopic phenotypes at a younger age but no documentation of these laboratory values (Supplementary Table 1).

CARD11 mutations interfere with NF-κB and mTORC1 activation

The four heterozygous mutations detected in the patients affect markedly different domains of the CARD11 protein (UniProtKB Q9BXL7) (Fig. 1b). L194P and dup183_196 are found in the coiled coil (CC) domain. E57D is in the N-terminal CARD domain, and R975W is in the C-terminal GUK domain. None were found in public polymorphism databases (for example, ExAC). Notably, Goodnow and colleagues noted elevated IgE levels and severe eczema in the Card11<sup>mutm</sup> (UNM) mouse strain, which harbors an ENU-mutagenesis-induced, homozygous Card11 mutation in the CC domain (p.Leu298Gln), raising the possibility that the patients’ CARD11 mutations might lead to hypomorphic activity. However, UNM heterozygous mice had no atopic phenotype, and the heterozygous parental carriers of patients with CARD11-associated severe combined immunodeficiency (SCID) were reported as clinically asymptomatic and did not have a history of atopy (A. Kulozik and P. Stepensky, personal communication), suggesting that haploinsufficiency was not a likely explanation for the patients’ presentation (Fig. 1c).

Two principal signaling pathways in which CARD11 participates are NF-κB and mTORC1 activation. We first assessed the effect of the four CARD11 mutations on NF-κB activation by transfecting wild-type and mutant CARD11 expression constructs into a CARD11-deficient Jurkat T cell line (JPM50.6) expressing a NF-κB-driven GFP reporter. As shown previously, the GOF mutant E134G, described in a family with BENTA disease, induced constitutive GFP expression in the absence of antigen receptor (AgR) ligation. When compared to wild type, all four CARD11 mutants induced little NF-κB activation upon stimulation with anti-CD3 and anti-CD28, confirming loss of function (Fig. 2a,b).
and Supplementary Fig. 1a). Furthermore, co-transfection of each mutant construct disrupted the ability of wild-type CARD11 to activate NF-κB (Fig. 2c–e and Supplementary Fig. 1b), suggesting dominant interference; the R975W mutant was least disruptive. Notably, disruption of NF-κB activation by wild-type CARD11 was not observed in co-transfections with empty vector, E134G or Q945X, a homozygous, functionally 'null' nonsense mutant described in an autosomal recessive SCID patient born to healthy, heterozygous parents (Supplementary Fig. 1c–h). Similar results were obtained upon transfection of Jurkat T cells expressing endogenous wild-type CARD11, as measured by an NF-κB-dependent luciferase assay (Fig. 2g,h and Supplementary Fig. 1i). All mutants were comparably expressed in transfected cells (Fig. 2).

Next, we examined mTORC1 activity by measuring phosphorylation of ribosomal protein S6 (p-S6) and found that all four hypomorphic CARD11 mutants resulted in significantly decreased mTORC1 activity at baseline (unstimulated) and upon anti-CD3 and anti-CD28 stimulation in Jurkat T cells (Fig. 2i,j and Supplementary Fig. 1j). Similar attenuation of p-S6 signaling was noted for each mutant, compared to cells transfected with ectopic wild-type CARD11 or empty vector, suggesting interference with endogenous wild-type CARD11 (Fig. 2k and Supplementary Fig. 1j,k). Q945X expression had little effect (Supplementary Fig. 1l). Taken together, these findings show that each CARD11 mutant is hypomorphic and reduces wild-type CARD11 activity in a heterozygous context, as illustrated by decreased downstream activation of both NF-κB and mTORC1.

Defective NF-κB activation is probably a result of impaired CBM complex assembly, as we observed substantially less association of CARD11 LOF mutants with BCL10 and MALT1 in JPM50.6 co-immunoprecipitations (co-IPs) after phorbol 12-myristate 13-acetate (PMA)–ionomycin stimulation (Fig. 3a). In fact, we recovered less CARD11 in BCL10 co-IPs from wild-type Jurkat cells transfected with CARD11 E57D, suggesting that assembly of the endogenous CBM complex was disrupted (Fig. 3b). Consistent with dominant interference, MALT1 protease activity, as measured by cleavage of the MALT1 substrate CYLD26, was also diminished in Jurkat T cells in the presence of CARD11 LOF mutants (Fig. 3c,d).

**CARD11-mutant patient T cells show low activation responses**

We next investigated CARD11-dependent signaling in primary patient T cells. PMA stimulation of patient lymphocytes revealed marked impairment of NF-κB activation, as indicated by reduced p65 phosphorylation and IκB degradation in T cells from families A, B and C (Fig. 3e). Similarly, mTORC1 activity (indicated by p-S6) was also attenuated in stimulated patient cells (Fig. 3e and Supplementary Fig. 2), whereas phosphorylation of AKT at S473 (a readout of mTORC2) was normal (Fig. 3e). Reduced p38 activity was also noted in some patient cells (Fig. 3e), whereas robust ERK activity was comparable across all subjects, consistent with normal ERK activity noted in the UNM mice. Patient B-I, who harbors the R975W alteration, which had the lowest NF-κB inhibitory activity, and who had the fewest comorbidities beyond atopy, had recently been treated with corticosteroids and methotrexate when blood was drawn, potentially complicating the assessment of T cell phenotype and function. Of note, milder NF-κB and mTORC1 signaling defects could also be detected in CD19+ B cells from some patients (Supplementary Fig. 3a). Overall, B cell development was generally unaffected in these patients (Supplementary Fig. 3b,c).

We also observed poor upregulation of key cell surface markers such as CD69, CD25 and CD98 after overnight stimulation with anti-CD3, particularly for patient A-I (Fig. 4a). Both peripheral blood mononuclear cells (PBMCs) (Fig. 4b, left) and sorted naive T cells (Supplementary Fig. 4a) from patient A-I showed decreased short-term proliferation upon anti-CD3 and anti-CD28 stimulation. PBMCs from subject B-I.2 also showed mildly decreased blastogenesis upon stimulation (Fig. 4b, middle), and subjects D-I, D-II.1 and D-II.2 showed a moderate defect (Fig. 4b, right). These data indicate that the activation and proliferation of CARD11-mutant patient T cells were variably impaired, consistent with defective activation of NF-κB and mTORC1.

Further flow cytometric phenotyping of patient PBMCs showed variability in CD45RA− memory T cells and circulating CXCR5+PD-1hi follicular helper T (cT\(_{\text{FH}}\)) cells in families A–C (Fig. 5a and Supplementary Table 1). Direct ex vivo (Fig. 5b and Supplementary Fig. 4b) and 5-d culture (Supplementary Fig. 4c) of patient T cells showed impaired T\(_{\text{FH}}\), normal interleukin 17–producing T helper (T\(_{\text{H}17}\)) cells and elevated T\(_{\text{H}2}\) cytokine production within the CD45RO+ compartment, mirroring the phenotype of UNM mice and, with the exception of IL-17 production, mice lacking mTORC1 and ASCT2 (refs. 27–30). Numbers of CD4+FOXP3+ regulatory T cells, whose diminished frequency is thought to contribute to the UNM mouse phenotype21, were normal in all subjects (Fig. 5c and Supplementary Table 1).

### Table 1 Summary of clinical data

| Subject | CARD11 alteration | Age (years) | Atopic dermatitis (AD) | Infections | Asthma | Other |
|---------|------------------|-------------|------------------------|------------|--------|-------|
| A-I     | L194P (C-C)      | 15          | Severe (SCORAD 76)     | Molluscum  | Yes    | Food allergy, recurrent respiratory distress |
| B-I     | R975W (GUK)      | 5           | Severe (SCORAD 74)     | Eczema herpeticum | Yes    | Food allergy, treated with methotrexate for AD |
| B-II.2  | R975W (GUK)      | 27          | Mild to moderate       | None       | No     | AD improved over time |
| C-I     | E57D (CARD)      | 16          | Severe                 | Pneumonia, molluscum | Yes    | Transient hypogammaglobulinemia of childhood, ulcerative colitis, stroke, short stature (<5% for height), food allergy, AD improved over time |
| C-II.1  | E57D (CARD)      | 51          | Severe                 | Pneumonia  | Yes    | AD improved over time, peripheral T cell lymphoma treated with autologous transplant |
| D-I     | 196dup           | 1.5         | Severe, erythodermic desquamating | Lumbar abscess, catheter related infection, CMV pneumonitis | No     | Eosinophilic coloprotctis, bilateral nystagmus, seizure, temporal syvian opercular dysplasia ASMA and actin-specific autoantibodies, prominent forehead, broad nose |
| D-II.1  | 196dup           | 21          | Moderate               | Pneumonia, pulmonary TB, bronchiectasis, boils, molluscum | No     | Scoliois, poor dentition, prominent forehead, broad nose, AD improved over time |
| D-II.2  | 196dup           | 15          | Severe                 | Pneumonia, bacteremia, superinfected varicella, Kaposis varicelliform, boils, molluscum | Severe | Poor dentition, dental fractures, chronic diarrhea, prominent forehead, broad nose, AD improved over time |

SCORAD, scoring atopic dermatitis; SCORAD > 50 indicates severe atopic dermatitis.
Supplementary Table 1). The percentage of dividing regulatory T (Treg) cells (Ki-67+) was also comparable to controls (Fig. 5c), as was their frequency and suppressive function (Supplementary Fig. 5). However, aberrant co-expression of GATA3 in Treg cells from patients A-I and B-I suggests impaired GATA3 regulation (Fig. 5d), a finding associated with atopy in a variety of settings. Collectively, these results indicate that patient T cells harboring CARD11 LOF or dominant-negative (DN) mutations are hyporesponsive and skew toward a Th2 phenotype, consistent with their atopic predisposition.

CARD11 mutations result in poor upregulation of ASC2

CARD11 was recently shown to be critical for mTORC1 activation, at least in part as a facilitator of T cell antigen receptor (TCR)-induced upregulation and/or activation of ASCT2, an essential glutamine transporter required for extracellular glutamine import during cellular activation. Similarly to CARD11-deficient murine T cells, CARD11-mutant patient T cells showed reduced ASCT2 upregulation after TCR activation (Fig. 5e and Supplementary Data). Mice lacking ASCT2 also show defects in mTORC1 activation, impairments in Th1 differentiation and a Th2-skewed phenotype. However, the addition of exogenous glutamine could rescue mTORC1 signaling and reverse Th1 defects. Supplementing culture medium with exogenous glutamine boosted mTORC1 activation (p-S6) in patient A-I T cells, particularly in CD45RA+ naive T cells (Fig. 6a,b), without affecting NF-kB or ERK phosphorylation (data not shown). We then cultured naive patient A-I T cells in the presence of supplemented glutamine...
ideal for measuring biases for cell-intrinsic TH cell differentiation, we observed CD98 induction, but only with exogenous glutamine supplementation. TNF and STAT3 (IL-6) partially rescued defects in proliferation and cytokine production. These subjects mostly lack the CARD11 scaffold protein, which is required for T cell responses in these patients. CYLD, another component of the CBM complex, is also mutated in these patients, but its role in T cell function is not well understood.

**DISCUSSION**

Here we show that different heterozygous hypomorphic mutations in CARD11 can lead to severe atopic disease in humans, associated with weak, TH2-skewed T cell responses. These mutations could be a common cause of severe allergy in at risk populations. CARD11 is a key regulator of T cell signaling and its dysfunction in these patients likely contributes to the development of atopy.

Figure 3 Impaired CBM complex formation leads to defective signaling in CARD11-mutant patient T cells. (a) Immunoblot to detect association of CARD11-Flag, MALT1 and BCL10 in BCL10 immunoprecipitates from JPM50.6 cells transfected with EV, WT, E57D or L194P CARD11 mutants with or without 15 min PMA and ionomycin. Input lysates (bottom) were probed with antibodies to Flag and β-actin to confirm equivalent CARD11-Flag expression. Data are representative of 3 independent experiments. (b) Immunoblot analysis of BCL10 immunoprecipitates from WT Jurkat cells transfected with EV, WT or E57D CARD11 mutants with or without 15 min PMA and ionomycin stimulation. Data are representative of 2 independent experiments. (c) Immunoblot analysis of lysates from transfected Jurkat cells with or without 2 h PMA and ionomycin stimulation to detect MALT1-dependent cleavage of CYLD and CARD11-Flag expression. FL, full-length; ct, C-terminal fragment. (d) Spot densitometric quantification of the ratio of cleaved to full-length CYLD in immunoblots represented in c; data are mean ± s.d. of 3 independent experiments. Asterisks denote significance versus stimulated EV (E57D). P = 0.028; L194P P = 0.016. (e) Flow cytometric assay of CARD11-mutated patient CD4+ T cells compared with unaffected family members (WT/WT) and healthy controls (HC) stimulated with PMA for 20 min (2 ng/mL for p-S6 and p-AKT, 5 ng/mL for p-P65, 1xIκB, p-P38 and p-ERK in families A and B; 10 ng/mL in family C). NS, no stimulation. Data are representative of 3 (A-I), 2 (family B) or 1 (family C) independent experiments.

(3 mM) under a variety of stimulation conditions. We found that combinations of cytokines capable of activating NF-κB (IL-1β and TNF) and STAT3 (IL-6) partially rescued defects in proliferation and CD98 induction, but only with exogenous glutamine supplementation (Fig. S6c and Supplementary Fig. 6). While these conditions are not ideal for measuring biases for cell-intrinsic T cell differentiation, we did find that divided cells were more likely to produce IFN-γ upon glutamine supplementation (Fig. S6e). Together, these results suggest that T cell responses in these patients might be partially restored simply through glutamine supplementation.

**Figure 3** Impaired CBM complex formation leads to defective signaling in CARD11-mutant patient T cells. (a) Immunoblot to detect association of CARD11-Flag, MALT1 and BCL10 in BCL10 immunoprecipitates from JPM50.6 cells transfected with EV, WT, E57D or L194P CARD11 mutants with or without 15 min PMA and ionomycin. Input lysates (bottom) were probed with antibodies to Flag and β-actin to confirm equivalent CARD11-Flag expression. Data are representative of 3 independent experiments. (b) Immunoblot analysis of BCL10 immunoprecipitates from WT Jurkat cells transfected with EV, WT or E57D CARD11 mutants with or without 15 min PMA and ionomycin stimulation. Data are representative of 2 independent experiments. (c) Immunoblot analysis of lysates from transfected Jurkat cells with or without 2 h PMA and ionomycin stimulation to detect MALT1-dependent cleavage of CYLD and CARD11-Flag expression. FL, full-length; ct, C-terminal fragment. (d) Spot densitometric quantification of the ratio of cleaved to full-length CYLD in immunoblots represented in c; data are mean ± s.d. of 3 independent experiments. Asterisks denote significance versus stimulated EV (E57D). P = 0.028; L194P P = 0.016. (e) Flow cytometric assay of CARD11-mutated patient CD4+ T cells compared with unaffected family members (WT/WT) and healthy controls (HC) stimulated with PMA for 20 min (2 ng/mL for p-S6 and p-AKT, 5 ng/mL for p-P65, 1xIκB, p-P38 and p-ERK in families A and B; 10 ng/mL in family C). NS, no stimulation. Data are representative of 3 (A-I), 2 (family B) or 1 (family C) independent experiments.

A rt i c l e s

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of the CARD11 alterations in patients were located in the CC domain, a demonstrated hot spot for GOF and LOF mutations, which probably perturb intramolecular regulation via the linker, thereby enhancing or debilitating self-oligomerization of CARD11 required for signalosome assembly, respectively. Similarly, it is likely that the E57D alteration of the CARD11 mutants in transfection studies, suggesting more discrete roles for the NF-κB and mTORC1 pathways in preventing infection and atopy, respectively.

It remains unclear which CARD11 domains might be required for direct interaction and/or transcriptional regulation of ASCT2, the glutamine transporter connecting AgR engagement with mTORC1 activation. Attenuated p38 signaling in some CARD11-mutant patient cells was surprising, as CARD11 has not been specifically implicated in this MAPK pathway. Given that the MAGUK protein DLGH1 governs the TCR-driven "alternative p38 pathway", perhaps these CARD11 mutants interfere with related MAGUK family scaffold proteins in as yet undefined ways.
Problems with T cell proliferation and balanced Th cell differentiation in the CARD11-mutant patients probably result from defects in multiple TCR-driven signaling pathways that rely on CARD11. Disruption of TCR signal strength, which can occur in a variety of settings and TCR signaling pathways, has been associated with Th2 bias40, and this phenomenon probably contributes to the pathogenesis of these patients. Numerous studies have shown that disruption of NF-κB or mTORC1 signaling blocks proper T cell expansion, probably by compromising both the interleukin 2 (IL-2) signaling loop and metabolic reprogramming required for T cell proliferation. For these reasons, it was difficult to definitively characterize the effect of these CARD11 LOF mutations on Th cell differentiation, as typical unbiased in vitro conditions did not support T cell proliferation. However, recent work indicates that mTOR activity profoundly affects Th cell differentiation41. The attenuation of CARD11-dependent mTORC1 activation ostensibly contributes to impaired Th1 differentiation in our patients, allowing an mTORC2-dependent Th2 response to dominate. NF-κB signal blockade in mouse T cells was also shown to preferentially disrupt the Th1 response and permit Th2-driven allergic inflammation in vivo42. Whether reduced (but not absent)
Figure 6 Effect of glutamine supplementation and cytokines on TCR-induced proliferation and IFN-γ defects in a patient with CARD11 mutation. (a) p-S6 activation by PMA after addition of l-glutamine. PBMCs from the healthy control (HC) and patients with CARD11 mutations were allowed to rest in medium then PBS supplemented with glutamine and stimulated with PMA. The dashed lines indicate the maximum p-S6 activation peak (mean fluorescence intensity [MFI]) by PMA with 5 mM glutamine addition in HC. (b) ∆MFI for p-S6 in HC, n = 5; 3 independent experiments for A-I and 2 for B-I; mean ± s.e.m.). (c) Left, proliferation of CD4+ naive T cells isolated from patient A-I in serum-free medium stimulated with anti-CD3 and anti-CD28 and/or IL-6 for 5 d with glutamine supplementation. Right, surface activation marker expression with glutamine plus cytokines in patient A-I (gated on FSChiSSChi blasts). Data are representative of 3 experiments. (d) Top, partial restoration of impaired naive CD4+ T cell proliferation by anti-CD3 and anti-CD28 stimulation with excess glutamine and IL-1β, TNF-α and IL-6 in patient A-I. Bottom, proliferation-dependent rescue of IFN-γ expression. Dividing cells were gated into 3 groups on the basis of CellTrace Violet intensity (top left), and IFN-γ was measured by intracellular staining in each group (bottom). Data are representative of 2 independent experiments. (e) Percentages of proliferative CD3+CD8− blasts (top) and IFN-γ-producing CD3+CD8− blasts (bottom) with the treatments by increasing glutamine concentration. For each experiment: anti-CD3 and anti-CD28, HC (n = 3), A-I (n = 2); +IL-1β and TNF-α, HC (n = 3), A-I (n = 1); +IL-6: HC (n = 1), A-I (n = 1); +IL-1β, TNF-α or IL-6, HC (n = 5), A-I (n = 2).
IFN-γ production explains the atopic and skin infection phenotype entirely, as suggested in patients with hypomorphic IFNGR1 mutations, is not clear. An intrinsic T112 bias alone could drive the atopic phenotype and predispose to skin infection.

Recent work suggests CARD11-deficient NK-b2 stimulation is particularly important for T117 differentiation of mouse T cells in vitro under specific polarizing conditions. In vivo, CARD11-deficient mice fail to mount a T12 response capable of driving allergic inflammation, whereas UNM mice show decreased frequency of FOXP3+ Treg cells and a gradual accumulation of T12 cells, resulting in allergic disease. In contrast, we found that CARD11-mutated patients had normal numbers of T117 cells and Treg cells, although the patients' Treg cells appeared 'T12-like' on the basis of aberrant expression of GATA3, a recognized hallmark of atopic disease susceptibility. Further study is required to confirm a causal role of this observation. These disparate findings underscore the importance of identifying and characterizing 'CARD11-opathies' in humans and mice. Indeed, BENTA patients harboring GOF CARD11 mutations show no signs of allergic disease, even though adoptively transferred T cells expressing constitutively active CARD11 can drive allergic disease in mice.

Abnormal CARD11 signaling probably has intrinsic consequences for B cell fate as well. In contrast to the selective expansion of naive B cells seen in BENTA, we noted reduced numbers of CD19+ B cells in some CARD11 LOF patients. However, both GOF and LOF CARD11 mutations can reduce circulating memory B cells and affect immunoglobulin production in humans, suggesting that CARD11 is a critical gatekeeper for tuning proper BCR signals required for normal B cell maturation and differentiation. Of interest here, although IgE signaling normally forces murine B cells to terminally differentiate into short-lived plasmablasts prone to apoptosis, loss of key BCR signaling molecules facilitates the survival and subsequent development of IgE+ memory and long-lived plasma cells. Our findings imply that attenuated CARD11 signaling may mirror this phenomenon in humans, permitting an atypical accumulation of longer-lived IgE-secreting plasma cells that promote allergic disease, particularly in childhood.

Additional work is required to identify the key pathogenic events related to the atopic phenotype and infection. Nevertheless, it may be that disease severity generally lessens over time, consistent with the phenotype in aged UNM mice (C. Goodnow, personal communication). Notably, our findings suggest that exogenous glutamine could partially correct specific defects in CARD11-mutant T cell responses, including proliferation and IFN-γ secretion. Glutamine supplementation has been explored as a treatment for reducing allergic disease in low-birth-weight infants, with a promising decrease in atopic dermatitis reported. This simple therapeutic intervention may ameliorate disease in atopic patients harboring functional mutations in CARD11 or related genes by restoring normal glutamine uptake and mTORC1 signaling in activated T cells.

URLs: ANNOVAR, http://annovar.openbioinformatics.org/; DAVID, https://david.ncifcrf.gov; GeneCards, http://www.genecards.org.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.A.M., Yuan Zhang, M.A.W. and S.G. performed experiments with primary patient cells, J.R.S. and B.D. produced all CARD11 mutant constructs, J.R.S., E.R., S.A., K.V. and B.D. conducted cell transfection experiments. J.J.L., C.G.N., T.D., K.D.S., H.F.M. and J.D.M. were involved in clinical workup of patient A.-I. J.S., J.N. and S.D.R. performed sequence analysis on patient A.-I., J.K.A., P.J.H., P.R.R. and E.W.G. were involved in clinical care and sequence analysis of family B. Yu Zhang, B.K., M.A.C., N.R., S.G. and E.M. were involved in clinical workup of family C. A.P., M.O., E.P., A.R.R.P., G.D. and S.D. were involved in clinical care and the workup of family D. J.Z. and M.A.M. performed sequence analysis of family D. N.Y. performed regulatory T cell experiments. J.J.M. provided sequencing resources and data. N.J. provided patient care and information. C.A.M., M.A.W., J.R.S., A.L.S. and J.D.M. co-wrote the manuscript. E.W.G., A.L.S. and J.D.M. supervised the project. All authors discussed the results and contributed to the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Patients. Informed consent was obtained, and the study was approved by the Institutional Review Boards of National Jewish Health and Yale University. Patients evaluated at NIH were enrolled in clinicaltrials.gov identifier NCT00557895. Informed consent, including consent to publish photographs, was obtained from all participating patients.

Whole-exome sequencing (WES). WES was performed using the Ion Torrent AmpliSeq RDY Exome Kit (Life Technologies) and the Ion Chef and Proton instruments (Life Technologies). Briefly, 100 ng gDNA was used as the starting material for the AmpliSeq RDY Exome amplification step following the manufacturer's protocol. Library templates were clonally amplified and enriched using the Ion Chef and the Ion PI Hi-Q Chef Kit (Chef package version IC.4.4.2, Life Technologies), following the manufacturer's protocol. Enriched, templated Ion Sphere Particles were sequenced on the Ion Proton sequencer using the Ion PI chip v3 (Life Technologies).

Bioinformatics analysis. Read mapping and variant calling were performed using the Ion Torrent Suite software v4.4.2. In short, sequencing reads were mapped against the UCSC hg19 reference genome using the Torrent Mapping Alignment Program (TMAP) map4 algorithm. SNPs and indels were called by the Torrent Variant Caller plugin (v.4.414-1) using the ‘Generic-Proton-Germ Line – Low Stringency’ configuration. Only reads that were unambiguously mapped were used for variant calling. Variants were annotated using ANNOVAR. Data mining, biological interpretation, and candidate gene discovery were performed using various online tools including The Database for Annotation, Visualization and Integrated Discovery (DAVID), and GeneCards. Target coverage was evaluated using the Torrent Coverage Analysis plugin (v.4.414-1), and the output was further evaluated using in-house, custom Perl scripts (available on request).

Sanger sequencing. CARD11 Sanger sequencing was performed to confirm WES-detected variants and to screen family members. gDNA was PCR-amplified using GoTaq polymerase (Promega) and exon specific primers. Amplicons were bi-directly sequenced using the Big Dye Terminator version 1.1 cycle sequencing kit and an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems).

Ex vivo Treg cell and cytokine analysis. Patient and healthy control (HC) PBMCs were purified by Ficoll density gradient centrifugation, washed with RPMI 1640 plus penicillin, streptomycin, and l-glutamine (Gibco) along with 10% FBS (R10, Sigma-Aldrich) and filtered through a 48-µm strainer. Cells were resuspended to a concentration of 1 × 10^6 cells/mL and aliquoted (1 mL/well) into 48-well plates. PMAs (final concentration 20 ng/mL), ionomycin (1 µM) and brefeldin A (5 µg/mL, Sigma-Aldrich) were added before incubation at 37 °C for 5–6 h. After incubation, cells were washed with FACS buffer and stained with LIVE/DEAD Blue (Life Technologies). Intracellular staining was performed using BD CytoFix/CytoPerm (BD Biosciences) reagents according to the manufacturer’s instructions using antibodies to CD3 (UCHT1, Beckman Coulter), IFN-γ, IL-2 (MQ1-17H12, BioLegend), IL-4 (8D4-8, BD Biosciences), CD45RO (UCHL1, Beckman Coulter), and CD45RO (UCHL1, Beckman Coulter).

Treg cell suppression assay. CD4+ T cells were isolated by negative selection using magnetic beads (Miltenyi Biotec) as per manufacturer’s protocol. Cells were cultured in X-VIVO-15 medium (Lonza) in the presence of plate-bound antibody to CD3 (1 µg/mL, OKT3, BioLegend) and soluble antibody to CD28 (0.5 µg/mL, L293, BD Biosciences) for 5 d, with recombinant human IL-6 (20 ng/mL, Peprotech), IL-1β (10 ng/mL, Peprotech), tumor necrosis factor-α (TNF-α, 10ng/mL, R&D), and l-glutamine (Gln) as indicated. On day 5, the cells were stimulated with 20 ng/mL PMA and 1 µM ionomycin for 5 h. Brefeldin A (10 µg/mL) was added after 1 h. Cells were stained with LIVE/DEAD Blue (Life Technologies), then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) per the manufacturer’s protocol and stained with antibodies against CD3 (UCHT1 or SP34-2, BD Biosciences), CD4 (RPA-T4, BD Biosciences), CD8 (SK1, BD Biosciences), IFNγ (B27, BD Biosciences), IL-4 (8D4-8, BD Biosciences) and CD45RO (UCHL1, Beckman Coulter).

Plasmid DNA cloning. The human pUNO-CARD11 plasmid (Invivogen) was modified to include a 3’ 3X FLAG tag using annealed, overlapping oligonucleotides encoding the tag and inserted via BamHI (5’) and Nhel (3’) overhangs. Single point mutations were introduced into the wild-type CARD11 construct by site-directed mutagenesis, using specific primers for linear amplification using 2x Pwo DNA polymerase (Roche) and subsequent digestion with DpnI to destroy methylated template DNA (ThermoFisher Scientific). A 42-bp fragment encoding amino acids 183–196 duplication was generated by overlap extension PCR and ligated into a BsrGI site in pUNO-CARD11 using a rapid dephosphorylation/ligation kit (Roche). Mutations were confirmed by Sanger sequencing. All plasmids were purified using a GenElute HP Plasmid Maxi Prep Kit (Sigma) from transformed competent DH5α E. coli (New England Biolabs) selected with blasticidin (InvivoGen).

Cell transfection assays. The wild-type Jurkat T cell line (clone E6.1) was obtained from ATCC (TIB-152), CARD11-deficient Jurkat cells (IP50.6) were originally provided by X. Lin (MD Anderson Cancer Center); for authentication, absence of CARD11 expression was periodically monitored by immunoblotting.
Neither cell line tested positive for mycoplasma contamination. Cell lines were cultured in complete RPMI 1640 (Lonza) supplemented with 10% FCS (Sigma), 2 mM glutamine, and 100 U/ml each of penicillin and streptomycin (Life Technologies). JPM50.6 cells (4–5 × 10^6/cuvette) were electroporated with 5 µg plasmid DNA in 0.4 mL RPMI plus 10% FBS (no antibiotics) using a BTX Electroporator (BTX Harvard Apparatus: 260 V, 950 µF). For luciferase assays, Jurkat cells were simultaneously transfected with 5 µg NF-kB-dependent firefly luciferase reporter plasmid (pNF-kB-Luc), and 0.25 µg of Renilla luciferase plasmid (pRL-TK) for transfection normalization. A portion of these cells were stimulated 24 h after transfection with 1 µg/ml anti-CD3 + anti-CD28 (BD Biosciences). NF-kB activity was measured 24 h later via detection of kB-GFP reporter expression in JPM50.6 cells using an Accuri C6 flow cytometer (BD), or by dual luciferase assay in Jurkat T cells (Promega) using a Tecan Infinite M200 Multimode Microplate Reader. Relative NF-kB activation in Jurkat transfectants was calculated by normalizing the relative ratio of firefly to Renilla luciferase signals. p-S6 was measured 24 h after stimulation via intracellular flow cytometry as previously described (26), using an Alexa Fluor 488–conjugated anti-p-S6 Ab (2F9, Cell Signaling Technology) and an Accuri C6 cytometer.

**Immunoprecipitations and immunoblotting.** Transfected cell lysates were prepared in 1% NP-40 lysis buffer as previously described (27). To verify CARD11 expression, lysates (5–20 µg) were separated on 4–20% Tris-Glycine SDS gels (Bio-Rad), transferred to nitrocellulose (TransBlot Turbo, Bio-Rad). Blocked membranes were probed with the following antibodies: anti-FLAG (M2) and anti-β-actin (AC-15, Sigma); anti-CARD11 (1D12, Cell Signaling Technology); anti-BCL10 (A-6), anti-MALT1, (H-300), anti-CYLD (E-10, Santa Cruz). Antibodies were detected using HRP-conjugated secondary Abs (Southern Biotech) and Pierce ECL (Thermo Scientific). To assay MALT1-dependent cleavage of CYLD, transfected cells were stimulated with 50 ng/mL PMA and 1 µM ionomycin for 2 h before lysis. For CBM complex IPs, cells were stimulated for 15 min with PMA/ionomycin as above, lysed in 0.1% Triton X-100 buffer (20 mM Tris, pH 8, 150 mM NaCl, 5 mM NaF, 5 mM sodium glycerophosphate, 1 mM DTT + Protease Complete/PhosStop inhibitors (Roche)), and incubated overnight with 2 µg anti-BCL10 (A-6) at 4 °C. Protein G Sepharose beads (20 µL, Sigma) were then added and rocked for 2 h at 4 °C. Beads were washed three times in lysis buffer and boiled before SDS-PAGE and immunoblotting.

Primary human naive CD4+ T cells were isolated from PBMCs using negative selection MACS microbeads (Miltenyi Biotec) per the manufacturer’s protocol. Naive cells were stimulated in coated anti-CD3 (OKT3, eBioscience) plate for 48 h in RPMI plus 10% FBS before lysis and SDS-PAGE. Anti-ASCT2 antibody (D7C12, Cell Signaling Technology) was used for detection. Full-length immunoblots are shown in Supplementary Data.

**Statistics.** Sample size was dictated by the small number of patients identified with CARD11 mutations (8 patients, 4 mutations). For Jurkat cell transfections, paired, one-way Student’s t-tests were used to test whether GFP or p-S6 signals (percentage positive and mean fluorescence intensity (MFI)) induced by each putative LOF CARD11 mutant were significantly less than wild-type. Variance (s.d. for percentage positive, s.e.m. for MFI) was comparable for each transfected construct. P values are included in each figure legend (except Fig. 2j, which are as follows: vs. EV (unstimulated): E57D \( P = 0.015 \); L194 \( P = 0.015 \); R975W = 0.014; dup183_196 = 0.02; vs. EV (stimulated): E57D \( P = 0.004 \); L194 \( P = 0.0014 \); R975W \( P = 0.02 \), dup183_196 \( P = 0.006 \); vs. WT (unstimulated): E57D \( P = 0.032 \); L194 \( P = 0.002 \); R975W \( P = 0.005 \); dup183_196 \( P = 5.3 \times 10^{-4} \); vs. WT (stimulated): E57D \( P = 0.001 \); L194 \( P = 0.013 \); R975W \( P = 0.002 \); dup183_196 \( P = 0.009 \). There are no P values for Figures 4–6.

**Data availability.** The data sets generated and analyzed during the current study are available from the corresponding author on reasonable request. Exome and genome sequencing data have been deposited in dbGaP under accession phs001369.v1.p1.
Corrigendum: Germline hypomorphic \textit{CARD11} mutations in severe atopic disease

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In the version of this article initially published online, the name of author Neil Romberg appeared incorrectly as Neil D Romberg, and the affiliation of author Nina Jones was incorrect and should have appeared as Clinical Research Directorate/Clinical Monitoring Research Program, Leidos Biomedical Research, Inc., NCI Campus at Frederick, Frederick, Maryland, USA. In addition, the following sentences were omitted from the Acknowledgments: “This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organizations imply endorsement by the US government.” These errors have been corrected in the print, PDF and HTML versions of this article.