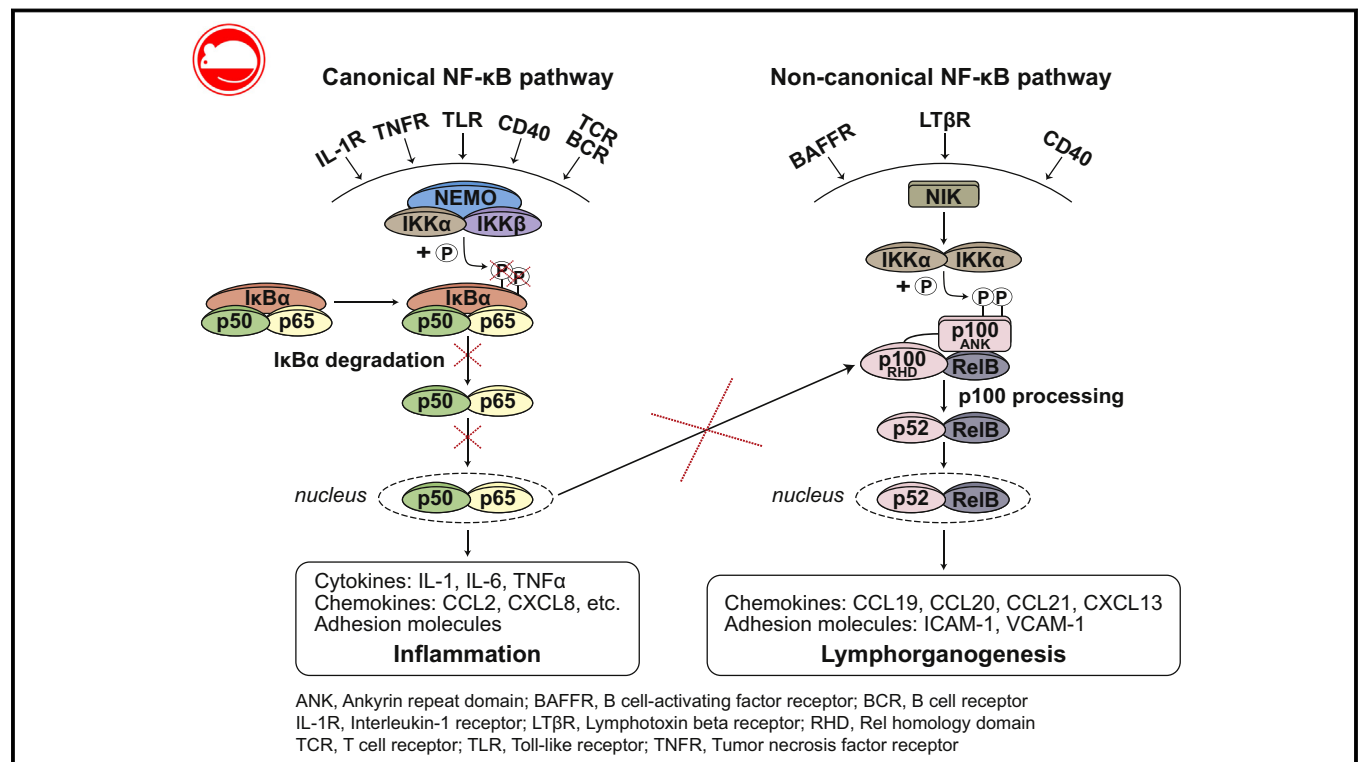


Mechanisms of genotype-phenotype correlation in autosomal dominant anhidrotic ectodermal dysplasia with immune deficiency

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GRAPHICAL ABSTRACT



Background: Autosomal dominant anhidrotic ectodermal dysplasia with immune deficiency (AD EDA-ID) is caused by heterozygous point mutations at or close to serine 32 and serine 36 or N-terminal truncations in IκBα that impair its

phosphorylation and degradation and thus activation of the canonical nuclear factor κ light chain enhancer of activated B cells (NF-κB) pathway. The outcome of hematopoietic stem cell transplantation is poor in patients with AD EDA-ID despite

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achievement of chimerism. Mice heterozygous for the serine 32I mutation in I κ B α have impaired noncanonical NF- κ B activity and defective lymphorganogenesis.

Objective: We sought to establish genotype-phenotype correlation in patients with AD EDA-ID.

Methods: A disease severity scoring system was devised.

Stability of I κ B α mutants was examined in transfected cells.

Immunologic, biochemical, and gene expression analyses were performed to evaluate canonical and noncanonical NF- κ B signaling in skin-derived fibroblasts.

Results: Disease severity was greater in patients with I κ B α point mutations than in those with truncation mutations. I κ B α point mutants were expressed at significantly higher levels in transfectants compared with truncation mutants. Canonical NF- κ B–dependent IL-6 secretion and upregulation of the NF- κ B subunit 2/p100 and RELB proto-oncogene, NF- κ B subunit (RelB) components of the noncanonical NF- κ B pathway were diminished significantly more in patients with point mutations compared with those with truncations. Noncanonical NF- κ B–driven generation of the transcriptionally active p100 cleavage product p52 and upregulation of *CCL20*, intercellular adhesion molecule 1 (*ICAM1*), and vascular cell adhesion molecule 1 (*VCAM1*), which are important for lymphorganogenesis, were diminished significantly more in LPS plus α -lymphotoxin β receptor–stimulated fibroblasts from patients with point mutations compared with those with truncations.

Conclusions: I κ B α point mutants accumulate at higher levels compared with truncation mutants and are associated with more severe disease and greater impairment of canonical and noncanonical NF- κ B activity in patients with AD EDA-ID. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: Ectodermal dysplasia with immune deficiency, NF- κ B inhibitor α , canonical nuclear factor κ B pathway, noncanonical nuclear factor κ B pathway, lymphorganogenesis, hematopoietic stem cell transplantation

Primary immunodeficiency diseases (PIDs) are often monogenic, with more than 300 genes implicated to date.^{1,2} There is a wide phenotypic heterogeneity in the presentation, age of onset, and outcome of individual PIDs. This heterogeneity reflects in part the influence of the genotype. Genotype-phenotype correlations have been important in predicting the severity, outcome, and custom tailoring of the treatment of a number of PIDs.³⁻⁷ Inhibitor of κ B (I κ B) proteins inhibit the transcription factor nuclear factor κ light chain enhancer of activated B cells (NF- κ B).⁸ Heterozygous mutations in the I κ B family member NF- κ B inhibitor α (I κ B α) cause autosomal dominant

Abbreviations used

AD EDA-ID:	Autosomal dominant anhidrotic ectodermal dysplasia with immune deficiency
DN:	Dominant negative
FKPM:	Fragments per kilobase of exon per million reads
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
HSCT:	Hematopoietic stem cell transplantation
I κ B α :	NF- κ B inhibitor α
ICAM-1:	Intercellular adhesion molecule 1
IKK:	I κ B kinase
LN:	Lymph node
LTo:	Lymphoid tissue organizer
LT β R:	Lymphotoxin β receptor
NF- κ B:	Nuclear factor κ light chain enhancer of activated B cells
NF- κ B1:	NF- κ B subtype 1
NF- κ B2:	NF- κ B subunit 2
NIK:	NF- κ B inducing kinase
PID:	Primary immunodeficiency disease
qPCR:	Quantitative PCR
RelB:	RELB proto-oncogene, NF- κ B subunit
RNA-Seq:	RNA sequencing
S32:	Serine 32
S36:	Serine 36
SLO:	Secondary lymphoid organ
TLR:	Toll-like receptor
VCAM-1:	Vascular cell adhesion molecule 1
WT:	Wild-type

anhidrotic ectodermal dysplasia with immune deficiency (AD EDA-ID), which is characterized by sparse hair, conical teeth, reduced number of sweat glands, and susceptibility to severe infections.⁹ The literature indicates a spectrum of disease in patients with AD EDA-ID, with variable severity and patient outcomes after hematopoietic stem cell transplantation (HSCT), but a genotype-phenotype correlation has not been established.

The NF- κ B family of transcription factors includes RelA (p65); RELB proto-oncogene, NF- κ B subunit (RelB); c-Rel; NF- κ B subtype 1 (NF- κ B1) (p50/p105); and NF- κ B subunit 2 (NF- κ B2; p52/p100) and plays a critical role in innate and adaptive immunity.^{8,10,11} In resting cells NF- κ B is retained in the cytoplasm through association with members of the I κ B family of proteins. Two distinct pathways leading to NF- κ B activation and nuclear translocation have been identified: a canonical and noncanonical NF- κ B pathway.¹² The canonical or classical NF- κ B pathway is used by a broad range of cell-surface receptors, including Toll-like receptors (TLRs), IL-1 receptor, TNF- α

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receptor, antigen receptors, and others.^{8,11} Receptor ligation causes activation of the I κ B kinase (IKK) complex, which consists of the catalytic subunits IKK α and IKK β and the scaffolding protein IKK γ /NF- κ B essential modulator. This results in phosphorylation of I κ B α at serine 32 (S32) and serine 36 (S36), priming it for ubiquitination and proteasomal degradation. The released p50:p65 complex translocates into the nucleus, where it induces transcription of genes that mediate the inflammatory response.^{13,14} The noncanonical NF- κ B pathway is used by a limited range of cell-surface receptors, including lymphotoxin β receptor (LT β R), B-cell activating factor receptor, and CD40. Receptor ligation drives NF- κ B inducing kinase (NIK) to phosphorylate IKK α .¹⁵ IKK α subsequently phosphorylates NF- κ B2/p100, which is in complex with RelB and contains an I κ B-like domain that prevents nuclear translocation of the p100:RelB complex in resting cells. Phosphorylated p100 undergoes polyubiquitination and proteasomal processing to p52. The p52:RelB dimers translocate to the nucleus and activate transcription of genes that include chemokines and adhesion molecules involved in lymphorganogenesis.^{15,16} There is crosstalk between the 2 NF- κ B pathways: NIK-dependent activation of IKK α has been reported to cause I κ B α phosphorylation and degradation.¹⁷ Importantly, transcription of p100 and RelB, as well as I κ B α , is regulated by p50:p65.^{18–21}

Two types of human I κ B α mutations have been described in patients with AD EDA-ID: mutations that result in a single amino acid substitution at or adjacent to the S32 and S36 phosphorylation sites (point mutations) and mutations that introduce a premature stop codon and give rise to N-terminally truncated I κ B α proteins through reinitiation of translation at downstream ATG sites (truncation mutations). Both types of mutations impair signal-induced phosphorylation of I κ B α and protect the mutant protein from subsequent ubiquitination and proteasomal degradation. By sequestering the NF- κ B transcription factors p50 and p65 in the cytoplasm, the nondegradable I κ B α mutant protein interferes with activation of the canonical NF- κ B pathway. Ten patients with AD EDA-ID carrying 8 I κ B α mutations have been reported to date,^{22–31} and more cases are known to exist or have been submitted for publication.³² TLR-driven cytokine production is impaired in these patients. Circulating T- and B-cell numbers are high, but the percentages of memory T and B cells are decreased. *In vitro* T-cell function varies from decreased to intact. In transfection studies, all I κ B α mutants, except W11X, inhibited NF- κ B activation more than wild-type (WT) I κ B α and thus have been demonstrated to function as dominant negative (DN). A majority of patients with AD EDA-ID reported have undergone HSCT. However, despite good donor cell engraftment, the outcome of HSCT has been poor, with persistent recurrent and chronic bacterial and viral infections, poor antibody responses to immunizations, and need for immunoglobulin replacement, suggesting a contribution from nonhematopoietic cells to residual disease after transplantation.^{32–34}

A knock-in mouse heterozygous for the S32I mutation in I κ B α shows ectodermal dysplasia, impaired phosphorylation and degradation of the mutant I κ B α protein, and diminished response to TLR ligation.³⁵ Strikingly, the S32I mutant completely lacks lymph nodes (LNs), Peyer patches, marginal zone B cells, and follicular dendritic cells and does not form germinal centers, features typical of defective noncanonical NF- κ B signaling that have remained largely unaddressed in patients with AD

EDA-ID.^{17,36–47} WT->S32I I κ B α chimeras did not form proper lymphoid organs and reconstitute immune function despite excellent donor cell engraftment, paralleling the failure of HSCT to cure patients with AD EDA-ID. In contrast, S32I I κ B α ->Rag2^{-/-} bone marrow chimeras developed proper lymphoid organs. These results suggest that defective stromal cell function can contribute to immunodeficiency in patients with AD EDA-ID and might explain the poor outcome of HSCT in patients with this disease.

The mechanism by which mutations in I κ B α also affect the noncanonical NF- κ B pathway in patients with AD EDA-ID is not well understood. In this study we analyzed 7 published and 2 unreported cases of AD EDA-ID.^{22–28} We demonstrate that I κ B α point mutants are expressed at higher levels and impair the canonical and noncanonical NF- κ B pathway more severely than I κ B α truncation mutants and thus result in more severe disease. Our study establishes a genotype-phenotype correlation in patients with AD EDA-ID, demonstrates a defect in the noncanonical NF- κ B signaling in patients with AD EDA-ID, and has important implications for the management of these patients.

METHODS

Cell lines

Skin fibroblast cell lines were established from skin punch biopsy specimens obtained from patients and 3 adult healthy donors after securing informed consent by using protocols approved by the ethics committees of the centers participating in the studies and in compliance with the Helsinki declaration. Fibroblast lines were cultured in Dulbecco modified Eagle medium (Life Technologies, Carlsbad, Calif) supplemented with 15% FBS (Gemini Bio-Products, Broderick, Calif), 10 mmol/L HEPES buffer (Sigma, St Louis, Mo), 100 U/mL potassium penicillin, and 100 μ g/mL streptomycin sulfate (Lonza, Walkersville, Md).

The HEK293T cell line (ATCC CRL-3216) was obtained from the American Type Culture Collection and cultured in Dulbecco modified Eagle medium supplemented with 10% FBS, 10 mmol/L HEPES buffer, 100 U/mL potassium penicillin, and 100 μ g/mL streptomycin sulfate. Before and during transfection experiments, HEK293T cells were cultured in antibiotic-free medium.

Plasmids and targeted mutagenesis

An expression vector containing cDNA encoding C-terminally FLAG-tagged human I κ B α (pCMV3-NFKBIA-C-FLAG; HG12045-CF) was obtained from Sino Biological (Beijing, China). Targeted mutagenesis was performed with the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, Calif), according to the manufacturer's instructions, together with custom mutagenic primers: Q9X_F, 5'-CGGCC GAGCGCCCCCTAGGAGTGGGCCATGGAGG-3'; Q9X_R, 5'-CCTCCATG GCCACTCCTAGGGGCGCTCGGCCG-3'; W11X_F, 5'-CCGAGCGCCC CCAGGAGTAGGCCATGGAGGGCC-3'; W11X_R, 5'-GGCCCTCCA TGGCCTACTCCTGGGGGCGCTCGG-3'; E14X_F, 5'-CCCAGGAGTGG GCCATGTAGGGCCCCCGCGACGG-3'; E14X_R, 5'-CCGTCGCGGGG GCCCTACATGGCCCACTCCTGGG-3'; S32I_F, 5'-GGACGACCGCCAC GACATCGGCCCTGGACTCC-3'; S32I_R, 5'-GGAGTCCAGGCCGATGTC GTGGCGGTCTGTC-3'; G33V_F, 5'-CGACCGCCACGACAGCGTCTCTG GACTCCATGAAAG-3'; G33V_R, 5'-CTTTCATGGAGTCCAGGACGCT GTCGTGGCGGTCTG-3'; S36Y_F, 5'-CGACAGCGGCCCTGGACTACATG AAAGACGAGG-3'; S36Y_R, 5'-CCTCGTCTTTCATGTAGTCCAG GCGCTGTGTCG-3'; M37K_F, 5'-GCGGCTGGACTCCAAGAAAGACGA GGAGTACG-3'; and M37K_R, 5'-CGTACTCCTCGTCTTCTTGGAGTC CAGGCCG-3'. This results in the following changes in I κ B α (RefSeq NM_020529) at the nucleotide and protein levels, respectively:

c.25C>T (p.Q9X), c.32G>A (p.W11X), c.40G>T (p.E14X), c.95G>T (p.S32I), c.98G>T (p.G33V), c.107C>A (p.S36Y), c.110T>A (p.M37K). Successful mutagenesis was confirmed by means of Sanger sequencing of plasmid DNA.

Transient transfection of HEK293T cells

The day before transfection, 4.5×10^5 HEK293T cells were seeded per well of a 6-well plate in antibiotic-free medium. Transfections were carried out with FuGENE 6 transfection reagent (Promega, Madison, Wis) at a 3:1 FuGENE/DNA ratio. Three micrograms of WT or mutant pCMV3-NFKBIA-C-FLAG were transfected per well. Cells were harvested 24 hours after transfection and used in immunoblotting, as described below.

Semiquantitative RT-PCR from transfectants

Total RNA of HEK293T cells transiently transfected with WT or mutant pCMV3-NFKBIA-C-FLAG was extracted by using TRIzol reagent (Life Technologies), according to the manufacturer's instructions. For each sample, 500 ng of RNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, Calif). The following primers were used for RT-PCR: I κ B α _F, 5'-CAATGCTCAGGAGCCCTGTAA-3'; FLAG_R, 5'-CTTATCGTCGTCATCCTTGAATC-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH)_F, 5'-TCGACAGTCAGCCG CATCTT-3'; and GAPDH_R, 5'-CAAATGAGCCCCAGCCTTCTC-3'. The reverse primer was designed to bind to the FLAG tag sequence (GATTA-CAAGGATGACGACGATAAG) to specifically amplify ectopically expressed I κ B α transcripts. Thirty-five PCR cycles were performed with an annealing temperature of 60°C. For analysis, PCR products were loaded on a 1% agarose gel.

Fibroblast stimulation

Primary skin fibroblasts from healthy control subjects and patients with AD EDA-ID were grown to about 80% confluency. Cells were harvested by using trypsinization, reseeded at a density of 100,000 cells per well (6-well format), and incubated overnight. Fibroblasts were treated with medium, 1 μ g/mL LPS (InvivoGen, San Diego, Calif), or 1 μ g/mL LPS plus 5 μ g/mL α -LT β R agonistic mAb (clone 31G4D8; BioLegend, San Diego, Calif) for 48 hours. IL-6 levels in the supernatants of LPS-stimulated cells were determined 24 hours after stimulation by means of ELISA (eBioscience, San Diego, Calif).

Immunoblotting

Samples were lysed in Western blot buffer (50 mmol/L Tris [pH 7.5], 100 mmol/L NaCl, and 1% Triton X-100) containing Protease Inhibitor Cocktail (Roche, Indianapolis, Ind), and lysates were clarified by spinning for 15 minutes at 20,000g. Total protein was quantified by using the BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, Ill), according to the manufacturer's instructions. Lysates containing equal amounts of protein were resolved by means of SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against the FLAG-tag (F3165, Sigma-Aldrich, St Louis, Mo), I κ B α (sc-371; Santa Cruz Biotechnology, Dallas, Tex), p100/p52 (#4882; Cell Signaling Technology, Danvers, Mass), GAPDH (#2118, Cell Signaling Technology), and RelB (sc-226, Santa Cruz Biotechnology), according to standard protocols. Band intensity was quantified with ImageJ software (National Institutes of Health, Bethesda, Md).

RNA sequencing analysis of LPS plus α -LT β R-stimulated fibroblasts

Fibroblasts from healthy control subjects and patients with AD EDA-ID were treated with medium or 1 μ g/mL LPS plus 5 μ g/mL α -LT β R agonistic mAb for 48 hours. RNA was isolated with the TRIzol Plus RNA Purification Kit (Life Technologies), according to the manufacturer's instructions.

On-column DNase digestion was performed with the PureLink DNase Set (Invitrogen, Carlsbad, Calif). Libraries were prepared by using Illumina TruSeq Stranded mRNA Sample Prep Kits (Illumina, San Diego, Calif) from 500 ng of purified total RNA, according to the manufacturer's protocol. The finished dsDNA libraries were quantified with a Qubit fluorometer (Thermo Fisher Scientific, Waltham, Mass), Agilent TapeStation 2200 (Agilent Technologies), and quantitative RT-PCR by using the Kapa Biosystems (Wilmington, Mass) Library Quantification Kit, according to the manufacturer's protocols. Uniquely indexed libraries were pooled in equimolar ratios and sequenced on an Illumina NextSeq500 (Illumina) with single-end 75bp reads by the Dana-Farber Cancer Institute Molecular Biology Core Facilities. Sequence reads were aligned to the hg19 reference genome assembly, and gene counts were quantified by using STAR (version 2.5.1b) and differential expression testing was performed by using DESeq2 (version 1.10.1) as part of the VIPER analysis pipeline (<https://bitbucket.org/cfce/viper/>). Normalized gene counts were expressed as fragments per kilobase of exon per million reads (FPKM). For further analysis, we used genes significantly (adjusted $P < .05$) upregulated or downregulated by at least 2-fold in LPS plus α -LT β R-stimulated fibroblasts from healthy donors compared with unstimulated cells. FPKM values were log₂-transformed, and z scores were calculated. Signaling components and molecular targets of NF- κ B were identified by comparing genes significantly upregulated or downregulated 2-fold in LPS plus α -LT β R-stimulated fibroblasts from healthy donors with online databases of NF- κ B target genes (<https://www.bu.edu/nf-kb/gene-resources/target-genes/>), as well as genes included in commercially available NF- κ B-specific gene arrays (TaqMan Array Human NFKB Pathway, catalog no. 4414095; Thermo Fisher, Waltham, Mass; RT² Profiler PCR Array Human NF- κ B Signaling Pathway and Signaling Targets, catalog no. PAHS-025Z and PAHS-225Z, Qiagen, Germantown, Md). Heat maps representing z scores of log₂-transformed FPKM values were done in R software (<https://www.r-project.org>) by using the pheatmap and RColorBrewer packages.

Quantitative PCR analysis of CCL20, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1 mRNA levels

RNA was isolated from fibroblasts treated with medium or 1 μ g/mL LPS plus 5 μ g/mL α -LT β R agonistic mAb for 48 hours. For each sample, 100 ng of RNA were reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad Laboratories). TaqMan primer/probes for human CCL20, intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1), and GAPDH were purchased from Thermo Fisher Scientific. Experiments were run on an Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, Foster City, Calif). Data were analyzed by using the $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

Statistical analyses were performed with Prism 7 software (GraphPad Software, La Jolla, Calif). Significance was calculated by using 2-tailed t tests.

RESULTS

Patients with AD EDA-ID with point mutations in I κ B α have more severe disease than patients with truncation mutations and a poor outcome after HSCT

We analyzed 9 patients with AD EDA-ID, including 7 published cases and 2 unreported cases (Table I).²²⁻²⁸ Six patients have 4 different heterozygous point mutations at or adjacent to the S32 and S36 phosphorylation sites, leading to single amino acid substitutions: 3 with p.S32I and 1 each with p.G33V, p.S36Y, and p.M37K (Table I). Three patients each have a heterozygous N-terminal nonsense mutation, p.Q9X, p.W11X, and p.E14X,

TABLE I. Clinical characteristics

Patient	IκBα mutation	Disease score*	SLOs		HSCT (age)	Outcome	Reference
			Tonsils	Palpable LNs			
1	S32I	15/15	Absent	Absent	Yes (1 y)	Recurrent infections, on IVIG; alive 17 y after HSCT	Courtois et al, 2003 ²²
2	S32I	14/15	Absent	Absent	Yes (7 y)	Recurrent infections, on IVIG; alive 3 y after HSCT	Unpublished
3	S32I	15/15	Absent	Absent	Yes (2.5 y)	Recurrent infections, on IVIG; deceased 4 mo after HSCT	Janssen et al, 2004 ²³
4	G33V	14/15	Absent	Absent	Yes (2 y)	Recurrent infections, on IVIG; alive 1 year after HSCT	Unpublished
5	S36Y	11/15	Very small	Very small	Yes (6 y)	GvHD and autoimmune thrombocytopenia; deceased 7 mo after HSCT with sepsis	Yoshioka et al, 2013 ²⁸
6	M37K	12/15	Absent	Absent	Yes (4.8 y)	Recurrent infections, on SCIG; deceased 18 mo after HSCT with sepsis	Schimke et al, 2013 ²⁷
7	Q9X	6/12	Yes	Yes	Yes (15 mo)	Healthy 5 y after HSCT, off IVIG	Ohnishi et al, 2012 ²⁶
8	W11X	4/15	Yes	Yes	No	Healthy, on IVIG	McDonald et al, 2007 ²⁴
9	E14X	4/12	Yes	Yes	Yes (8 mo)	Deceased day 31 after HSCT with sepsis	Lopez-Granados et al, 2008 ²⁵

GvHD, Graft-versus-host disease; IVIG, intravenous immunoglobulin replacement therapy; SCIG, subcutaneous immunoglobulin replacement therapy.

*Disease scoring is based on 5 clinical and laboratory criteria, each ranging in score from 0 to 3, according to the severity of the abnormality (Table II). For patients 7 and 9, data were not available on one of the criteria, resulting in a maximum disease score of 12.

TABLE II. Disease severity scoring system for AD EDA-ID

Score	Infections	Ectodermal dysplasia: Systems affected*	Antibody responses†	Lymphocytes/μL	Memory T and/or B cells‡
0	None	None	Normal	Normal	Normal
1	Mucosal	Dental only	Polysaccharide (PS) deficient	10,000-20,000	Modest decrease
2	Visceral and/or blood borne	2	Protein antigen deficient	20,000-30,000	Severe decrease
3	Opportunistic	≥3	PS and protein antigen deficient	>30,000	Absent

*Ectodermal systems evaluated include hair, teeth, nails, and sweat glands.

†Deficient antibody response is defined as absent or unprotective antibody titers and/or an increase in antibody titers of less than 4-fold after immunization.

‡Modest decrease = values between 2 and 3 SDs less than the mean for age; severe decrease = values of greater than 3 SDs less than the mean for age but greater than zero.

respectively, which is expected to give rise to N-terminally truncated IκBα proteins that use alternative methionine start sites, presumably M13 and M37 for Q9X and W11X and M37 for E14X.²⁴⁻²⁶

To assess the severity of AD EDA-ID, we developed a disease scoring system in consultation with 3 experts in primary immunodeficiency who were not participants in the study. Scoring is based on 5 clinical and laboratory criteria, each of which was assigned a score from 0 to 3 according to the severity of the abnormality (Table II). The criteria included type of infections (mucosal, visceral, blood borne, and opportunistic), number of systems exhibiting features of EDA, spectrum of antibody deficiency (deficiency of response to polysaccharide antigens, protein antigens, or both), magnitude of the increase in circulating lymphocyte count, and severity of the reduction in CD3⁺CD45RO⁺ memory T-cell and/or CD19⁺CD27⁺ B-cell numbers. The maximum total score is 15. In 2 patients (patients 7 and 9 in Table I) only 4 of the 5 criteria were evaluated, resulting in a maximum score of 12. Because not all criteria were evaluated in all patients, a relative disease severity score was calculated as the percentage of the maximum achievable score in each patient. Disease severity score was significantly higher in the 6 patients with IκBα point mutations compared with the 3 patients with IκBα truncation mutations (Fig 1, A, and Table I). The breakdown of the individual scores for each of the 2 groups and for each of the patients is shown

in Fig 1, B, and Table E1 in this article's Online Repository at www.jacionline.org.

Five of the 6 patients with IκBα point mutations lacked visible tonsils and had no palpable LNs. Small tonsils and LNs were reported present in only 1 of the 6 patients with IκBα point mutations. In contrast, tonsils and LNs were present in all 3 patients with IκBα truncation mutations (Table I). All 6 patients with IκBα point mutations had undergone HSCT because of severe disease (Table I). Three of the 6 died within 18 months after HSCT and continued to have recurrent infections until their death and required immunoglobulin replacement therapy. All 3 patients undergoing transplantation with IκBα point mutations who are currently alive continue to have recurrent infections and require immunoglobulin replacement. All 6 patients achieved excellent donor cell engraftment (>90%) in T-cell, B-cell, and myeloid cell lineages in the peripheral blood. Thus HSCT did not result in clinical improvement in 6 of 6 patients with IκBα point mutations despite excellent donor cell engraftment. Of the 3 patients with truncation mutations, the patient with the Q9X mutation underwent transplantation at 15 months of age and is healthy more than 5 years after HSCT. The patient with the E14X mutation underwent transplantation at 7 months of age but died from sepsis in the immediate posttransplantation period, precluding evaluation of the outcome of HSCT. The patient with the W11X mutation, now 22 years of age, is doing well on immunoglobulin replacement therapy and has not undergone HSCT.

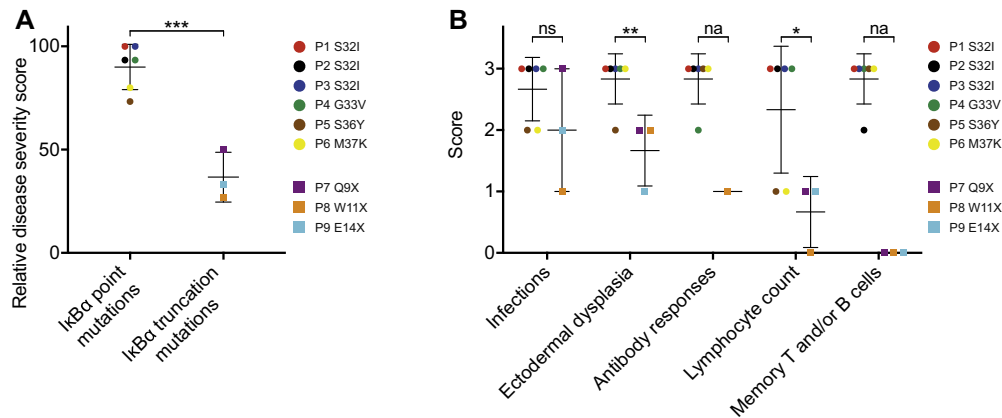


FIG 1. Relative disease severity score in patients with AD EDA-ID. **A**, Cumulative disease severity score expressed as a percentage of the maximal score based on the evaluable disease criteria listed in Table II. **B**, Individual disease category scores for patients with $\text{IkB}\alpha$ point mutations and patients with $\text{IkB}\alpha$ truncations. * $P < .05$, ** $P < .01$, and *** $P < .001$. na, Not applicable; ns, not significant.

$\text{IkB}\alpha$ point mutants are expressed at higher levels compared with $\text{IkB}\alpha$ N-terminally truncated mutants

Degradation-resistant $\text{IkB}\alpha$ mutant proteins sequester p50:p65 in the cytoplasm, impairing NF- κ B activation. Thus the level of degradation-resistant $\text{IkB}\alpha$ mutant protein in the cell is expected to determine the extent of impairment of NF- κ B activation. We hypothesized that the levels of $\text{IkB}\alpha$ point mutants, which are associated with significantly more severe disease, would be greater than those of $\text{IkB}\alpha$ truncation mutants. Immunoblotting for $\text{IkB}\alpha$ was performed with an antibody directed to the C-terminus of $\text{IkB}\alpha$ in lysates from skin-derived fibroblasts. Fibroblasts were available from 4 patients with point mutations (2 with S32I, 1 with G33V, and 1 with M37K) and 2 patients with truncations (Q9X and W11X). All patients' fibroblasts were studied simultaneously, except those from the patient with the W11X mutation, because of delay in our ability to grow these cells from frozen aliquots. As previously reported,^{24,26} fibroblasts from the 2 patients with the Q9X and W11X $\text{IkB}\alpha$ truncation mutations demonstrated an $\text{IkB}\alpha$ band of normal size that represents the product of the WT allele (Fig 2, A). In addition, 2 fainter lower-molecular-weight $\text{IkB}\alpha$ bands were detected in these fibroblasts. According to their molecular weight, the translation start sites of these products are likely to be M13 and M37, respectively. The mean ratio of the sum of the truncated $\text{IkB}\alpha$ products to WT $\text{IkB}\alpha$ in the 2 patients was 0.4 ± 0.2 ($n = 3$).

As previously reported,^{22,27,28} only 1 $\text{IkB}\alpha$ band was observed in fibroblast lysates from patients with point mutations, as would be expected for mutant proteins with a single amino acid substitution. The intensity of this band was similar to the band in healthy control subjects. Because $\text{IkB}\alpha$ is under the control of canonical NF- κ B signaling,^{48,49} it is likely that the contribution of the product of the WT $\text{IkB}\alpha$ allele to the total $\text{IkB}\alpha$ level is less than that of the degradation resistant product of the mutant $\text{IkB}\alpha$ allele.

Measurement of $\text{IkB}\alpha$ levels in cultured fibroblasts might not reflect the intrinsic stability of the mutant proteins because of the genetic heterogeneity of the fibroblast donors, the effect on $\text{IkB}\alpha$ expression of chronic stimulation of the fibroblast line by potential NF- κ B activators in FCS, and the inability to directly determine the level of WT and mutant $\text{IkB}\alpha$ in fibroblasts from

patients with $\text{IkB}\alpha$ point mutations. HEK293T cells were transiently transfected with expression vectors encoding C-terminally FLAG-tagged WT or mutant $\text{IkB}\alpha$ to assess the stability of $\text{IkB}\alpha$ mutant proteins. Twenty-four hours after transfection, cells were harvested, and lysates were immunoblotted with an antibody to the FLAG tag. In this approach cells of a uniform genetic background are examined under conditions of short-term culture, and the expression of the FLAG-tagged point mutants is directly measurable. With the exception of M37K $\text{IkB}\alpha$, which was expressed at levels not significantly different from those of WT $\text{IkB}\alpha$, all $\text{IkB}\alpha$ mutant proteins showed a significant decrease in expression compared with WT $\text{IkB}\alpha$ (Fig 2, B and C). Importantly, expression levels of the 3 $\text{IkB}\alpha$ truncation mutants Q9X, W11X, and E14X were significantly reduced compared with those of the 4 point mutants S32I, G33V, S36Y, and M37K. Reduced expression levels of the mutant proteins did not result from mRNA instability, as evaluated by using semiquantitative RT-PCR of $\text{IkB}\alpha$ -FLAG mRNA (Fig 2, D). These results strongly suggest that $\text{IkB}\alpha$ point mutants are significantly more stable than $\text{IkB}\alpha$ truncation mutants.

$\text{IkB}\alpha$ point mutants impair LPS-driven IL-6 secretion and accumulate after LPS stimulation significantly more than $\text{IkB}\alpha$ truncation mutants

Impaired production of inflammatory cytokines after activation of the canonical NF- κ B pathway in patients with AD EDA-ID has been documented in individual case reports.²²⁻²⁸ These reports measured the production of different cytokines (IL-1 and/or IL-6 and/or TNF- α) by different cell types (PBMcs, monocytes, fibroblasts) using different modes of stimulation (LPS and/or TNF- α and/or IL-1), precluding useful comparison of inflammatory cytokine production in the patients. We compared secretion of the same cytokine (IL-6) by a single cell source (skin-derived fibroblasts) after stimulation with the same agent (LPS). IL-6 secretion was robustly induced by LPS stimulation in fibroblasts from healthy control subjects (Fig 3, A). In contrast, LPS-driven IL-6 production was virtually abolished in all 4 patients with point mutations studied (2 with S32I, 1 with G33V, and 1 with M37K) but was partially preserved in the 2 patients

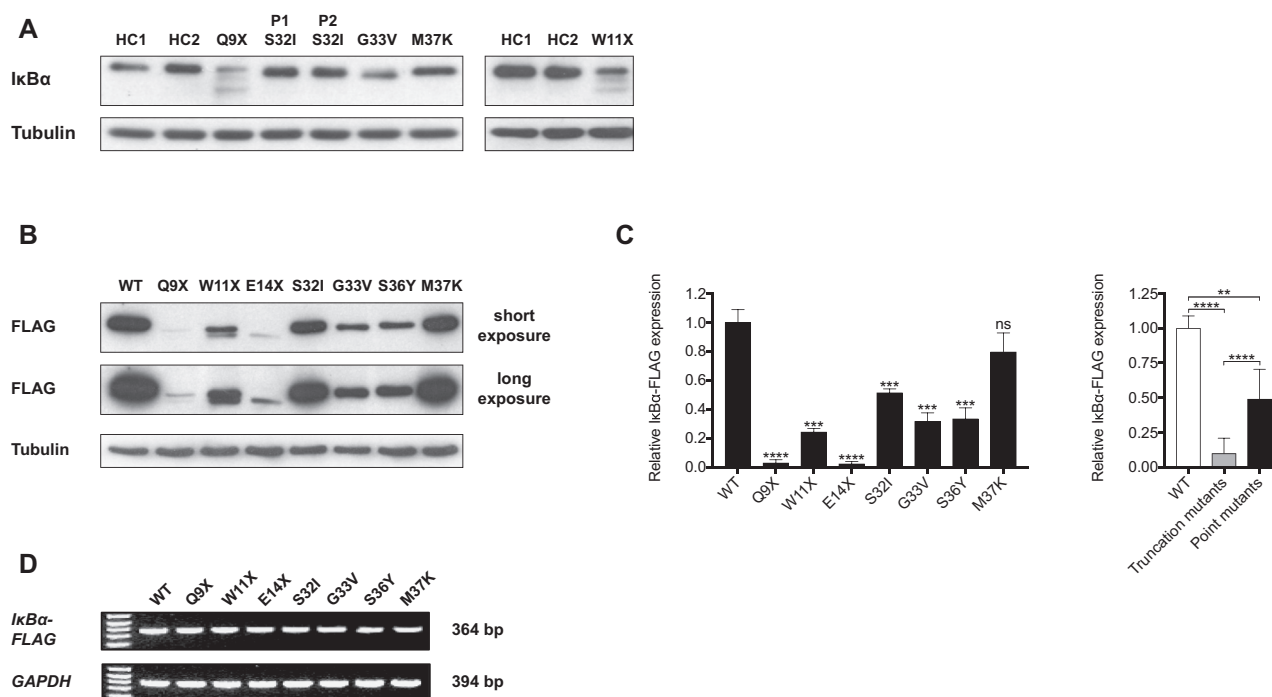


FIG 2. Expression of IκBα in fibroblasts and transfectants. **A**, Representative immunoblot of IκBα expression in cultured skin-derived fibroblasts from healthy control subjects (HC) and patients with AD EDA-ID (P). P1 S32I and P2 S32I are patients 1 and 3 in Table I. **B** and **C**, Representative immunoblot (Fig 2, B) and quantitative analysis (Fig 2, C) of the expression of FLAG-tagged WT and mutant IκBα in transfected HEK293T cells. Results in the left panel of Fig 2, C, are expressed as the mean ratio of IκBα to tubulin of each mutant analyzed relative to the mean of WT IκBα set at 1.0. Results in the right panel of Fig 2, C, are expressed as the mean ratio of IκBα to tubulin of pooled IκBα truncation mutants and pooled IκBα point mutants relative to the mean of WT IκBα set at 1.0. **D**, RT-PCR analysis of mRNA encoding FLAG-tagged WT and mutant IκBα. Amounts of cDNA used were in the linear range determined for mRNA encoding FLAG-tagged WT IκBα. Data are representative of 3 independent experiments. Columns and bars represent the mean ± SDs of at least 3 independent experiments. ***P* < .01, ****P* < .001, and *****P* < .0001. ns, Not significant.

with truncation mutations studied (Q9X and W11X). These results suggest that IκBα point mutants cause a significantly greater impairment of canonical NF-κB activation compared with IκBα truncation mutants.

Canonical NF-κB activation results in rapid degradation of IκBα, followed by IκBα resynthesis.^{48,49} The marked reduction of LPS-driven IL-6 secretion in fibroblasts from patients with IκBα truncation mutations, in which the level of the mutant was approximately half that of WT protein before LPS stimulation, prompted us to examine whether LPS activation alters the mutant/WT IκBα ratio, given the resistance of the mutants to stimulus-induced degradation. LPS stimulation for 48 hours caused a significant decrease in WT IκBα levels in normal fibroblasts (Fig 3, B and C). Similarly, WT IκBα levels significantly decreased in LPS-stimulated fibroblasts from patients with Q9X and W11X IκBα truncation mutations, whereas, in contrast, the level of truncated mutant IκBα significantly increased (Fig 3, B and C). This resulted in a significant change of the truncation mutant to WT IκBα ratio from approximately 0.4 in unstimulated cells to approximately 4 (*n* = 3) in stimulated cells (Fig 3, D). Therefore these results provide a rationale for how IκBα truncation mutants can function as DN despite their lower expression compared with WT IκBα. The 2 fainter bands representing the IκBα truncated products in unstimulated fibroblasts detected in Fig 2, A, are poorly evident in the representative experiment

shown in Fig 3, B, in which samples from all available patients were loaded on the same gel. However, these 2 bands were evident in other experiments (see Fig E1 in this article's Online Repository at www.jacionline.org). Of note, only levels of the IκBα truncated product with lower molecular weight, which likely lacks the S32 and S36 phosphorylation sites, increased after stimulation, whereas the higher-molecular-weight truncated product, which likely retains S32 and S36, might be subject to degradation (see Fig E1).

LPS stimulation caused a significant (1.5 ± 0.3 -fold, *n* = 3) increase in the level of immunoreactive IκBα protein in fibroblasts from patients with IκBα point mutations. Because of the inability to differentiate WT IκBα from point mutant IκBα, we were unable to determine the effect of LPS stimulation on the point mutant/WT IκBα ratio. The WT IκBα protein is expected to be expressed at a lower level than the IκBα point mutant at baseline because of impaired NF-κB canonical activity, resulting in an expected point mutant/WT IκBα ratio of greater than 1 at baseline. After LPS stimulation, the WT but not the mutant protein would be degraded, resulting in a further increase in the point mutant/WT IκBα ratio. This ratio would be expected to be higher than that of the truncation mutant/WT ratio because of the higher stability of the point mutants, providing a likely explanation for the more severe impairment of NF-κB activation in patients with IκBα point mutations.

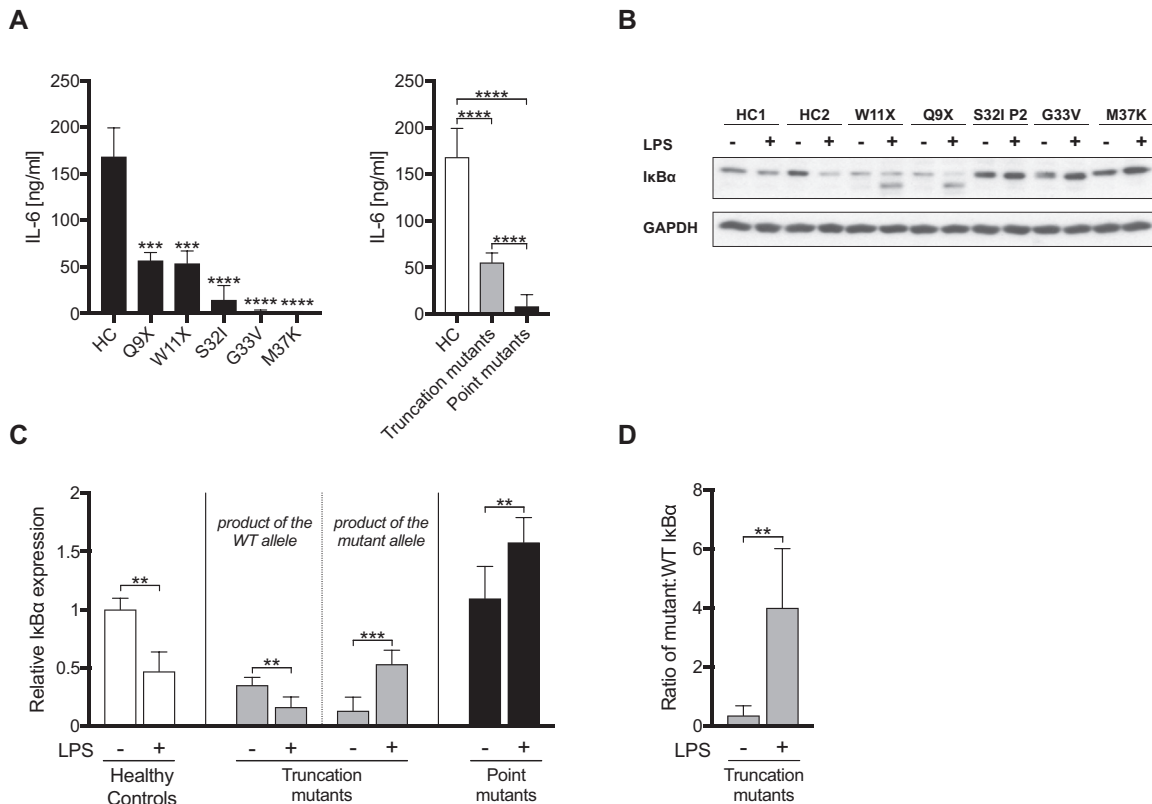


FIG 3. IκBα point mutants exert a stronger DN effect on IL-6 secretion and accumulate to a greater extent after LPS stimulation than IκBα truncation mutants. **A**, LPS-driven IL-6 secretion by fibroblasts. *Left panel*, Mean of 3 determinations on each of 2 healthy control subjects (HC), 2 patients with S32I, and individual patients with the other IκBα mutations. *Right panel*, Pooled results showing mean IL-6 secretion by fibroblasts from HCs (n = 2), patients with IκBα truncation mutations (n = 2), and patients with IκBα point mutations (n = 4). **B** and **C**, Effect of LPS stimulation on IκBα levels: representative (Fig 3, B) and quantitative (Fig 3, C) immunoblot analysis of IκBα expression in fibroblasts from 2 HCs and patients with AD EDA-ID before and after stimulation with LPS. Results from only 1 patient with an S32I mutation are shown in Fig 3, B, because of the limited number of lanes in the gel, but results from both patients with S32I mutations studied are included in the quantitative analysis, which includes 2 control subjects, 2 patients with IκBα truncation mutations, and 4 patients with IκBα point mutations. Results in Fig 3, C, are expressed as the mean ratio of IκBα to GAPDH relative to the mean of unstimulated HC set at 1.0. **D**, Mean ratio of mutant to WT IκBα in unstimulated and LPS-stimulated fibroblasts from patients with AD EDA-ID with truncating mutations in IκBα. Columns and bars represent the means ± SDs of 3 independent experiments. ***P* < .01, ****P* < .001, and *****P* < .0001.

IκBα point mutants impair noncanonical NF-κB activation to a greater extent than do IκBα truncation mutants

Defective development of tonsils and peripheral LNs in some patients with AD EDA-ID suggests a defect in noncanonical NF-κB signaling. This hypothesis is supported by our previous observation that noncanonical NF-κB signaling is defective in mouse embryonic fibroblasts derived from mutant mice heterozygous for the S32I mutation.³⁵ Furthermore, genetic deletion of many signaling components of the noncanonical NF-κB pathway in mice results in defective lymphorganogenesis, disrupted architecture of secondary lymphoid organs (SLO), or both.^{17,36-47} Noncanonical NF-κB signaling in stromal lymphoid tissue organizer (LTo) cells is critical for lymphorganogenesis and activated on binding of lymphotoxin β (LTβ) expressed by hematopoietic lymphoid tissue inducer cells to the LTβR on LTo cells.⁴⁷ Noncanonical NF-κB signaling depends on IKKα-driven phosphorylation of the cytoplasmically located NF-κB2 (p100) and its

cleavage to generate the transcriptionally active p52 product, which, in complex with the NF-κB family member RelB, translocates into the nucleus and activates gene transcription.¹⁵ Importantly, both p100 and RelB are critical for the development of SLOs^{46,50-53} and their expression is under the transcriptional control of the canonical NF-κB pathway, which thus regulates the amount of p100 and RelB available for signaling through the noncanonical NF-κB pathway.^{19,20} We examined the upregulation of p100 and RelB after stimulation of the canonical NF-κB pathway by LPS in fibroblasts used as surrogates for stromal cells. As expected, LPS stimulation upregulated p100 and RelB expression in normal fibroblasts (Fig 4, A). LPS-driven upregulation of p100 and RelB expression was significantly reduced in fibroblasts from the 5 patients with AD EDA-ID studied compared with normal fibroblasts (Fig 4, A-C). Importantly, upregulation of p100 and RelB expression was reduced significantly more in the 3 patients with the point mutations S32I, G33V, and M37K compared with the 2 patients with the truncation mutations Q9X and W11X (Fig 4, A-C). These results demonstrate that

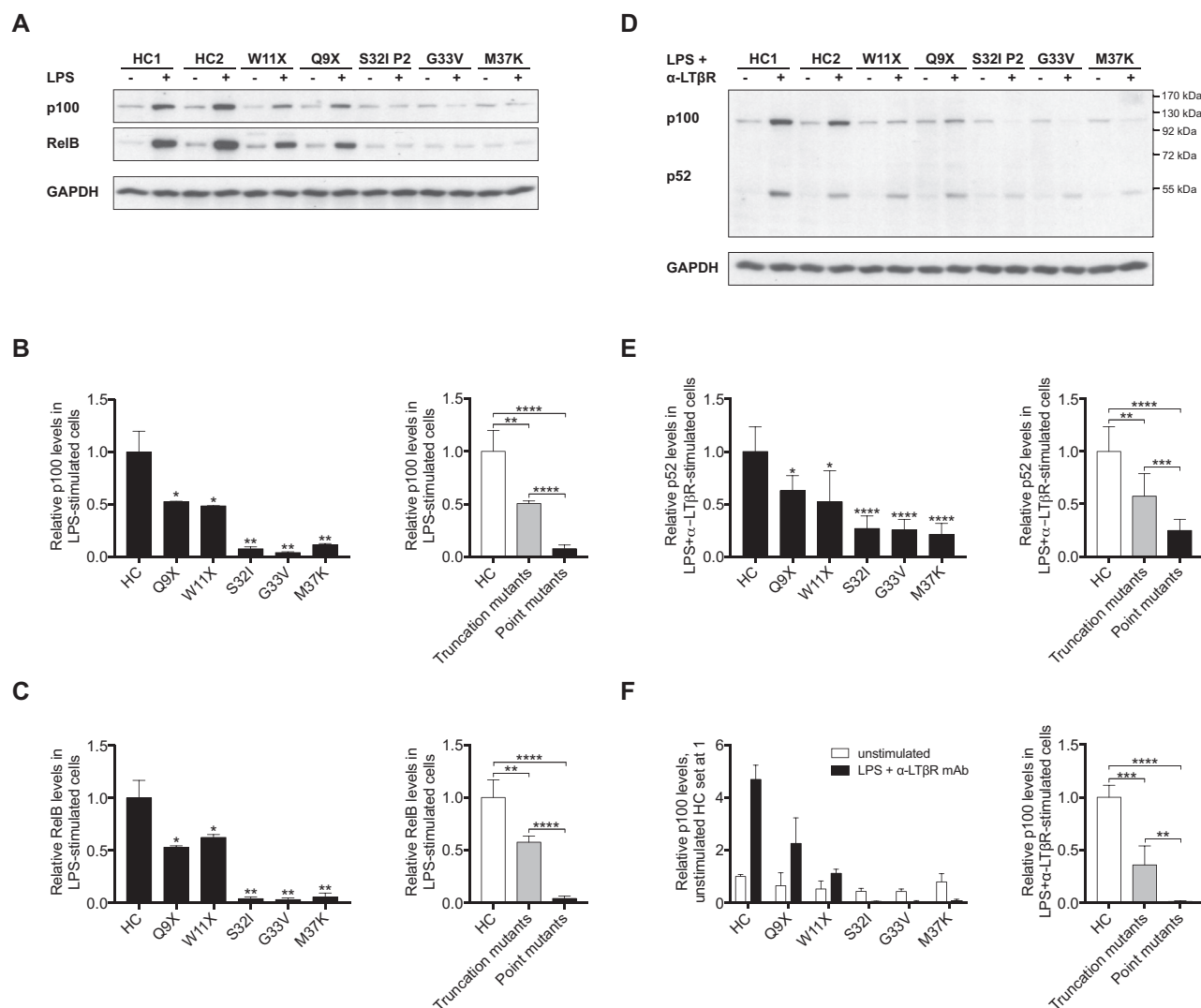


FIG 4. $\text{I}\kappa\text{B}\alpha$ point mutants impair more severely LPS-driven expression of NF- κB /p100 and RelB and activation of the noncanonical NF- κB pathway than $\text{I}\kappa\text{B}\alpha$ truncation mutants. **A**, Representative immunoblot analysis of p100 and RelB levels in fibroblasts from 2 healthy control subjects (HC) and patients with AD EDA-ID (P) before and after stimulation with LPS for 48 hours. **B** and **C**, Quantitative analysis of expression of p100 (Fig 4, B) and RelB (Fig 4, C) in LPS-stimulated fibroblasts from 2 HCs and individual patients (left panels) and pooled results representing the mean p100 and RelB expression levels in fibroblasts from patients with $\text{I}\kappa\text{B}\alpha$ truncation mutations (n = 2) and $\text{I}\kappa\text{B}\alpha$ point mutations (n = 4; right panels). Results are expressed as the mean ratio of p100 and RelB to GAPDH relative to the mean of LPS-stimulated healthy controls set at 1.0. **D**, Representative immunoblot analysis of p100 and p52 levels in fibroblasts from 2 HCs and patients with AD EDA-ID before and after stimulation with LPS plus anti-LT β R mAb for 48 hours. **E** and **F**, Quantitative analysis of expression of p52 (Fig 4, E) and p100 (Fig 4, F) in LPS plus anti-LT β R mAb-stimulated fibroblasts from HCs and individual patients (left panels) and pooled results representing the mean p52 and p100 expression levels in fibroblasts from HCs (n = 2) and patients with $\text{I}\kappa\text{B}\alpha$ truncation mutations (n = 2) and $\text{I}\kappa\text{B}\alpha$ point mutations (n = 4; right panels). Results are expressed as the ratio of p52 and p100 to GAPDH relative to the mean of LPS plus anti-LT β R mAb-stimulated (Fig 4, E) or unstimulated (Fig 4, F) HC set at 1.0. Columns and bars represent means $\pm$ SDs of at least 3 independent experiments. * P < .05, ** P < .01, *** P < .001, and **** P < .0001.

upregulation of the noncanonical NF- κB pathway family members p100 and RelB after canonical NF- κB signaling is impaired in fibroblasts from patients with AD EDA-ID and that residual canonical NF- κB activity in patients with truncation mutations correlates with their ability to upregulate expression of these 2 proteins.

Generation of p52 was measured by stimulating fibroblasts with LPS and an agonistic mAb to the LT β R for 48 hours and then

immunoblotting cellular lysates for p52 and p100. Negligible amounts of p52 were detected in unstimulated fibroblasts from patients and control subjects (Fig 4, D). Stimulation with LPS and anti-LT β R mAb resulted in the appearance of a strong p52 band in healthy control subjects, indicating LT β R-driven, noncanonical, NF- κB -mediated cleavage of p100. Fibroblasts from patients with the Q9X and W11X truncation mutations showed a partial

but significant decrease in p52 accumulation after stimulation compared with that seen in healthy control subjects (Fig 4, D-F). p52 accumulation was diminished significantly more in patients with I κ B α point mutations (approximately 80%) than in those with truncations (approximately 50%). Of note, after LPS plus anti-LT β R mAb stimulation, p100 levels robustly increased in normal fibroblasts, as observed with LPS stimulation alone, but increased significantly less in fibroblasts from patients with I κ B α truncation mutations and became virtually undetectable in fibroblasts from patients with I κ B α point mutations (Fig 4, D and F). These results suggest that the patients' cells can cleave p100 but do not produce it and replenish it normally to provide sufficient substrates for p52 generation.

Induction of genes encoding chemokines and adhesion molecules important for lymphorganogenesis is impaired more severely in patients with AD EDA-ID with I κ B α point mutations than I κ B α truncation mutations

During the development of SLOs, both the canonical and noncanonical NF- κ B pathways synergize in stromal LTo cells that receive an LT β signal to induce the expression of chemokines and adhesion molecules important for lymphorganogenesis.^{36,38-40,47} These include the chemokines CCL19, CCL20, CCL21, CXCL12, and CXCL13 and the adhesion molecules ICAM-1 and VCAM-1.^{47,54} Expression of these molecules is either fully or partially dependent on the noncanonical NF- κ B pathway.^{16,55-57} RNA sequencing (RNA-Seq) analysis of normal fibroblasts ($n = 3$) revealed that LPS plus anti-LT β R mAb stimulation upregulated the expression of 619 genes and downregulated the expression of 289 genes at a fold change of greater than 2 and a false discovery rate of less than 0.05 (Fig 5, A). The numbers of upregulated and downregulated genes were substantially lower in LPS plus anti-LT β R mAb-stimulated fibroblasts from patients with I κ B α truncation mutations (Q9X and W11X, $n = 2$) compared with those from control subjects ($n = 3$) and were drastically reduced in patients with I κ B α point mutations (S32I and G33V, $n = 2$), in whom only 4 genes were significantly upregulated and no gene was significantly downregulated (Fig 5, A). A heat map of known targets of NF- κ B, the expression of which was significantly altered by LPS plus anti-LT β R stimulation, is shown in Fig 5, B. The corresponding heat map, including all genes with expression that was significantly altered by LPS plus anti-LT β R stimulation, is shown in Fig E2, A, in this article's Online Repository at www.jacionline.org.

Of the genes known to be implicated in lymphorganogenesis, *CCL20*, *ICAM1*, and *VCAM1* were upregulated more than 2-fold in LPS plus anti-LT β R mAb-stimulated normal fibroblasts (Fig 5, B and C). Upregulation of all 3 genes was partially decreased in fibroblasts from patients with I κ B α truncation mutations and virtually nil in patients with I κ B α point mutations (Fig 5, B and C). Importantly, upregulation of *CCL20*, *ICAM1*, and *VCAM1* was more severely impaired in patients with I κ B α point mutations than in patients with I κ B α truncation mutations (Fig 5, B and C). To validate these results, we performed quantitative PCR (qPCR) analysis of the mRNA (Fig 5, D). Upregulation of *CCL20*, *ICAM1*, and *VCAM1* mRNA levels was significantly impaired in patients with I κ B α point mutations

($n = 3$) compared with healthy control subjects ($n = 3$), confirming the RNA-Seq results. Expression levels of *CCL20*, *ICAM1*, and *VCAM1* mRNA in LPS plus anti-LT β R mAb-stimulated fibroblasts from patients with I κ B α truncation mutations ($n = 2$) were reduced compared with those in healthy control subjects ($n = 3$), but the difference was not significant. These results demonstrate that expression of chemokines and adhesion molecules important for lymphorganogenesis is more severely impaired in patients with AD EDA-ID with I κ B α point mutations than in patients with I κ B α truncation mutations, thus providing a mechanistic understanding of the absence of LNs and apparently poor response to HSCT seen in patients with I κ B α point mutations. As expected, LPS plus anti-LT β R mAb stimulation strongly upregulated expression of *NFKB1A*, *NFKB2*, and *RELB* in normal fibroblasts but substantially less so in fibroblasts from patients with AD EDA-ID (see Fig E2, B).

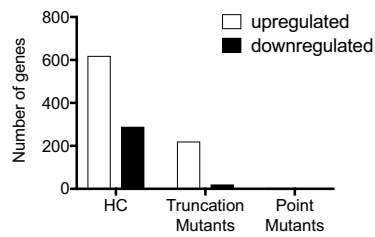
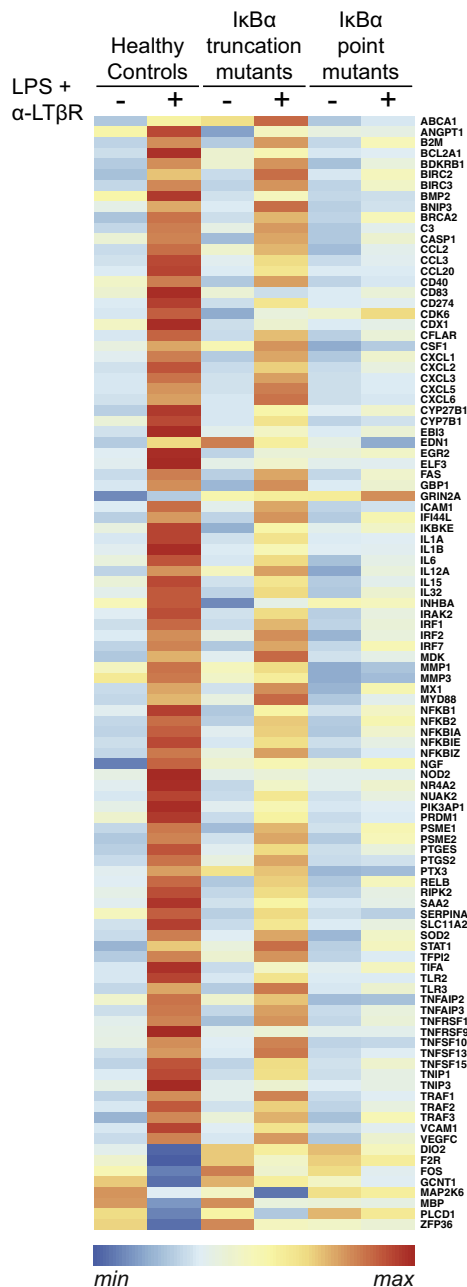
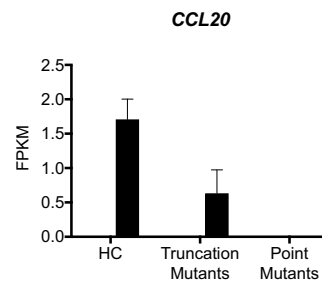
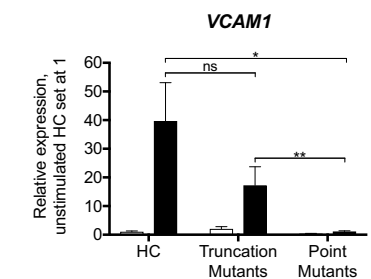
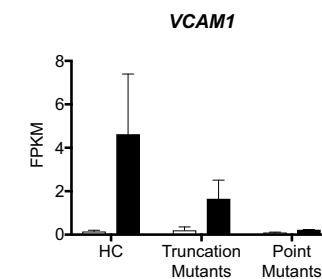
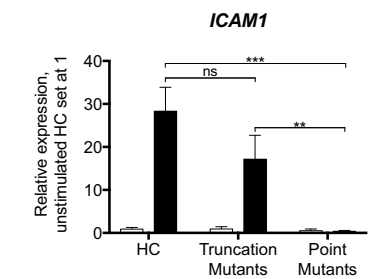
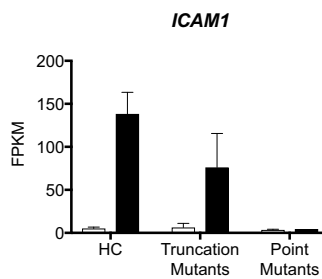
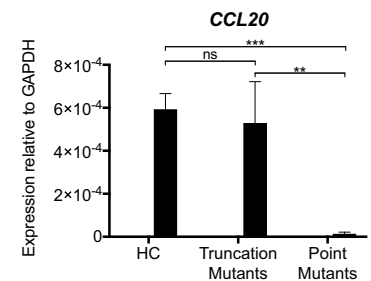
DISCUSSION

We established a robust genotype-phenotype correlation in patients with AD EDA-ID and demonstrated that I κ B α point mutants are expressed at higher levels, impair more severely canonical and noncanonical NF- κ B signaling, and result in more severe disease than I κ B α truncation mutants. Noncanonical NF- κ B-dependent genes important for lymphorganogenesis were inducible in patients with I κ B α truncation mutations but absent in patients with I κ B α point mutations, providing a potential explanation for their virtual lack of tonsils and LNs and their poor immune reconstitution after HSCT.

The disease severity score was significantly greater in the 6 patients with I κ B α point mutations compared with the 3 patients with I κ B α N-terminal truncation mutations, demonstrating that I κ B α point mutations are associated with more severe disease compared with I κ B α truncation mutations in patients with AD EDA-ID. The outcome of HSCT was poor in all 6 patients with point mutations in I κ B α despite excellent donor cell chimerism, suggesting that a defect in stromal cells might be at play.

Using fibroblasts from the patients, we were able to demonstrate lower expression of the truncated I κ B α compared with WT I κ B α . However, we were not able to determine the contribution of WT I κ B α versus point mutant I κ B α . Using HEK293T cells transfected with I κ B α mutants, we observed that truncation and point mutants were expressed at significantly lower levels than WT I κ B α , suggesting that both types of mutants, and more so the truncation mutants, are intrinsically unstable. However, we cannot rule out a contribution of inefficient translation from downstream ATG sites to the decreased expression of the truncation mutants. In addition, expression of the truncated I κ B α mutants compared with WT I κ B α was relatively lower in transfected cells than in fibroblasts from the patients.

Consistent with their higher expression levels, I κ B α point mutants were associated with more significant reduction in LPS-driven IL-6 secretion and upregulation of p100 in fibroblasts, which are dependent on the canonical NF- κ B pathway. Although pathways other than the canonical NF- κ B pathway can be activated by LPS, LPS induction of IL-6 and p100 are strictly dependent on NF- κ B.^{58,59} Impairment of IL-6 secretion exceeded what would have been expected from the baseline ratio of mutant to WT I κ B α for both patients with point and those with truncation mutations. The decrease in the level of WT I κ B α

A**B****C****D**

□ unstimulated
■ LPS + α -LT β R mAb

□ unstimulated
■ LPS + α -LT β R mAb

48 hours after LPS stimulation suggests that upregulation of WT I κ B α is outweighed by its degradation. The marked increase in the truncation mutant/WT I κ B α ratio in LPS-stimulated cells explains the DN effect exerted by these mutants despite their lower expression compared with WT protein in unstimulated cells. We previously reported that the W11X mutant did not exert a DN effect on canonical NF- κ B signaling when transiently cotransfected with WT I κ B α and an NF- κ B reporter into HEK293T cells, which were then stimulated with TNF- α for 6 hours.²⁴ In that study the short stimulation time and low expression level of the unstable mutant protein and endogenous WT I κ B α might have masked the potential of the mutant to exert a DN effect.

For the first time, we show that the noncanonical NF- κ B pathway is impaired in patients with AD EDA-ID. We demonstrate that upregulation of p100 and RelB expression, 2 key components of this pathway known to be transcriptionally regulated by the canonical NF- κ B pathway, is deficient in LPS-stimulated fibroblasts from these patients. The defect was more severe in patients with point mutations than in those with truncation mutations, paralleling the relative magnitude of the defect in LPS-driven IL-6 secretion. As expected from their lower content of the p100 substrate, generation of the transcriptionally active product p52, which is dependent on activation of the noncanonical NF- κ B pathway, was deficient in LPS plus anti-LT β R mAb-stimulated fibroblasts from these patients. The defect was more severe in patients with I κ B α point mutations than in those with I κ B α truncation mutations, a reflection of the relative level of p100 in these patients. It should be noted that a defect in the noncanonical NF- κ B pathway in the patients is an extrinsic one because of the decreased ability to generate p100, a critical substrate for IKK α . In fact, although the level of p52 after activation with LPS plus anti-LT β R is lower in the patients, the p52/p100 ratio is higher, suggesting that the intrinsic ability to activate NIK and IKK α , which is essential for p100 phosphorylation and degradation, is intact. It is likely that the higher p52/p100 ratio is a reflection of the limited amount of substrate rather than an intrinsically more active noncanonical NF- κ B activation.

Defective signaling through the noncanonical NF- κ B pathway in LPS plus anti-LT β R mAb-stimulated fibroblasts from patients with AD EDA-ID was associated with defective regulation of gene expression, as demonstrated by using RNA-Seq analysis. Importantly, 3 genes critical for lymphorganogenesis, *CCL20*, *ICAM1*, and *VCAM1*, were significantly upregulated in LPS plus anti-LT β R mAb-stimulated normal fibroblasts. Several

other genes in human LTOs are important for lymphorganogenesis. These include *CCL19*, *CCL21*, and *CXCL13*.⁶⁰ Failure to detect upregulation of genes other than *CCL20*, *ICAM1*, and *VCAM1* that are important and essential for lymphorganogenesis is likely because fibroblasts are an imperfect surrogate for the stromal LTO cells. qPCR analysis confirmed that upregulation of *CCL20*, *ICAM1*, and *VCAM1* was abolished in patients with I κ B α point mutations. Notably, expression of these 3 genes in fibroblasts from patients with I κ B α truncation mutations, although lower for *VCAM1* and *ICAM1*, was not significantly different from healthy control subjects. This finding suggests that the residual NF- κ B pathway signaling in patients with I κ B α truncation mutations is sufficient for the expression of these genes and is consistent with the presence of LNs and tonsils in these patients. Taken together, our results suggest defective activation of stromal organizer cells in patients with AD EDA-ID as a possibility.

Severely defective activation of the noncanonical NF- κ B pathway in stromal cells from patients with I κ B α point mutations likely explains the absence of visible tonsils and the lack of detectable peripheral LNs in these patients, as well as their poor outcome after HSCT. SLOs are critical for adaptive immunity because they provide a scaffold for the interactions of antigen-bearing dendritic cells derived from peripheral tissues with recirculating antigen-specific T cells, which are essential for T-cell immunity. They also provide a scaffold for the interaction of B cells with T helper cells, which is important for T cell-dependent antibody responses, and with antigen-presenting cells in the splenic marginal zone, which is important for type II T cell-independent antibody responses to polysaccharide antigens.

Our study establishes a genotype-phenotype correlation in patients with AD EDA-ID that has important implications for their management. Higher levels of mutant protein in patients with I κ B α point mutations might explain the more severe disease, impaired development of SLOs, and poor response of these patients to HSCT. Given the severe functional defect in the noncanonical pathway in patients with point mutations, strategies aimed at correcting the stromal cell niche essential for lymphorganogenesis need to be considered in these patients.

We thank Drs J. Orange, L. D. Notarangelo, and F. Bonilla for their valuable advice in devising the AD EDA-ID scoring system and Ms B. Cangemi for technical assistance.

FIG 5. I κ B α point mutants impair the induction of genes important for lymphorganogenesis more severely than I κ B α truncation mutants. **A**, Number of genes significantly ($P < .05$) upregulated or downregulated by 2-fold or more in LPS plus anti-LT β R mAb-stimulated fibroblasts from healthy control subjects ($n = 3$) and patients with I κ B α truncation ($n = 2$) and point ($n = 2$) mutations. **B**, Heat map analysis of NF- κ B-regulated genes with expression that was significantly altered by LPS plus anti-LT β R stimulation of normal fibroblasts. **C**, RNA-Seq analysis of the expression of *CCL20*, *ICAM1*, and *VCAM1* mRNA in unstimulated and LPS plus anti-LT β R mAb-stimulated fibroblasts from healthy control subjects ($n = 3$) and patients with I κ B α truncation ($n = 2$) and point ($n = 2$) mutations. Results are expressed as FPKM. Columns and bars represent means $\pm$ SDs for each of the 3 groups. **D**, qPCR analysis of the expression of *CCL20*, *ICAM1*, and *VCAM1* mRNA in unstimulated and LPS plus anti-LT β R mAb-stimulated fibroblasts from healthy control subjects ($n = 3$) and patients with I κ B α truncation ($n = 2$) and point mutations ($n = 3$). Results for *ICAM1* and *VCAM1* are expressed as the ratio of the gene of interest to *GAPDH* as calculated by using the $2^{-\Delta\Delta C_t}$ method relative to that of unstimulated healthy controls set at 1.0. Because *CCL20* was undetectable in unstimulated fibroblasts, results are expressed as the ratio of *CCL20* to *GAPDH* as calculated by using the $2^{-\Delta C_t}$ method. Columns and bars in Fig 5, D, represent the means $\pm$ SDs of 3 independent experiments. * $P < .05$, ** $P < .01$, and *** $P < .001$. ns, Not significant.

Key messages

- **IkB α point mutations are associated with more severe disease compared with IkB α truncation mutations in patients with AD EDA-ID.**
- **IkB α point mutants are expressed at significantly higher levels in fibroblasts and transfectants compared with IkB α truncation mutants.**
- **IkB α point mutations are associated with greater impairment of canonical and noncanonical NF- κ B activity and severely defective expression of genes important for lymphorganogenesis compared with IkB α truncation mutations.**

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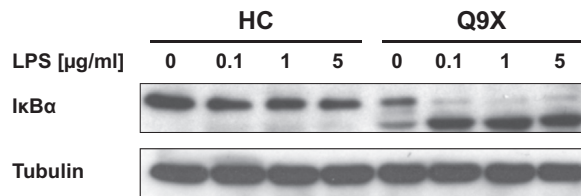


FIG E1. Effect of LPS stimulation on I κ B α levels in the patient with the I κ B α Q9X truncating mutation. Representative I κ B α immunoblot of fibroblasts from a healthy control subject (*HC*) and the patient. Two lower-molecular-weight I κ B α bands are evident in unstimulated fibroblasts from the patient; only the band with the lowest molecular weight increased in intensity after stimulation with increasing concentrations of LPS.

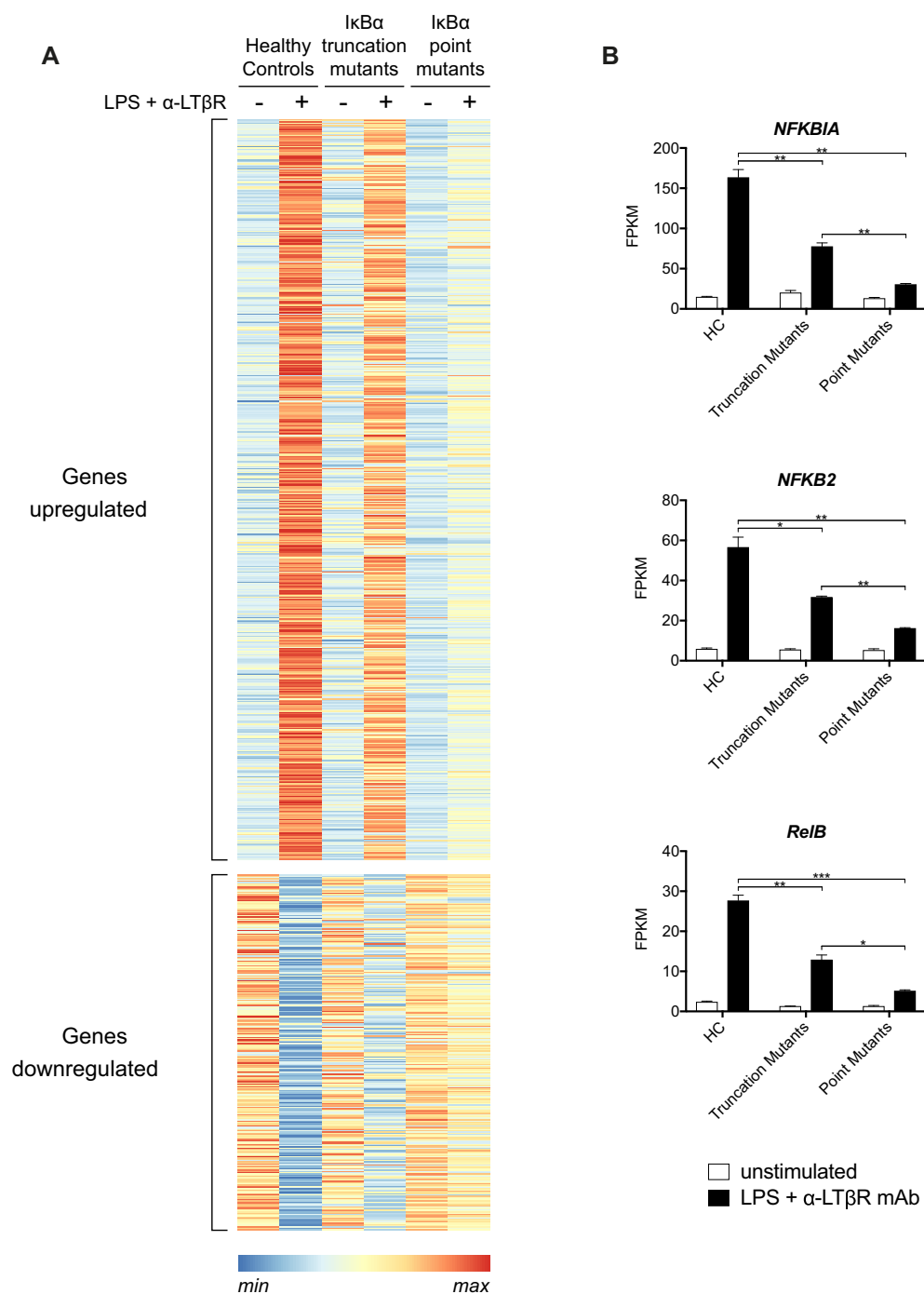


FIG E2. I κ B α point mutants impair more than I κ B α truncation mutants the induction of various genes, including those regulated by NF- κ B. **A**, Heat map of genes the expression of which was significantly altered by LPS plus anti-LT β R stimulation of normal fibroblasts. **B**, RNA-Seq analysis of the expression of *NFKB1A*, *NFKB2*, and *RELB* mRNA in unstimulated and LPS plus anti-LT β R mAb-stimulated fibroblasts from HCs ($n = 3$) and patients with I κ B α truncation ($n = 2$) and point ($n = 2$) mutations. Results are expressed as FPKM. Columns and bars represent means $\pm$ SDs for each of the 3 groups. * $P < .05$, ** $P < .01$, and *** $P < .001$.

TABLE E1. Breakdown of the disease severity score of the patients

Patient	Infections	Ectodermal dysplasia:			Memory T and/or B cells	Cumulative score
		Systems affected	Antibody responses	Lymphocytes/ μ L		
1	3	3	3	3	3	15/15
2	3	3	3	3	2	14/15
3	3	3	3	3	3	15/15
4	3	3	2	3	3	14/15
5	2	2	3	1	3	11/15
6	2	3	3	1	3	12/15
7	3	2	NA	1	0	6/12
8	1	2	1	0	0	4/15
9	2	1	NA	1	0	4/12

NA, Not available.