

Decreased T-cell receptor signaling through CARD11 differentially compromises forkhead box protein 3–positive regulatory versus T_H2 effector cells to cause allergy

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Background: Allergy, the most common disease of immune dysregulation, has a substantial genetic component that is poorly understood. Although complete disruption of T-cell receptor (TCR) signaling causes profound immunodeficiency, little is known about the consequences of inherited genetic variants that cause partial quantitative decreases in particular TCR-signaling pathways, despite their potential to dysregulate immune responses and cause immunopathology.

Objective: We sought to elucidate how an inherited decrease in TCR signaling through CARD11, a critical scaffold protein that signals to nuclear factor κ B (NF- κ B) transcription factors, results in spontaneous selective accumulation of large numbers of T_H2 cells. **Methods:** “Unmodulated” mice carry a *Card11* single nucleotide variant that decreases but does not abolish TCR/CD28 signaling to induce targets of NF- κ B. The consequences of this mutation on T-cell subset formation *in vivo* were examined, and its effects within effector versus regulatory T-cell subsets were dissected by the adoptive transfer of wild-type cells and by the examination of forkhead box protein 3 (*Foxp3*)–deficient unmodulated mice.

Results: Unlike the pathology-free boundary points of complete *Card11* sufficiency or deficiency, unmodulated mice have a specific allergic condition characterized by increased IgE levels and dermatitis. The single nucleotide variant partially decreases both the frequency of Foxp3⁺ regulatory T cells and the efficiency of effector T-cell formation *in vivo*. These intermediate effects combine to cause a gradual and selective expansion of T_H2 cells.

Conclusions: Inherited reduction in the efficiency of TCR–NF- κ B signaling has graded effects on T-cell activation and Foxp3⁺ regulatory T-cell suppression that result in selective T_H2 dysregulation and allergic disease. (J Allergy Clin Immunol 2011;■■■:■■■–■■■.)

Key words: *Card11*, genetic variation, T-cell receptor signaling, nuclear factor κ B, T_H1/T_H2, regulatory T cells, dermatitis, IgE

Atopic dermatitis, rhinitis, asthma, food allergy, and anaphylaxis are allergic diseases thought to share immunopathogenic mechanisms. The production of IgE in response to ubiquitous environmental antigens is the best predictor for allergy, and because class-switch recombination to IgE depends on production of IL-4 and IL-13 by T_H2 cells,¹ disordered T_H2 differentiation is thought to be central to its pathogenesis.² Compared with other isotypes, serum IgE concentrations are normally very low, and IL-4–producing T_H2 effector/memory cells are likewise normally infrequent *in vivo*.³ These observations imply that T_H2 differentiation is normally kept under tight and specific regulatory control; however, the nature of this control and the ways in which it becomes selectively subverted in atopic subjects remain poorly understood.⁴

The forkhead box protein 3 (Foxp3)–positive CD25⁺ regulatory T (Treg)–cell subset has emerged as an essential mediator of immune regulation and homeostasis^{5–8} and, on dysregulation, as a candidate to explain allergy.^{4,9} Null mutations in *FOXP3* block the formation of this subset and result in a rare Mendelian disease known as immunodysregulation, polyendocrinopathy, and enteropathy, X-linked (IPEX) syndrome (OMIM #304790),^{10–12} which includes the clinical and histologic hallmarks of severe eczema, food allergy, and high levels of plasma IgE.¹³ However, these allergic manifestations arise as part of a distinct genetic syndrome that also comprises severe intestinal inflammation, autoimmune type 1 diabetes, thyroid disease, lymphoproliferation, and autoimmune cytopenias occurring early in life, which confers a grave prognosis. Whether impaired Treg cell function can also explain the selective T_H2 dysregulation and more limited spectrum of pathology that characterizes common allergic disease remains unclear.

Single nucleotide variants (SNVs) represent the major form of human genetic variation and typically act singly or in combination to cause partial quantitative changes in gene product activity (“hypomorphic alleles”) rather than null alleles or “knock-outs.”¹⁴ A great number of genes are required for the events after T-cell antigen receptor engagement, and these events govern many critical T-cell processes, including T-cell differentiation,

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Abbreviations used

Foxp3: Forkhead box protein 3
 IRF4: Interferon regulatory factor 4
 NF- κ B: Nuclear factor κ B
 PE: Phycoerythrin
 PKC: Protein kinase C
 SNV: Single nucleotide variant
 TCR: T-cell receptor
 Teff: Effector T
 Treg: Regulatory T
 ZAP-70: ζ Chain-associated protein, 70 kd

selection, Treg cell formation, and peripheral activation and effector formation. It follows that SNVs affecting signaling components downstream of T-cell receptor (TCR)/CD28 represent a likely set of candidates for the genetic predisposition to immunopathology. However, although the consequences of gene knock-outs in many TCR-signaling components are known, almost nothing is known about the consequences of SNVs causing partial hypomorphic alleles. A straightforward extrapolation from knock-out mutations would predict that partial defects might diminish both T-cell activation and Treg cell function and cancel one another out in a "one step forward, one step backward" manner. Nevertheless, the possibility that quantitative variation will have paradoxical outcomes is illustrated by the central TCR-signaling component, ζ chain-associated protein, 70 kd (ZAP-70), tyrosine kinase, in which null alleles result in severe immunodeficiency but SNVs that diminish but do not abolish the enzyme's activity partially decrease thymic Treg cell formation and negative selection while preserving sufficient T-cell activation to cause autoimmunity.¹⁵⁻¹⁷ If we are to interpret patterns of human genetic variation revealed by genome-wide resequencing and association studies, it will be essential to test experimentally the consequences of graded decreases in different TCR signaling pathways.

Downstream of ZAP-70, TCR-signaling events that activate the transcription factor nuclear factor κ B (NF- κ B) are essential for T-cell activation and Treg cell formation and involve numerous genes and proteins. TCR and CD28 signaling activates protein kinase C (PKC) θ to phosphorylate the signaling scaffold protein CARD11 (caspase recruitment domain, family member 11, also known as *Carma1*), which recruits Bcl10 and other proteins to activate the I κ B kinase and trigger nuclear translocation of NF- κ B. Null mutations in *Card11* or *Bcl10* completely disrupt TCR-CD28 signaling to NF- κ B and abolish T-cell activation to cause profound immunodeficiency in the absence of any allergic or autoimmune pathology, despite simultaneously causing a complete absence of Foxp3⁺ Treg cells.¹⁸⁻²¹ Although the effects of complete disruption of TCR-NF- κ B signaling on T-cell activation cancel out the effects on Foxp3⁺ Treg cells, it is not known how these opposing functions of TCR-NF- κ B signaling will behave in response to partial defects in the pathway, which are likely to be more common.

Here we examine the cellular basis of spontaneous skin disease resembling atopic dermatitis and increased IgE production in mice carrying an SNV in *Card11* that decreases TCR-NF- κ B signaling.²² We show that the SNV partially decreases both the frequency of Foxp3⁺ Treg cells and the efficiency of T-cell activation and effector cell formation but that these opposing effects do not cancel one another out. Instead, they combine to produce a gradual and selective expansion of T_H2 cells and allergic pathology without autoimmunity

or generalized inflammation. Our results establish that the TCR-NF- κ B signaling pathway is sensitive to inherited variation that decreases its activity, manifesting as selective T_H2 dysregulation corresponding to the spectrum of common allergic disease.

METHODS

Male Foxp3^{null} mice on the C57BL/6 background²³ were analyzed at 15 to 25 days of age, and Foxp3^{+/-null} carrier females were used for breeding. Unmodulated mice were generated by means of ENU mutagenesis on the C57BL/6 background and have been described previously.²² Apart from Foxp3^{null} pups, all mice were at least 6 weeks of age at the time of analysis, and as far as possible, mice of different genotypes were age and sex matched within experiments and often littermates. Mice were monitored daily for dermatitis, which typically presented as reddening and thickening of the skin on the ear, neck, and face accompanied by scratching of the affected areas. All animal procedures were approved by the Australian National University Animal Ethics and Experimentation Committee.

Ex Vivo stimulation and intracellular staining

Lymphocytes from pooled lymph nodes (cervical, inguinal, axillary, and brachial) or peripheral blood (briefly pretreated with Tris ammonium chloride buffer to lyse erythrocytes) were suspended in RPMI media containing 10% FCS (Gibco, Carlsbad, Calif). Cells were plated at 5×10^5 cells per well in 96-well tissue-culture plates in 200 μ L of media containing phorbol myristate acetate (50 ng/mL; Sigma, St Louis, Mo), ionomycin (500 ng/mL, Sigma), and GolgiStop (1:1,000; BD, Franklin Lakes, NJ) and left to accumulate cytokines for 4 hours at 37°C. After stimulation, cells were harvested, washed, and surface stained for 20 minutes at 4°C with Alexa Fluor 700-conjugated anti-mouse CD4 (clone RM4-5; BioLegend, San Diego, Calif) and Pacific Blue-conjugated anti-mouse CD44 (clone IM7, BioLegend). Cells were fixed and permeabilized with the eBioscience intracellular staining kit and then stained for 20 minutes at 4°C with allophycocyanin-conjugated anti-mouse IFN- γ (clone XMGI.2; eBioscience, San Diego, Calif), phycoerythrin (PE) Cy7-conjugated anti-mouse IL-4 (clone BVD6-24G2, eBioscience), fluorescein isothiocyanate-conjugated anti-mouse IL-17A (clone TC11-18H10.1, BioLegend), fluorescein isothiocyanate-conjugated anti-mouse Foxp3 (clone FJK-16a, eBioscience), Alexa Fluor 488-conjugated anti-mouse interferon regulatory factor 4 (IRF4; clone IRF4.3E4, BioLegend), and/or Alexa Fluor 488-conjugated anti-mouse GATA3 (clone L50-823, BD). After staining, cells were washed and then read on an LSR II flow cytometer (BD). FlowJo software (TreeStar, Inc, Ashland, Ore) was used for analysis.

IgE ELISA

Plates were coated overnight with 1 μ g/mL anti-IgE capture antibody (clone R35-72, BD). The capture antibody was removed, plates were blocked with 1% BSA and washed, and then plasma samples (diluted 1:20) and standards were added, followed by a 1-hour incubation at 37°C. Plates were then washed, and 2 μ g/mL biotinylated anti-IgE detection antibody (clone R35-118, BD) was added. Binding was detected by means of subsequent incubation with streptavidin-conjugated alkaline phosphatase (Vector Laboratories, Burlingame, Calif), followed by the addition of phosphatase substrate tablets (Sigma) in phosphatase/glycine buffer. Plates were developed for 1 hour at 37°C, and optical densities at 405 nm were measured with a plate reader (Molecular Devices, Eugene, Ore). IgE quantification was calculated according to calibration curves based on the optical densities for a series of dilutions of a control IgE isotype antibody of known concentration (clone C38-2, BD).

Overnight stimulation

Splenocytes from *Card11^{unim}* or wild-type mice were plated at 1×10^6 cells per well in 96-well tissue culture plates precoated with 1 μ g/mL anti-mouse CD3 (clone 145-2C11, BD) in 200 μ L of media containing 10 μ g/mL soluble anti-mouse CD28 (clone 37.51, BD). After stimulation overnight, cells were harvested, washed, surface stained for 20 minutes at 4°C with Alexa Fluor

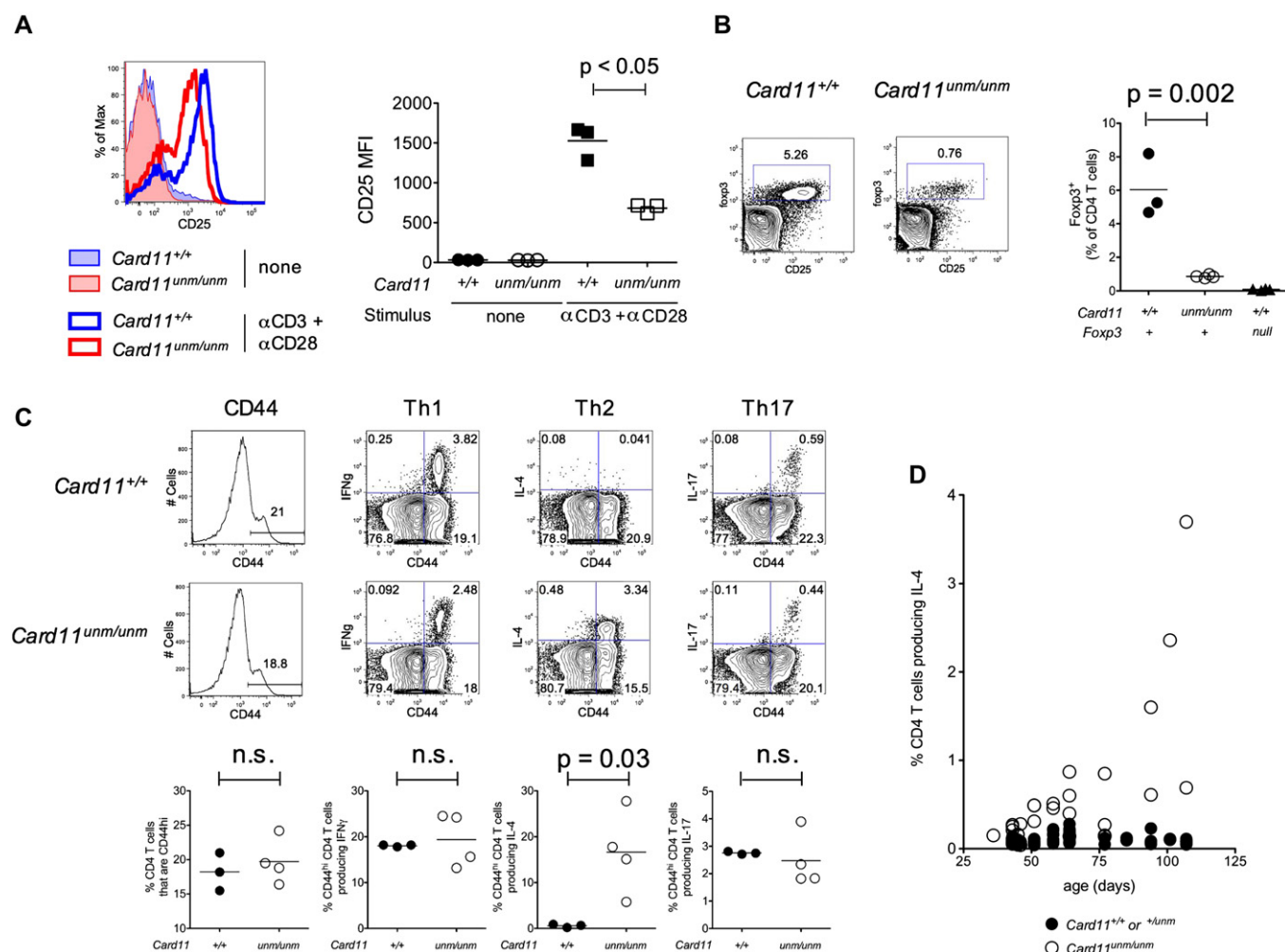


FIG 1. A, Histograms and plotted median fluorescence intensity (MFI) of CD25 expression on CD4⁺ T cells from *Card11*^{unm} or wild-type mice after overnight CD3 plus CD28 stimulation. Replicates are from 3 separate stimulated wells. B, Treg cell abundance in lymph nodes from wild-type or *Card11*^{unm} mice determined by means of intracellular staining for Foxp3 gated on CD4⁺ T cells. C, Expression of CD44, IFN- γ , IL-4, and IL-17 by lymph node CD4⁺ T cells from *Card11*^{unm} and wild-type mice after 4 hours' *ex vivo* phorbol 12-myristate 13-acetate plus ionomycin stimulation and intracellular staining. D, IL-4-producing CD4⁺ T-cell frequency in blood from *Card11*^{unm} mice of a range of ages and their wild-type or heterozygous littermates detected by means of intracellular staining as above. n.s., Not significant.

700-conjugated anti-mouse CD4 (clone RM4-5, BioLegend) and PE-conjugated anti-mouse CD25 (clone PC61; Caltag, Burlingame, Calif), and then read on the flow cytometer.

T-cell adoptive transfer

Total CD4⁺ T cells (CD4⁺), Treg cells (CD4⁺CD25⁺), or effector T (Teff) cells (CD4⁺CD25⁺CD62L⁺CD44⁺) were sorted from the spleens of CD45.1 donor mice by using a FACSARIA cell sorter (BD). Total CD4⁺ T cells (10⁶) or Treg or Teff cells (10⁵) were suspended in 200 μ L of PBS and injected intravenously into the tail veins of recipient mice. Recipients were then monitored for dermatitis and bled retro-orbitally to monitor the T-cell phenotype. CD4⁺ T-cell differentiation was assessed by means of *ex vivo* stimulation and intracellular staining (as previously described), and donor cells were identified by means of positive staining with PE-conjugated anti-mouse CD45.1 (clone A20, BD).

Statistical analysis

Unpaired *t* tests were used to compare 2 groups of data. Where there were more than 2 groups, 1-way ANOVA followed by the Bonferroni selected-pairs

posttest was applied. The α value for significance was set at .05. GraphPad Prism (GraphPad Software, Inc, La Jolla, Calif) was used for statistical analysis.

RESULTS

Selective TH2 cell expansion and partial Treg cell deficiency caused by an inherited reduction in TCR/CD28-NF- κ B signaling

"Unmodulated" mice are homozygous for an SNV (*Card11*^{unm}) that causes an amino acid substitution in the coiled-coil domain of CARD11,²² a critical intracellular scaffolding protein for signaling by TCR and CD28 to activate canonical NF- κ B transcription factors.^{19,24,25} Whereas the *Card11* knockout abolishes TCR/CD28-induced expression of NF- κ B target genes, such as *Il2ra* (encoding CD25),^{19,24,25} the *Card11*^{unm} mutation is hypomorphic²² and decreases but does not abolish signaling through this pathway (Fig 1, A).

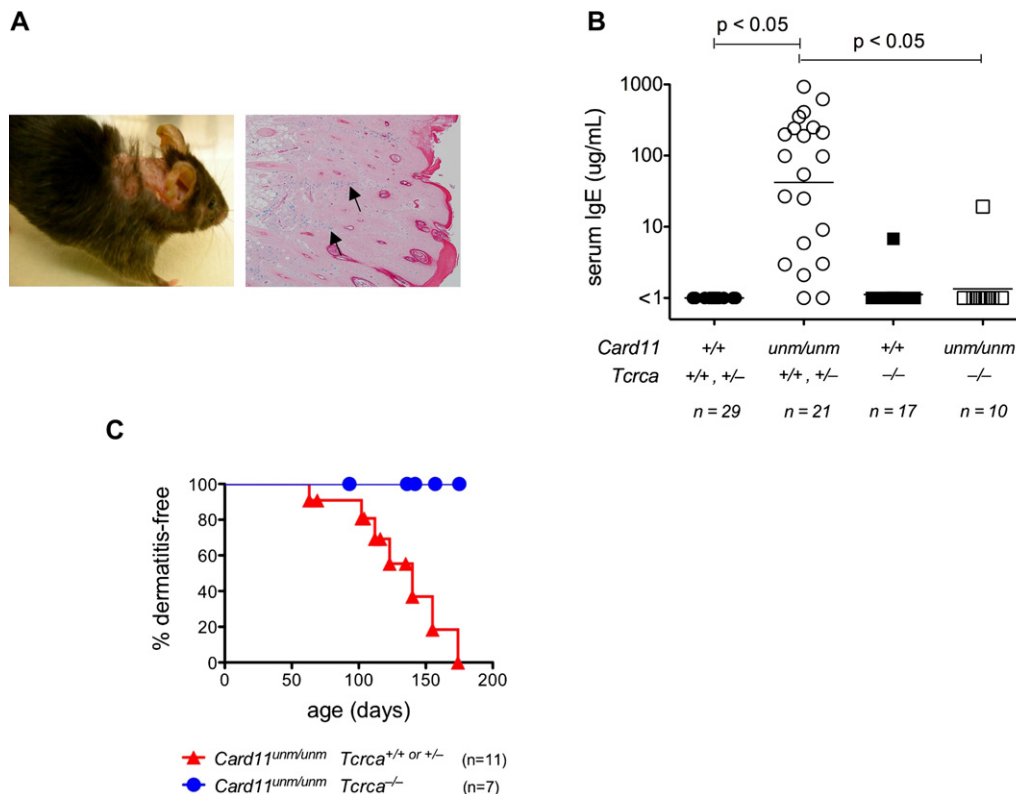


FIG 2. **A**, Macroscopic appearance and histology of an affected *Card11*^{unm} mouse showing pruritic skin lesion and mast cell infiltrates (arrows) revealed by Alcian blue staining. **B**, Plasma IgE concentrations in $\alpha\beta$ T cell-sufficient or $\alpha\beta$ T cell-deficient mice carrying either wild-type or mutant alleles of *Card11*, as quantified by means of ELISA. **C**, Incidence of dermatitis in $\alpha\beta$ T cell-sufficient or $\alpha\beta$ T cell-deficient *Card11*^{unm} mice.

Development and thymic selection of conventional CD4⁺ and CD8⁺ T cells proceeds normally in unmodulated mice,^{22,23} whereas the formation of Foxp3⁺ Treg cells is compromised, and these cells occur in the periphery at about one seventh the frequency found in wild-type control animals (Fig 1, B). A decrease in the Foxp3⁺ Treg cell frequency is also observed in the thymuses of *Card11*^{unm} mice (see Fig E1 in this article's Online Repository at www.jacionline.org). The reduction in Treg cell frequency in *Card11*^{unm} mice contrasts with the complete absence of this subset in *Card11*-knockout animals,²¹ which is consistent with the hypomorphic nature of *Card11*^{unm}. Foxp3⁺ Treg cells in *Card11*^{unm} mice express reduced levels of CD25 and cytotoxic T lymphocyte-associated antigen 4 but normal or slightly increased levels of Foxp3, IRF4, and glucocorticoid-induced TNF-related receptor (see Fig E1).

In contrast to the massive expansion of activated T cells seen in mice lacking Foxp3⁺ Treg cells,²³ *Card11*^{unm} mice do not experience generalized T-cell activation and have no significant increase in the percentages of CD44^{hi}CD4⁺ T cells and CD44^{hi}CD4⁺ T cells producing IFN- γ or IL-17. However, *Card11*^{unm} mice had a dramatically increased frequency of IL-4-producing CD4⁺ T cells (Fig 1, C). The IL-4⁺ T-cell population gradually increases with age, accounting for between 0.5% and 4% of CD4⁺ T cells in 100-day-old mutant animals but less than 0.1% in control mice (Fig 1, D). These IL-4⁺CD4⁺ T cells express high levels of GATA3, the master transcriptional regulator of T_H2 cells (see Fig E2 in this article's Online Repository at

www.jacionline.org). In *Card11*^{unm} mice carrying an OT-II TCR transgene, an expanded IL-4⁺ population likewise develops, but this expanded population derives exclusively from CD4⁺ T cells bearing endogenously rearranged (V β 5⁻) TCRs (see Fig E3 in this article's Online Repository at www.jacionline.org), suggesting the need for specific antigenic stimulation rather than a generalized T-cell activation into T_H2 cells.

T_H2 cell expansion leads to selective allergic disease

The gradual and selective expansion of T_H2 cells in *Card11*^{unm} mice precedes the development of allergic disease. This is characterized by a substantial increase in plasma IgE levels and erosive dermatitis of the neck and ear with prominent acanthosis, parakeratosis, and hyperkeratotic scale and heavy infiltration by mast cells (Fig 2, A and B). The dermatitis is highly penetrant but of relatively late onset, with a median incidence at approximately 130 days of age (Fig 2, C). However, we could not find evidence of colitis, diabetes, or other inflammatory lesions typical of complete Treg cell deficiency. Moreover, in contrast to mice harboring a partial defect in TCR signaling at the more proximal point, ZAP-70,¹⁷ test results for antinuclear autoantibodies were uniformly negative (data not shown). When crossed to a *Tcrca*-null allele, $\alpha\beta$ T-cell deficiency abolished both excessive IgE production and dermatitis in *Card11*^{unm} mice (Fig 2, B and C). By contrast, B-cell deficiency introduced by crossing to a *Cd79a*-null allele eliminated IgE production but did not prevent dermatitis (data not shown). Hence $\alpha\beta$ T cells are required to

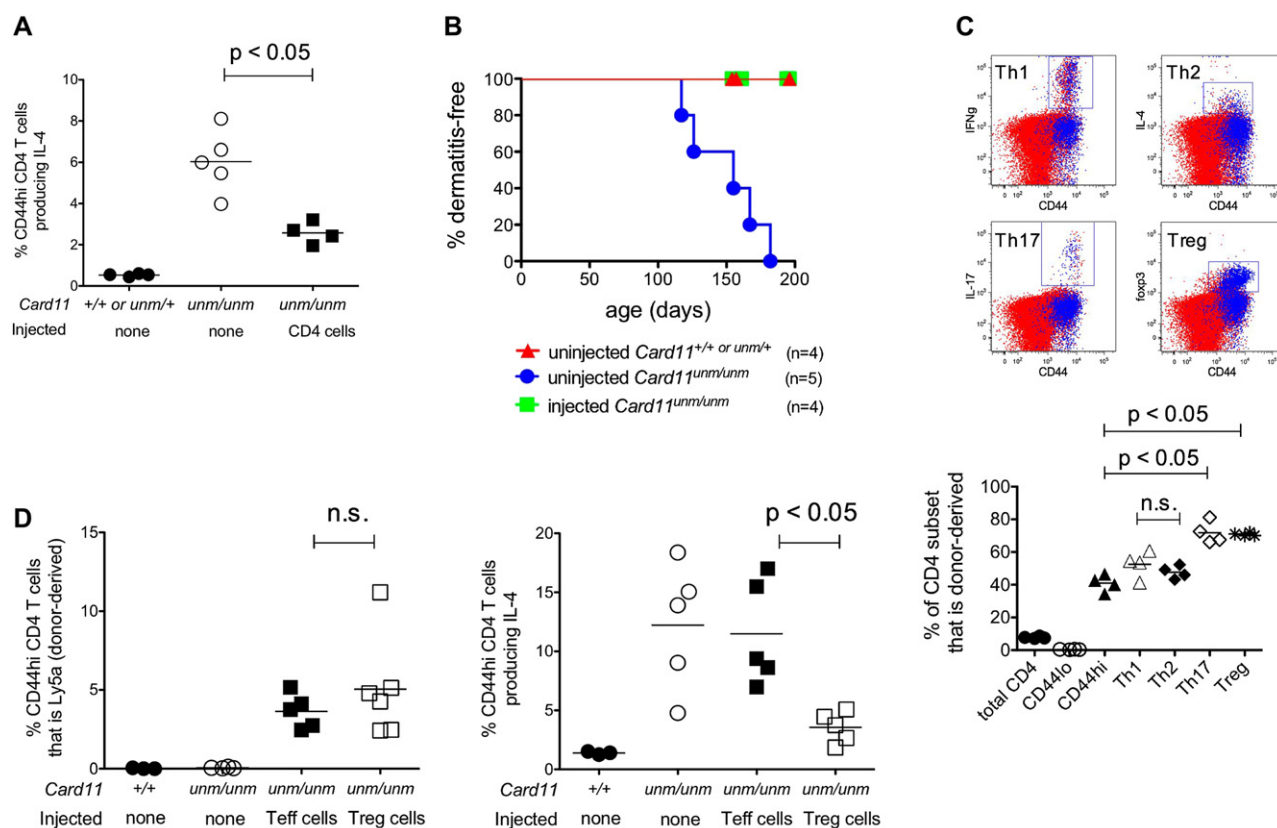


FIG 3. **A** and **B**, Percentage of IL-4-producing cells among lymph node CD44^{hi}CD4⁺ T cells (Fig 3, **A**) or incidence of dermatitis (Fig 3, **B**) in *Card11*^{unnm} mice that were either untreated or injected intravenously with 10⁶ sorted CD4⁺ T cells from a wild-type Ly5a donor 10 weeks prior, as measured previously. **C**, Representative plots (*upper*) and quantitation (*lower*) showing the representation of donor-derived (Ly5a⁺, shown in blue) and recipient-derived (Ly5b⁺, shown in red) cells among lymph node CD44^{lo}, CD44^{hi}, Th1, Th2, Th17, or Treg cell subsets in the recipient mice described in Fig 3, **A**. **D**, Representation of donor-derived (Ly5a⁺) cells (*left*) or total IL-4-producing cells (*right*) among the CD44^{hi}CD4⁺ T-cell compartment in the peripheral blood of *Card11*^{unnm} mice that were either untreated or injected intravenously with 10⁶ sorted Treg (CD4⁺CD25⁺) or Teff (CD4⁺CD25⁻CD44^{hi}CD62L^{lo}) cells 10 weeks prior. Data are representative of 2 independent experiments. *n.s.*, Not significant.

drive increased IgE production and dermatitis as 2 independent downstream events.

Transfer of wild-type Treg cells prevents T_H2 expansion

We next investigated whether reduced Treg cell function is responsible for the T_H2 cell expansion in *Card11*^{unnm} mice by reconstituting this subset. First, we adoptively transferred CD4⁺ T cells that were FACS sorted from congenically marked wild-type donors into young *Card11*^{unnm} recipients and monitored them for T_H2 cell accumulation and dermatitis. Transfer of 10⁶ unfractionated wild-type CD4⁺ T cells was sufficient to significantly reduce T_H2 cell expansion assessed 10 weeks after transfer (Fig 3, **A**). Moreover, whereas all untreated control animals succumbed to dermatitis by 150 to 200 days of age, recipients of wild-type CD4⁺ T cells showed no signs of disease (Fig 3, **B**).

Donor-derived wild-type cells (blue dots in Fig 3, **C**) formed a very low proportion of CD44^{lo}CD4⁺ T cells in the *Card11*^{unnm} recipients, as expected given that a relatively small number of donor T cells were being diluted into a large recipient population. In contrast, donor-derived cells accounted for approximately 40%

of the CD44^{hi}CD4⁺ T-cell subset (Fig 3, **C**), representing considerable expansion beyond the 10⁶ input cells and indicating that they had undergone extensive proliferation into effector/memory cells. Although normal frequencies of CD44^{hi} T cells are present in unmanipulated *Card11*^{unnm} mice, the repopulation of this subset by wild-type cells could be explained by a relative defect in *Card11*^{unnm} T-cell activation or effector/memory cell persistence that becomes apparent under competitive repopulation conditions. Repopulation by wild-type T cells occurred comparably in the Th1 and Th2 subsets of CD44^{hi}CD4⁺ cells (Fig 3, **C**). In contrast, repopulation by wild-type cells was more extreme in the Th17 and Treg cell subsets, in which donor cells comprised approximately 70%. Repopulation of the Foxp3⁺ Treg cell population by donor cells increased the frequency of Treg cells relative to that seen in uninjected *Card11*^{unnm} mice, although not to the frequency seen in wild-type control animals (see Fig E4 in this article's Online Repository at www.jacionline.org).

To test whether the transferred Teff or Treg cell populations were responsible for the ameliorated T_H2 phenotype in the recipient mice, we repeated the transfer experiment using fractionated wild-type Treg (CD4⁺CD25⁺) or Teff (CD4⁺CD25⁻CD44^{hi}CD62L^{lo}) donor T cells. After transfer, both populations retained their respective

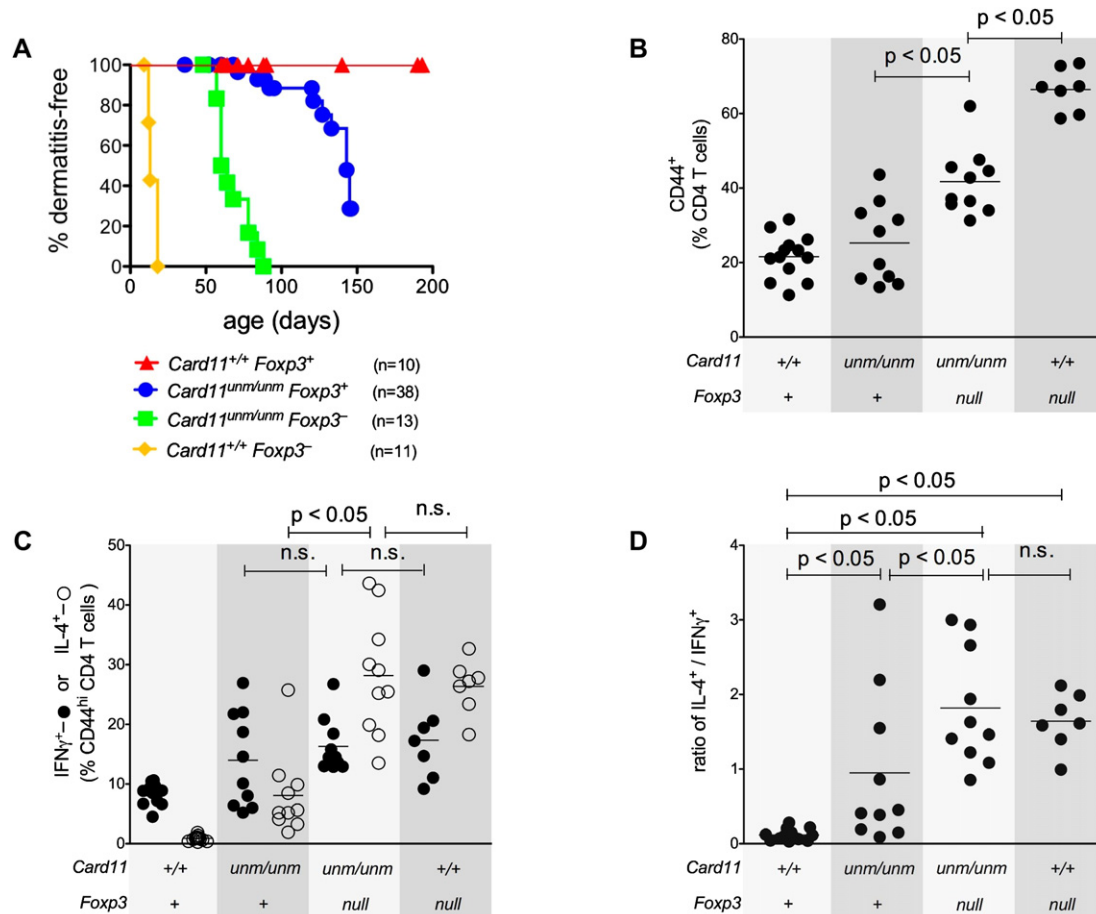


FIG 4. A, Incidence of dermatitis in *Card11*^{unm}*Foxp3*^{null} mice compared with single-mutant and wild-type control animals. B–D, Quantitation of CD4⁺ T-cell expression of CD44, IFN-γ, and IL-4 or the ratio of IL-4⁺ cells/IFN-γ⁺ cells from mice of the 4 genotypes measured by means of *ex vivo* stimulation and intracellular cytokine staining, as previously described. Data are collated from 4 independent experiments. n.s., Not significant.

Foxp3⁺ and *Foxp3*⁻ phenotypes (data not shown) and became similarly engrafted into the recipients' CD44^{hi}CD4⁺ T-cell compartments (Fig 3, D). T_H2 cell accumulation in *Card11*^{unm} recipients was unaffected by transfer of wild-type Teff cells, but it was suppressed by the transfer of wild-type Treg cells. Thus T_H2 cells accumulate because of the partial Treg cell deficiency in *Card11*^{unm} mice.

Differentiating the effect of decreased TCR–CD28–CARD11–NF-κB signaling within Teff cells from its effect on Treg cells

In *Foxp3*^{null} mice there is massive T-cell activation and differentiation into T_H1 and T_H2 cells, autoimmunity, and early lethality, whereas *Card11*^{unm} animals have normal proportions of activated T cells with a selectively expanded T_H2 population and late-onset allergic disease without autoimmunity. These disparate phenotypes might be explained by the presence of some Treg cells in *Card11*^{unm} mice, by the decrease in TCR/CD28 signaling for T-cell activation and Teff cell formation caused by *Card11*^{unm}, or both. To dissect the contributions of these effects, we bred *Card11*^{unm}*Foxp3*^{null} double mutants and compared them with single-mutant control animals in terms of disease severity

(measured based on dermatitis incidence) and CD4⁺ T-cell activation/differentiation state (Fig 4 and see Fig E5 in this article's Online Repository at www.jacionline.org).

The effect of the remaining Treg cells in *Card11*^{unm} mice was illuminated by comparing the 2 groups of mice matched for the *Card11* defect: *Card11*^{unm}*Foxp3*⁺ mice with a reduced Treg cell frequency versus *Card11*^{unm}*Foxp3*^{null} mice with complete Treg cell deficiency. Dermatitis occurred at a younger age in *Card11*^{unm}*Foxp3*^{null} double mutants (Fig 4, A, green curve) than in *Card11*^{unm}*Foxp3*⁺ mice (Fig 4, A, blue curve). *Card11*^{unm}*Foxp3*^{null} animals with complete Treg cell deficiency had a significantly increased proportion of CD44^{hi} T cells (Fig 4, B), and the proportion of CD44^{hi} cells that produced IL-4, but not IFN-γ, was also increased. Consequently, although there was some variation between mice, the absence of unmodulated Treg cells led to a significant increase in the average ratio of IL-4 to IFN-γ producers in *Card11*^{unm}*Foxp3*^{null} versus *Card11*^{unm}*Foxp3*⁺ mice (Fig 4, D). These results indicate that the remaining Treg cells in *Card11*^{unm} animals play an active role in restraining T-cell cytokine production and delaying disease but do not account for the T_H2 bias because their removal does not normalize this bias but might in fact exaggerate it.

The effect of the *Card11*^{unm} mutation within Teff cells was illuminated by comparing the 2 groups of mice with no Treg cells:

Card11⁺*Foxp3*^{null} mice with normal TCR–NF-κB signaling in Teff cells and *Card11*^{unm}*Foxp3*^{null} double mutants with lower TCR–NF-κB signaling. Whereas *Card11*⁺*Foxp3*^{null} mice had dermatitis and the typical *scurfy* phenotype by 15 days of age (Fig 4, A, yellow curve), the median onset of dermatitis was delayed to approximately 70 days of age in *Card11*^{unm}*Foxp3*^{null} mice (Fig 4, A, green curve). Moreover, whereas approximately 70% of CD4⁺ T cells were CD44^{hi} in *Card11*⁺*Foxp3*^{null} mice, this was decreased to approximately 40% in *Card11*^{unm}*Foxp3*^{null} mice (Fig 4, B). Despite this amelioration of the generalized T-cell activation by *Card11*^{unm}, the proportion and ratio of CD44^{hi}CD4⁺ T cells that produced IFN-γ or IL-4 did not change between *Card11*^{unm}*Foxp3*^{null} and *Card11*⁺*Foxp3*^{null} mice (Fig 4, C). Thus in the complete absence of Treg cells, *Card11*^{unm} reduces the efficiency of Teff cell formation but does not change the T_H2/T_H1 bias of the effectors that form.

These results, together with those of the cell-transfer experiments, indicate that reduced Treg cell function is sufficient to explain the preferential dysregulation of T_H2 cells in *Card11*^{unm} animals. This is supported by a consideration of T_H2/T_H1 ratios in mice of the different genotypes (Fig 4, D). Wild-type mice showed a T_H1-dominated state with a 1:6 bias of IL-4 producers to IFN-γ producers. In contrast, the T_H2/T_H1 ratio was significantly shifted toward T_H2 in each of the *Card11*^{unm}*Foxp3*⁺, *Card11*^{unm}*Foxp3*^{null}, and *Card11*⁺*Foxp3*^{null} mice. In other words a change toward a T_H2 bias was observed in mice of all 3 genotypes that have Treg cell deficits, including completely Treg cell-deficient mice with wild-type *Card11*.

DISCUSSION

How can quantitative genetic variation predispose to the selective T_H2 dysregulation of allergy? Extrapolating from knockout mutations, partial defects in the complex signaling pathway between the TCR and NF-κB might be predicted to diminish both T-cell activation and Treg cell function and cancel one another out in a “one step forward, one step backward” manner. However, here we show that an intermediate reduction in TCR–NF-κB signaling through *Card11* confers partial and non-counterbalancing defects in Teff and Treg cells, leading to selective T_H2 cell expansion and allergic dermatitis. We demonstrate that the selective T_H2 dysregulation can be explained as the product of 2 opposing effects of decreased TCR–NF-κB signaling: (1) partial Teff cell deficiency that has an equal effect slowing the accumulation of T_H1, T_H2, and T_H17 effectors and (2) partial Treg cell deficiency interfering with normal Treg cell function sufficiently to allow dramatically increased T_H2 cell accumulation and dermatitis but preserving enough Treg cell function to prevent lymphoproliferation, colitis, and autoimmunity. Our results establish that the opposing functions of the TCR/CD28 signaling pathway are prone to imbalance resulting from genetic variation that decreases its activity, with the imbalance presenting as a selective T_H2 cell accumulation disease corresponding to the spectrum of common allergy.

Card11^{unm} mice have partial Treg cell deficiency, which leads to spontaneous T_H2 cell expansion and a late-onset allergic disorder comprising selective IgE hyperproduction, mastocytosis, and dermatitis (Figs 1 and 2). T_H2 cell formation in *Card11*^{unm} mice appears to be antigen driven and not a general effect on all CD4⁺ T cells because OVA-specific T cells did not adopt a T_H2 phenotype (see Fig E3). Although it will be important to map the

specificity of the emergent T_H2 cells, this result, together with the size of the response in the absence of active immunization, suggests that Treg cells normally actively restrain T_H2 differentiation in response to either self-antigens or commensals in the steady state.

Despite their spontaneous disease phenotype, *Card11*^{unm} mice are protected from the extreme lymphoproliferation, T-cell hyperactivation, autoimmunity, and early death of *Foxp3*^{null} mice because *Card11*^{unm} reduces the efficiency of Teff cell accumulation and retains some Treg cell activity (Figs 3 and 4). This contrasts with the *Card11*-null allele, which completely abolishes both T-cell activation and Treg cell formation and has no spontaneous immunopathological consequences.¹⁹ Our results therefore demonstrate how a partial defect in a pathway with pleiotropic functions, TCR/CD28 signaling to NF-κB, can produce an outcome that is shaped by unequal titration of opposing effects and that could not have been predicted from null alleles that completely disrupt the pathway.

CARD11 is one of many proteins required for normal TCR–CD28 signaling to activate NF-κB transcription factors and promote the activation and differentiation of Teff cells and the development and maintenance of Treg cells. TCR and CD28 signaling act through a cascade of tyrosine kinases, adaptor molecules, G proteins, and phospholipase Cγ1. These activate PKCθ, which then phosphorylates CARD11 and triggers an allosteric change that brings together Bcl10, MALT1 (mucosa associated lymphoid tissue lymphoma translocation gene 1), TAK1 (TGF-beta activated kinase 1, or mitogen-activated protein kinase kinase kinase 7), IKKγ (inhibitor of κB kinase γ), and other proteins that collectively activate the IκB kinase and trigger nuclear translocation of canonical NF-κB transcription factors.²⁶ Among the direct target genes activated by NF-κB in T cells are *Il2*, encoding the cytokine and T-cell growth factor IL-2, and *Il2ra*, encoding the α chain of the IL-2 receptor. Null mutations in *Cd28*,^{27,28} *Prkcg* (PKCθ),^{29,30} the c-Rel subunit of NF-κB,³¹ *Il2*,^{32,33} and *Il2ra*³³ decrease T-cell activation and the frequency of Treg cells and, in the case of *Il2* and *Il2ra*, cause an immunodysregulation, polyendocrinopathy, and enteropathy, X-linked-like syndrome of T-cell lymphoproliferation, multiorgan inflammation, and autoimmunity.^{34,35} These proteins provide numerous points at which subtle genetic variation could disproportionately reduce Treg cell function relative to Teff cell function to permit selective T_H2 overaccumulation and allergy.

Treg cells have been implicated in the regulation of T_H2 deviation. Wan and Flavell³⁶ reported that integration of a luciferase-containing construct into the *Foxp3* gene attenuated *Foxp3* expression 5- to 10-fold and led to a spontaneous outbreak of IL-4-producing CD4⁺ T cells (including the attenuated *Foxp3*⁺ cells themselves) and pathology. In that instance T_H2 pathology was interpreted as being the consequence of conversion of *Foxp3*⁺ Treg cells into T_H2 effector cells that in turn polarized other T cells to T_H2. Subsequently, Zheng et al³⁷ found that selective inactivation of *Irf4* within *Foxp3*⁺ cells resulted in spontaneous disease with plasma cell infiltration and selectively increased T_H2 cell frequencies and IgE titers.

Despite the observed T_H2 bias, both of these studies describe more severe syndromes of generalized T-cell activation and multiorgan inflammation than we observed in *Card11*^{unm} mice. Moreover, the mechanisms they demonstrate do not appear to account for the *Card11*^{unm} phenotype because expression of neither *Foxp3* nor *Irf4* was decreased in *Card11*^{unm} Treg cells (see Fig E1). In

contrast, our observation of a T_H2 bias in mice with wild-type Teff cells but completely lacking $Foxp3^+$ Treg cells ($Foxp3^{null}$ mice in Fig 4, C and D) suggests that the T_H2 bias in $Card11^{unm}$ mice can be explained by the Treg cell deficit alone, without the need to invoke particular qualitative defects in $Card11^{unm}$ Treg or Teff cells. This is supported by the observations in Fig 4, C and D, that (1) the proportion of activated cells that were of a T_H2 or T_H1 phenotype was similar between $Card11^{+/+}Foxp3^{null}$ and $Card11^{unm}Foxp3^{null}$ mice (arguing against a Teff cell-intrinsic bias caused by $Card11^{unm}$) and (2) the level of T_H2 bias was no greater in $Card11^{unm}Foxp3^+$ than $Card11^{unm}Foxp3^{null}$ mice (arguing against a Treg cell phenotype in $Card11^{unm}$ that is preferentially suppressive of T_H1 responses).

The relationship established here between decreased TCR-Card11-NF- κ B signaling, partial Treg cell deficiency, and selectively dysregulated accumulation of T_H2 cells *in vivo* provides a mechanistic basis to explain the growing number of inverse correlations between Treg cell numbers and allergy or atopy.³⁸⁻⁴⁰ Relative deficiency of Treg cells in cord blood predicts atopy later in childhood.⁴¹ Effective therapeutic maneuvers in patients with severe allergy are reported to correlate with increased Treg cell frequencies.⁴² Our findings invite future studies investigating associations between allergy, Treg cell frequency, and common or family-specific variation in the many genes encoding components of the TCR-CD28-CARD11-NF- κ B pathway. As a corollary, interventions that enhance TCR-NF- κ B signaling efficiency might be beneficial in the treatment or prevention of allergy.

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Key messages

- Graded decreases in particular TCR-signaling pathways can have paradoxical consequences that need to be tested experimentally to help interpret patterns of human genetic variation revealed by genome-wide resequencing and association studies.
- An inherited decrease in the efficiency of TCR-NF- κ B signaling causes graded decreases in T-cell activation and suppression by $Foxp3^+$ Treg cells that do not cancel one another out but instead result in selective T_H2 dysregulation and allergic disease.

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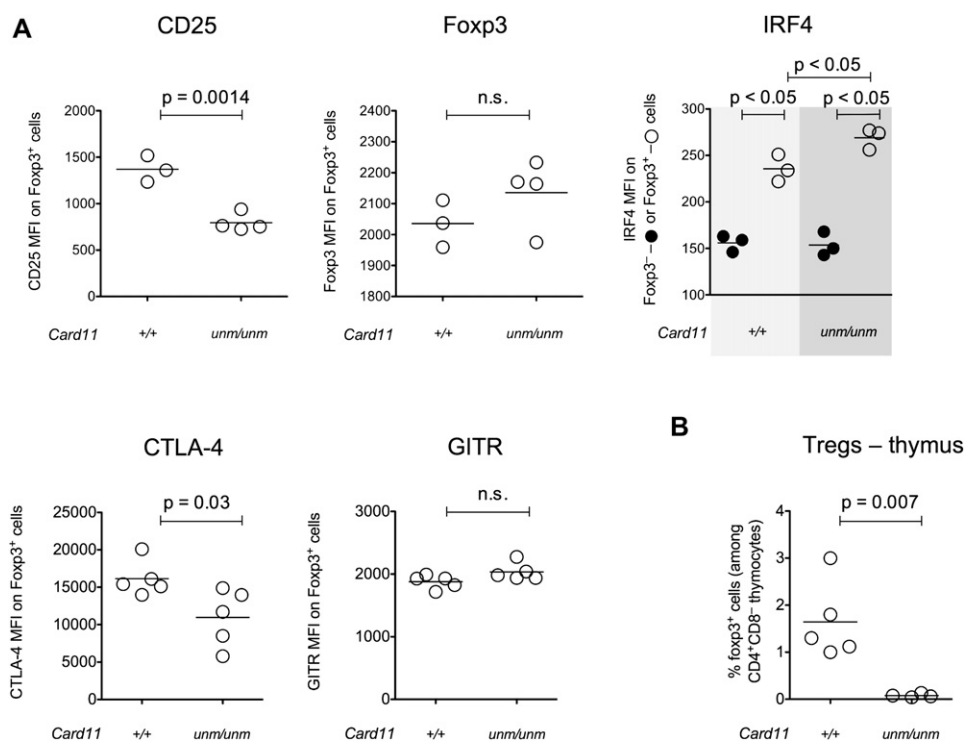


FIG E1. A, Plotted median fluorescence intensity (MFI) of CD25, Foxp3, IRF4, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), and glucocorticoid-induced TNF-related receptor (GITR) on lymph node Foxp3⁺CD4⁺ T cells from *Card11*^{unm} or wild-type mice measured by means intracellular staining. **B**, Frequency of Foxp3⁺ cells among CD4⁺CD8⁻ thymocytes from *Card11*^{unm} or wild-type mice. n.s., Not significant.

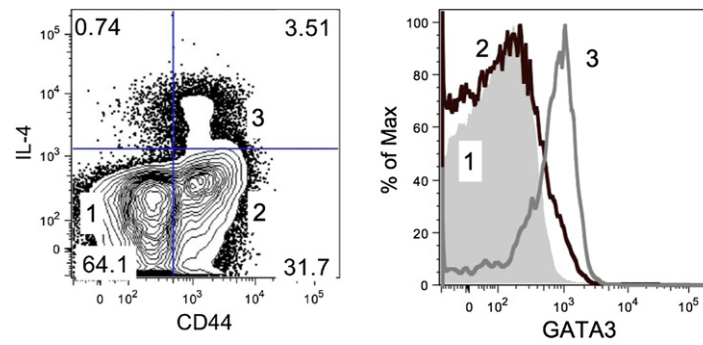


FIG E2. Expression of GATA3 on the CD40^{lo}IL-4⁻, CD44^{hi}IL-4⁻, or CD44^{hi}IL-4⁺CD4⁺ T-cell subsets (as indicated) from a *Card11^{unm}* mouse, as detected by means of *ex vivo* restimulation followed by intracellular cytokine staining.

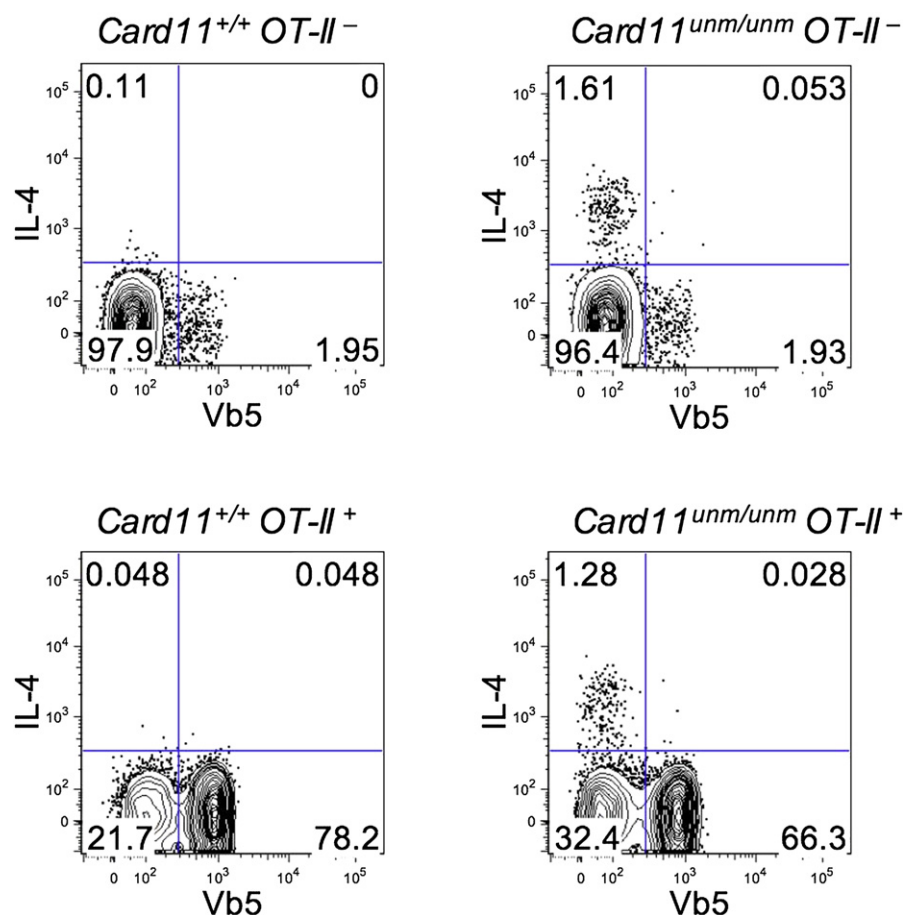


FIG E3. IL-4 production by CD4⁺ T cells from nontransgenic or OT-II TCR transgenic *Card11*^{unm} or control mice detected by means of *ex vivo* restimulation followed by intracellular cytokine staining. Cells expressing the transgenic TCR are Vβ5⁺.

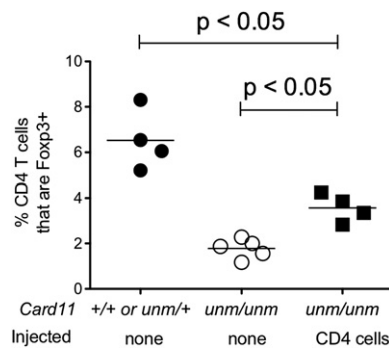


FIG E4. Reconstitution of the Foxp3⁺ population in *Card11*^{unm} mice after wild-type CD4⁺ T-cell transfer. Percentage of Foxp3⁺ cells among lymph node CD4⁺ T cells from mice that were either untreated or injected intravenously with 10⁶ sorted CD4⁺ T cells from a Ly5a donor 10 weeks prior.

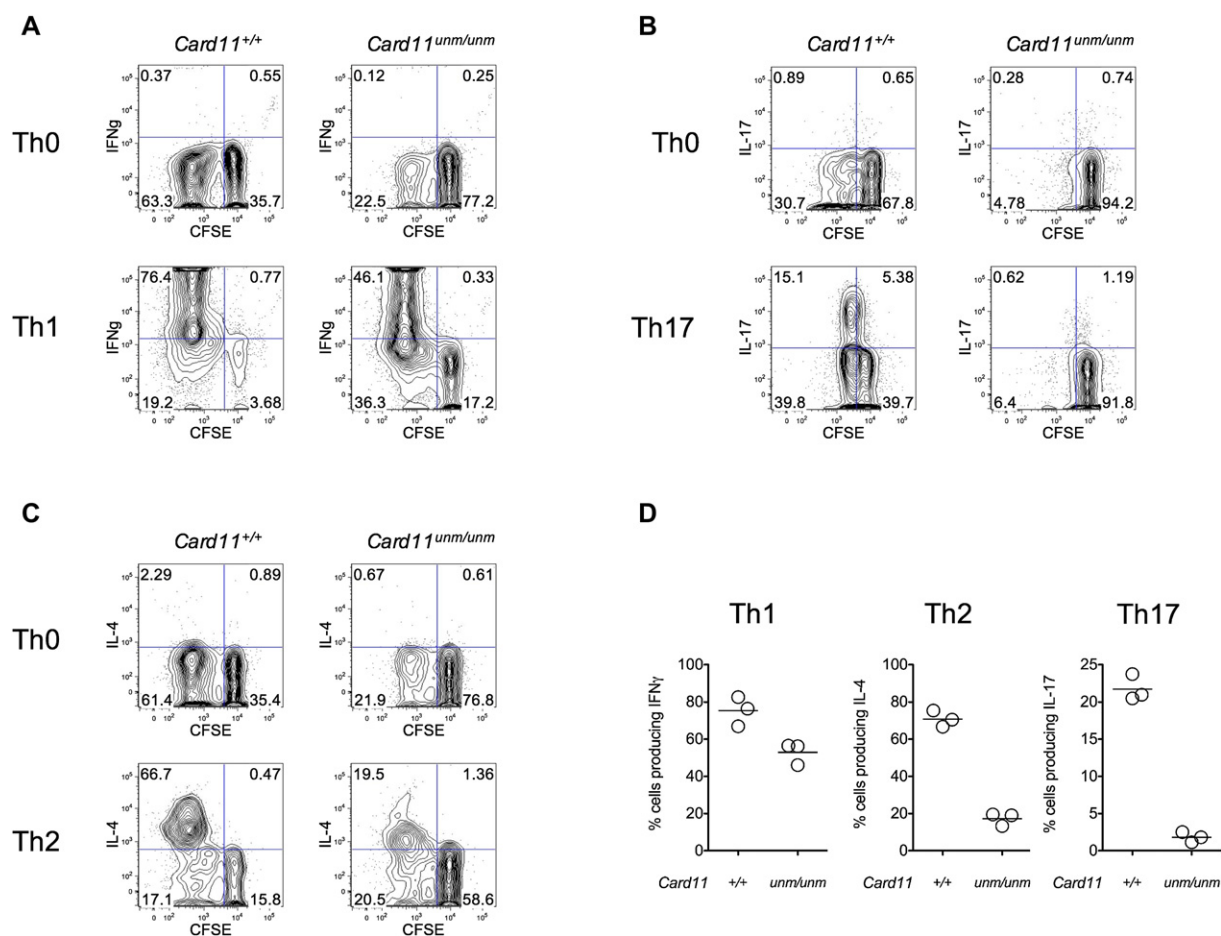


FIG E5. **A** and **B**, Dilution of carboxyfluorescein succinimidyl ester (CFSE) and production of IFN- γ and IL-4 by wild-type or *Card11*^{unm} cells after culture of sorted naive CD4⁺ splenocytes under T_H0-, T_H1-, or T_H2-polarizing conditions for 5 days. **C**, Dilution of CFSE and production of IL-17 by wild-type or *Card11*^{unm} cells after culture of sorted naive CD4⁺ splenocytes under T_H0- or T_H17-polarizing conditions for 3 days. **D**, Quantitation of the percentage of cytokine-producing cells at the end point of the T_H1, T_H2, or T_H17 cultures (3 mice per genotype).