Severe disease and greater impairment of NF-κB activation in IκBa point mutants versus truncation mutants in autosomal dominant anhidrotic ectodermal dysplasia with immune deficiency

Daniel Petersheim, MD[1,#,*], **Michel J. Massaad, PhD**[1,#], **Saetbyul Lee, PhD**[1], **Alessia Scarselli, MD**[2], **Caterina Cancrini, MD**[2], **Kunihiko Moriya, MD, PhD**[3], **Yoji Sasahara, MD, PhD**[3], **Arjan C. Lankester, MD, PhD**[4], **Morna Dorsey, MD**[5], **Daniela Di Giovanni, MD**[6], **Liliana Bezrodnik, MD**[6], **Hidenori Ohnishi, MD, PhD**[7], **Ryuta Nishikomori, MD**[8], **Kay Tanita, MD**[9], **Hirokazu Kanegane, MD, PhD**[9], **Tomohiro Morio, MD**[9], **Erwin W. Gelfand, MD**[10], **Ashish Jain, MD**[11], **Elizabeth Secord, MD, PhD**[12], **Capucine Picard, MD, PhD**[13], **Jean-Laurent Casanova, MD, PhD**[13,14], **Michael H. Albert, MD**[15], **Troy R. Torgerson, MD, PhD**[16], and **Raif S. Geha, MD**[1]

[1]Division of Immunology, Boston Children's Hospital, Harvard Medical School, Boston, MA
[2]Division of Immunology and Infectious Diseases, Department of Pediatrics, Bambino Gesù Children's Hospital, Rome, University of Rome Tor Vergata, Italy [3]Department of Pediatrics, Tohoku University, Japan [4]Leiden University Medical Center, The Netherlands [5]University of California, CA [6]Ricardo Gutiérrez Children's Hospital, Argentina [7]Department of Pediatrics, Gifu University, Japan [8]Department of Pediatrics, Kyoto University, Japan [9]Department of Pediatrics, Tokyo Medical and Dental University, Japan [10]Immunodeficiency Diagnosis and Treatment Program, Department of Pediatrics, National Jewish Health, Denver, CO [11]Merck Research Laboratories Boston, MA [12]Children's Hospital of Michigan, MI [13]Necker Hospital, Paris Descartes University, France [14]Rockefeller University, NY [15]Dr. von Hauner University Children's Hospital, Ludwig-Maximilians-University Munich, Germany [16]Seattle Children's Research Institute, WA

Abstract

Background—Autosomal dominant anhidrotic ectodermal dysplasia with immune deficiency (AD EDA-ID) is caused by heterozygous point mutations at or close to S32 and S36 or N-terminal truncations in IκBα that impair its phosphorylation and degradation, and thus activation of the canonical NF-κB pathway. The outcome of hematopoietic stem cell transplantation is poor in AD

Address correspondence to: Raif S. Geha, One Blackfan Circle, Boston, Massachusetts 02115, (617) 919-2482; raif.geha@childrens.harvard.edu.
#Equal contribution
*Current address: Department of Pediatrics, Dr. von Hauner Children's Hospital, Ludwig-Maximilians-University, Munich, Germany

EDA-ID despite achievement of chimerism. Mice heterozygous for the S32I mutation in IκBα have impaired non-canonical NF-κB activity and defective lymphorganogenesis.

Objective—To establish genotype-phenotype correlation in AD EDA-ID.

Methods—A disease severity scoring system was devised. Stability of IκBα mutants was examined in transfected cells. Immunological, biochemical, and gene expression analyses were performed to evaluate canonical and non-canonical NF-κB signaling in skin-derived fibroblasts.

Results—Disease severity was greater in patients with IκBα point mutations than in patients with truncation mutations. IκBα point mutants were expressed at significantly higher levels in transfectants compared to truncation mutants. Canonical NF-κB-dependent IL-6 secretion and upregulation of the NF-κB2/p100 and RelB components of the non-canonical NF-κB pathway were diminished significantly more in patients with point mutations compared to those with truncations. Non-canonical NF-κB-driven generation of the transcriptionally active p100 cleavage product p52, and upregulation of *CCL20*, *ICAM1* and *VCAM1*, important for lymphorganogenesis, were diminished significantly more in LPS+α-LTβR-stimulated fibroblasts from patients with point mutations compared to those with truncations.

Conclusions—IκBα point mutants accumulate at higher levels compared to truncation mutants and are associated with more severe disease and greater impairment of canonical and non-canonical NF-κB activity in AD EDA-ID.

Graphical abstract
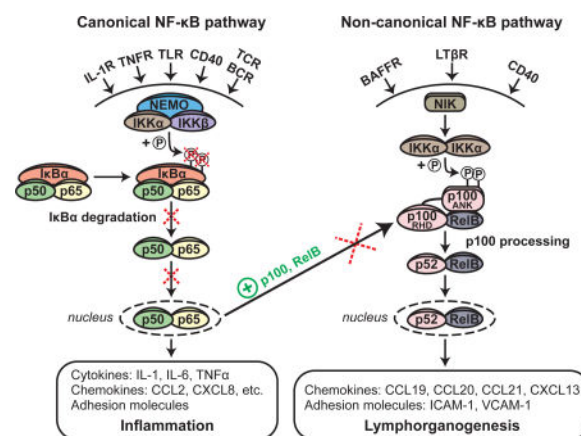
ANK, Ankyrin repeat domain; BAFFR, B cell-activating factor receptor; BCR, B cell receptor IL-1R, Interleukin-1 receptor; LTβR, Lymphotoxin beta receptor; RHD, Rel homology domain TCR, T cell receptor; TLR, Toll-like receptor; TNFR, Tumor necrosis factor receptor

Keywords

ectodermal dysplasia with immune deficiency; IκBα; canonical NF-κB pathway; non-canonical NF-κB pathway; lymphorganogenesis; hematopoietic stem cell transplantation

INTRODUCTION

Primary immunodeficiency diseases (PID) 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] The inhibitor of κB (IκB) proteins inhibit the transcription factor NF-κB (nuclear factor kappa light chain enhancer in activated B cells).[8] Heterozygous mutations in the IκB family member IκBα cause autosomal dominant anhidrotic ectodermal dysplasia with immune deficiency (AD EDA-ID), 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 AD EDA-ID with variable severity and patient outcome after hematopoietic stem cell transplantation (HSCT), but a genotype-phenotype correlation has not been established.

The NF-κB family of transcription factors includes RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105) and NF-κB2 (p52/p100) and plays a critical role in innate and adaptive immunity. [8, 10, 11] In resting cells, NF-κB is retained in the cytoplasm through association with members of the IκB family of proteins. Two distinct pathways leading to NF-κB activation and nuclear translocation have been identified: a canonical and a non-canonical NF-κB pathway.[12] The canonical, or classical, NF-κB pathway is used by a broad range of cell surface receptors, including toll-like receptors (TLRs), interleukin-1 (IL-1) receptor, tumor necrosis factor alpha (TNFα) receptor, antigen receptors, and others.[8, 11] Receptor ligation causes activation of the IκB kinase (IKK) complex, which consists of the catalytic subunits IKKα and IKKβ and the scaffolding protein IKKγ/NEMO. This results in the phosphorylation of IκBα at serines 32 (S32) and 36 (S36), priming it for ubiquitination and proteasomal degradation. The released p50:p65 complex translocates into the nucleus where it induces the transcription of genes that mediate the inflammatory response.[13, 14] The non-canonical NF-κB pathway is used by a limited range of cell-surface receptors, including lymphotoxin beta (LTβ) receptor, B cell activating factor (BAFF) receptor, and CD40. Receptor ligation drives NF-κB inducing kinase (NIK) to phosphorylate IKKα.[15] IKKα subsequently phosphorylates NF-κB2/p100, which is in complex with RelB and contains an IκB-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 two NF-κB pathways: NIK-dependent activation of IKKα has been reported to cause IκBα phosphorylation and degradation.[17] Importantly, transcription of p100 and RelB, as well as IκBα, is regulated by p50:p65.[18–21]

Two types of human IκBα mutations have been described in 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 IκBα proteins through re-initiation of translation at downstream ATG sites ('truncation mutations'). Both types of mutations impair signal-induced phosphorylation of IκBα and protect the mutant protein from subsequent ubiquitination and

proteasomal degradation. By sequestering the NF-κB transcription factors p50 and p65 in the cytoplasm, the non-degradable IκBα mutant protein interferes with activation of the canonical NF-κB pathway. Eight AD EDA-ID patients carrying six IκBα mutations have been reported to date.[22–29] and more cases are known to exist or have been submitted for publication.[30] 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 IκBα mutants, except W11X, inhibited NF-κB activation more than WT IκBα, and thus have been demonstrated to function as dominant negative (DN). A majority of AD EDA-ID cases 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 (Ig) replacement, suggesting a contribution from non-hematopoietic cells to residual disease after transplantation.[30–32]

A knock-in mouse heterozygous for the S32I mutation in IκBα shows ectodermal dysplasia, impaired phosphorylation and degradation of the mutant IκBα protein, and diminished response to TLR ligation.[33] Strikingly, the S32I mutant completely lacks lymph nodes (LNs), Peyer's patches, marginal zone B cells and follicular dendritic cells, and fails to form germinal centers, features typical of defective non-canonical NF-κB signaling that have remained largely unaddressed in AD EDA-ID patients.[17, 34–45] WT->S32I IκBα chimeras failed to form proper lymphoid organs and to reconstitute immune function despite excellent donor cell engraftment, paralleling the failure of HSCT to cure AD EDA-ID patients. In contrast, S32I IκBα->$Rag2^{-/-}$ bone marrow (BM) chimeras developed proper lymphoid organs. These results suggest that defective stromal cell function may contribute to the immunodeficiency in AD EDA-ID, and may explain the poor outcome of HSCT in this disease.

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

MATERIALS AND METHODS

Cell lines

Skin fibroblast cell lines were established from skin punch biopsies obtained from patients and three adult healthy donors after securing informed consent 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 DMEM (Life Technologies, Carlsbad, CA), supplemented with 15% FBS (Gemini Bio-Products, Broderick, CA), 10 mM HEPES buffer (Sigma, St. Louis, MO), 100 U/ml Potassium Penicillin and 100 µg/ml Streptomycin

Sulfate (Lonza, Walkersville, MD). HEK293T cell line (ATCC CRL-3216) was obtained from the American Type Culture collection and cultured in DMEM, supplemented with 10% FBS, 10 mM HEPES buffer, 100 U/ml Potassium Penicillin and 100 μg/ml Streptomycin Sulfate. Before and during transfection experiments, HEK293T cells were cultured in antibiotics-free medium.

Plasmids and targeted mutagenesis

An expression vector containing cDNA encoding C-terminally FLAG-tagged human IκBα (pCMV3-NFKBIA-C-FLAG; HG12045-CF) was obtained from Sino Biological Inc. (Beijing, China). Targeted mutagenesis was performed using Quik Change II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions together with custom mutagenic primers: Q9X_F: 5'-CGGCCGAGCGCCCCTAGGAGTGGGCCATGGAGG-3'; Q9X_R: 5'-CCTCCATGGCCCACTCCTAGGGGCGCTCGGCCG-3'; W11X_F: 5'-CCG-AGCGCCCCCAGGAGTAGGCCATGGAGGGCC-3'; W11X_R: 5'-GGCCCTCCATGG-CCTACTCCTGGGGGCGCTCGG-3'; E14X_F: 5'-CCCAGGAGTGGGCCATGTAGG-GCCCCCGCGACGG-3'; E14X_R: 5'-CCGTCGCGGGGGCCCTACATGGCCCAC-TCCTGGG-3'; S32I_F: 5'-GGACGACCGCCACGACATCGGCCTGGACTCC-3'; S32I_R: 5'-GGAGTCCAGGCCGATGTCGTGGCGGTCGTCC-3'; G33V_ F: 5'-CGACCGCCA-CGACAGCGTCCTGGACTCCATGAAAG-3'; G33V_R: 5'-CTTTCATGGAGTCCAGGA-CGCTGTCGTGGCGGTCG-3'; S36Y_F: 5'-CGACAGCGGCCTGGACTACATGAA-AGACGAGG-3'; S36Y_R: 5'-CCTCGTCTTTCATGTAGTCCAGGCCGCTGTCG-3'; M37K_F: 5'-GCGGCCTGGACTCCAAGAAAGACGAGGAGTACG-3'; M37K_R: 5'-CGT-ACTCCTCGTCTTTCTTGGAGTCCAGGCCGC-3'; resulting in the following changes in IκBα (RefSeq NM_020529) on 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 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 antibiotics-free medium. Transfections were carried out using FuGENE 6 transfection reagent (Promega, Madison, WI) at a 3:1 FuGENE:DNA ratio. Three μg of WT or mutant pCMV3-NFKBIA-C-FLAG were transfected per well. Cells were harvested 24 hours post-transfection and used in immunoblotting as described below.

Semi-quantitative RT-PCR from transfectants

Total RNA of HEK293T cells transiently transfected with WT or mutant pCMV3-NFKBIA-C-FLAG was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. For each sample, 500 ng of RNA were reverse transcribed using iScript cDNA synthesis kit (BioRad, Hercules, CA). The following primers were used for RT-PCR: IκBα_F: 5'-CAATGCTCAGGAGCCCTGTAA-3'; FLAG_R: 5'-CTTATCGTCGT CATCCTTGTAATC-3'; GAPDH_F: 5'-TCGACAGTCAGCCGCATCTT-3'; GAPDH_R: 5'-CAAATGAGCCCCAGCCTTCTC-3'. To specifically amplify ectopically expressed IκBα transcripts, the reverse primer was

designed to bind to the FLAG tag sequence (GATTACAAGGATGACGACGATAAG). 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 controls and AD EDA-ID patients were grown to about 80% confluency. Cells were harvested by trypsinization, re-seeded at a density of 100,000 cells per well (6-well format) and incubated overnight. Fibroblasts were treated with media, 1 μg/ml LPS (Invivogen, San Diego, CA) or 1 μg/ml LPS + 5 μg/ml α-LTβR agonistic mAb (clone 31G4D8, BioLegend, San Diego, CA) for 48 hours. IL-6 levels in the supernatants of LPS-stimulated cells were determined 24 hours post stimulation by ELISA (eBioscience).

Immunoblotting

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

RNA-Seq analysis of LPS+α-LTβR-stimulated fibroblasts

Fibroblasts from healthy controls and AD EDA-ID patients were treated with media or 1 μg/ml LPS + 5 μg/ml α-LTβR agonistic mAb for 48 hours. RNA was isolated using TRIzol Plus RNA Purification Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. On-column DNAse digestion was performed using PureLink DNAse Set (Invitrogen, Carlsbad, CA). Libraries were prepared using Illumina TruSeq Stranded mRNA sample preparation kits from 500 ng of purified total RNA according to the manufacturer's protocol. The finished dsDNA libraries were quantified by Qubit fluorometer, Agilent TapeStation 2200, and RT-qPCR using the Kapa Biosystems library quantification kit according to manufacturer's protocols. Uniquely indexed libraries were pooled in equimolar ratios and sequenced on an Illumina NextSeq500 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 using STAR (v2.5.1b) and differential expression testing was performed by DESeq2 (v1.10.1) 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-value<0.05) up or downregulated by at least two folds in LPS+α-LTβR stimulated fibroblasts from healthy donors compared to unstimulated cells. FPKM values were log2-transformed and Z-scores

were calculated. Signaling components and molecular targets of NF-κB were identified by comparing genes significantly up or downregulated by two folds in LPS+α-LTβR stimulated fibroblasts from healthy donors with online databases of NF-κB target genes (https://www.bu.edu/nf-kb/gene-resources/target-genes/) as well as genes included in commercially available NF-κB-specific gene arrays (TaqMan Array Human NFKB Pathway, Cat. 4414095, Thermo Fisher, Waltham, MA; RT^2 Profiler PCR Array Human NF-κB Signaling Pathway and Signaling Targets, Cat. PAHS-025Z and PAHS-225Z, Qiagen, Germantown, MD). Heatmaps representing Z-scores of log2-transformed FPKM values were done in R (https://www.r-project.org) using the pheatmap and RColorBrewer packages.

Quantitative PCR (qPCR) analysis of *CCL20*, *ICAM1*, and *VCAM1* mRNA levels

RNA was isolated from fibroblasts treated with media or 1 μg/ml LPS + 5 μg/ml α-LTβR agonistic mAb for 48 hours. For each sample, 100 ng of RNA were reverse transcribed using iScript cDNA synthesis kit (BioRad, Hercules, CA). TaqMan primer/probes for human *CCL20*, *ICAM1*, *VCAM1* and *GAPDH* were purchased from Thermo Fisher Scientific (Waltham, MA). Experiments were run on an Applied Biosystems 7300 Real-Time PCR system. Data was analyzed using the $2^{-\Delta Ct}$ method.

Statistical analysis

Statistical analyses were performed using Prism 7 software (GraphPad, La Jolla, CA). Significance was calculated by using two-tailed t tests.

RESULTS

AD EDA-ID patients with point mutations in IκBα have more severe disease than patients with truncation mutations and a poor outcome after HSCT

We analyzed nine AD EDA-ID patients, including seven of the eight published cases and two unreported cases (Table I). Six patients have four different heterozygous point mutations at or adjacent to the S32 and S36 phosphorylation sites, leading to single amino acid substitutions: three with p.S32I and one each with p.G33V, p.S36Y and p.M37K (Table I). Three patients have each a heterozygous N-terminal nonsense mutation, p.Q9X, p.W11X, and p.E14X, 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 three experts in primary immunodeficiency who were not participants in the study. Scoring is based on five 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 the antibody deficiency (deficiency of response to polysaccharide antigens, protein antigens, or both), magnitude of the elevation in the circulating lymphocyte count, and severity of the reduction in CD3$^+$CD45RO$^+$ memory T cells and/or CD19$^+$CD27$^+$ B cells. The maximum total score is 15. In two patients (Patients 7 and 9 in Table I) only four of the five criteria were evaluated,

resulting in a maximal 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 six patients with IκBα point mutations compared to the three patients with IκBα truncation mutations (Table 1 and Fig. 1, A). The breakdown of the individual scores for each of the two groups and for each of the patients are shown in Fig. 1, B and Supplemental Table E1.

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

IκBα point mutants are expressed at higher levels compared to IκBα N-terminally truncated mutants

Degradation-resistant IκBα mutant proteins sequester p50:p65 in the cytoplasm impairing NF-κB activation. Thus, the level of degradation-resistant IκBα mutant protein in the cell is expected to determine the extent of impairment of NF-κB activation. We hypothesized that the levels of IκBα point mutants, which are associated with significantly more severe disease, would be higher than those of IκBα truncation mutants. Immunoblotting for IκBα was performed using an antibody directed to the C-terminus of IκBα in lysates from skin-derived fibroblasts. Fibroblasts were available from four patients with point mutations (two S32I, one G33V and one M37K) and two patients with truncations (Q9X, 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 two patients with the Q9X and W11X IκBα truncation mutations demonstrated an IκBα band of normal size that represents the product of the WT allele (Fig. 2, A). In addition, two fainter lower molecular weight IκBα 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 IκBα products to WT IκBα in the two patients was 0.4±0.2 (n=3). As previously reported,[22, 27, 28] only one IκBα 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 normal controls. Since κBα is under control of canonical NF-κB signaling[46, 47], it is likely that the contribution of the product of the WT IκBα allele to the total IκBα level is less that of the degradation resistant product of the mutant IκBα allele.

Measurement of IκBα 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 IκBα expression of chronic stimulation of the fibroblast line by potential NF-κB activators in fetal calf serum, and the inability to directly determine the level of WT and mutant IκBα in fibroblasts from patients with IκBα point mutations. To assess the stability of IκBα mutant proteins, HEK293T cells were transiently transfected with expression vectors encoding C-terminally FLAG-tagged WT or mutant IκBα. Twenty-four hours post-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 IκBα, which was expressed at levels not significantly different from WT IκBα, all IκBα mutant proteins showed a significant decrease in expression compared to WT IκBα (Fig. 2, B and C). Importantly, the expression levels of the three IκBα truncation mutants Q9X, W11X and E14X were significantly reduced compared to those of the four point mutants S32I, G33V, S36Y and M37K. Reduced expression levels of the mutant proteins did not result from mRNA instability, as evaluated by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) of *IκBα-FLAG* mRNA (Fig. 2, D). These results strongly suggest that IκBα point mutants are significantly more stable than IκBα truncation mutants.

IκBα point mutants impair LPS-driven IL-6 secretion, and accumulate following LPS stimulation significantly more than IκBα truncation mutants

Impaired production of inflammatory cytokines following activation of the canonical NF-κB pathway in 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 TNFα) by different cell types (PBMCs, monocytes, fibroblasts) using different modes of stimulation (LPS and/or TNFα and/or IL-1), precluding useful comparison of inflammatory cytokine production in the patients. We compared the secretion of the same cytokine, IL-6, by a single cell source, skin-derived fibroblasts, following stimulation by the same agent, LPS. IL-6 secretion was robustly induced by LPS stimulation in fibroblasts from healthy controls (Fig. 3, A). In contrast, LPS-driven IL-6 production was virtually abolished in all four patients with point mutations studied (two S32I, one G33V, one M37K), but was partially preserved in the two patients with truncation mutations studied (Q9X, W11X). These results suggest that IκBα point mutants cause a significantly greater impairment of canonical NF-κB activation compared to IκBα truncation mutants.

Canonical NF-κB activation results in rapid degradation of IκBα followed by IκBα resynthesis.[46, 47] 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 ~ half that of WT protein prior to LPS stimulation, prompted us to examine whether LPS activation alters

the mutant to 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 in normal fibroblasts (Fig. 3, B and C). Similarly, WT IκBα 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 ~0.4 in unstimulated cells to ~4 (n=3) in stimulated cells (Fig. 3, D). These results, therefore, provide a rationale for how IκBα truncation mutants can function as DN despite their lower expression compared to WT IκBα. The two 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 two bands were evident in other experiments (Supplemental Fig. S1). Of note, only the IκBα truncated product with the lower molecular weight, which likely lacks the S32 and S36 phosphorylation sites, increased following stimulation; whereas the higher molecular weight truncated product, which likely retains S32 and S36, may be subject to degradation (Supplemental Fig. S1).

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 κBα protein is expected to be expressed at a lower level than the IκBα point mutant at baseline due to impaired NF-κB canonical activity, resulting in an expected point mutant:WT IκBα ratio of >1 at baseline. Following LPS stimulation, the WT but not the mutant protein would be degraded resulting in a further increase in the point mutant:WT κBα ratio. This ratio would be expected to be higher than that of the truncation mutant: WT ratio due to 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.

IκBα point mutants impair non-canonical 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 non-canonical NF-κB signaling. This hypothesis is supported by our previous observation that non-canonical NF-κB signaling is defective in mouse embryonic fibroblasts derived from mutant mice heterozygous for the S32I mutation.[33] Furthermore, genetic deletion of many signaling components of the non-canonical NF-κB pathway in mice results in defective lymphorganogenesis and/or disrupted architecture of secondary lymphoid organs (SLO).[17, 34–45] Non-canonical NF-κB signaling in stromal lymphoid tissue organizer (LTo) cells is critical for lymphorganogenesis and is activated upon binding of lymphotoxin beta (LTβ) expressed by hematopoietic lymphoid tissue inducer (LTi) cells to the LTβ receptor (LTβR) on LTo cells.[45] Non-canonical 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[44, 48–51] 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 non-canonical NF-κB-pathway.[19, 20] We examined the upregulation of p100 and RelB following 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 five AD EDA-ID patients studied compared to normal fibroblasts (Fig. 4, A–C). Importantly, upregulation of p100 and RelB expression was reduced significantly more in the three patients with the point mutations S32I, G33V and M37K compared to the two patients with the truncation mutations Q9X and W11X (Fig. 4, A–C). These results demonstrate that upregulation of the non-canonical NF-κB pathway family members p100 and RelB following canonical NF-κB signaling is impaired in fibroblasts from AD EDA-ID patients, and that residual canonical NF-κB activity in patients with truncation mutations correlates with their ability to upregulate expression of these two proteins.

The generation of p52 was measured by stimulating fibroblasts with LPS and an agonistic monoclonal antibody (mAb) to the LTβR for 48h, then immunoblotting cellular lysates for p52 and p100. Negligible amounts of p52 were detected in unstimulated fibroblasts from patients and controls (Fig. 4, D). Stimulation with LPS and anti-LTβR mAb resulted in the appearance of a strong p52 band in healthy controls, indicating LTβR-driven, non-canonical 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 following stimulation compared to healthy controls (Fig. 4, D–F). p52 accumulation was diminished significantly more in patients with IκBα point mutations (~80%) than in those with truncations (~50%). Of note, following LPS+anti-LTβR mAb stimulation, p100 levels robustly increased in normal fibroblasts, as observed with LPS stimulation alone, but increased significantly less in fibroblasts from patients with IκBα truncation mutations, and became virtually undetectable in fibroblasts from patients with κBα point mutations (Fig. 4, D–F). These results suggest that the patient cells can cleave p100, but fail to 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 AD EDA-ID patients with IκBα point mutations than IκBα truncation mutations

During the development of SLOs, both the canonical and non-canonical NF-κB pathways synergize in stromal LTo cells that receive an LTβ signal to induce the expression of chemokines and adhesion molecules important for lymphorganogenesis.[34, 36–38, 45] These include the chemokines CCL19, CCL20, CCL21, CXCL12 and CXCL13, and the adhesion molecules ICAM-1 and VCAM-1.[45, 52] Expression of these molecules is either fully or partially dependent on the non-canonical NF-κB-pathway.[16, 53–55] RNA-Seq analysis of normal fibroblasts (n=3) revealed that LPS+anti-LTβR mAb stimulation upregulated the expression of 619 genes, and downregulated the expression of 289 genes at a fold change >2, and FDR< 0.05 (Fig. 5, A). The numbers of upregulated and downregulated genes were substantially lower in LPS+anti-LTβR mAb stimulated fibroblasts from patients with IκBα truncation mutations (Q9X and W11X, n=2) compared to controls (n=3), and were

drastically reduced in patients with IκBα 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-κB the expression of which was significantly altered by LPS+anti-LTβR stimulation is shown in Fig. 5, B. The corresponding heat map including all genes the expression of which was significantly altered by LPS+anti-LTβR stimulation is shown in Supplemental Fig. S2, A.

Of the genes known to be implicated in lymphorganogenesis, *CCL20, ICAM1* and *VCAM1* were upregulated more than two fold in LPS+anti-LTβR mAb stimulated normal fibroblasts (Fig. 5, B and C). Upregulation of all three genes was partially decreased in fibroblasts from patients with IκBα truncation mutations and virtually nil in patients with IκBα point mutations (Fig. 5, B and C). Importantly, upregulation of *CCL20, ICAM1* and *VCAM1* was more severely impaired in patients with IκBα point mutations than in patients with IκBα truncation mutations (Fig. 5, B and C). To validate these results, we performed qPCR analysis of the mRNA (Fig. 5, D). Upregulation of *CCL20, ICAM1* and *VCAM1* mRNA levels was significantly impaired in patients with IκBα point mutations (n=3) compared to healthy controls (n=3), confirming the RNA-Seq results. The expression levels of *CCL20, ICAM1* and *VCAM1* mRNA in LPS+anti-LTβR mAb stimulated fibroblasts from patients with IκBα truncation mutations (n=2) were reduced compared to healthy controls (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 AD EDA-ID patients with IκBα point mutations than in patients with IκBα truncation mutations, thus providing a mechanistic understanding of the absence of lymph nodes and apparently poor response to HSCT seen in patients with IκBα point mutations. As expected, LPS+anti-LTβR mAb stimulation strongly upregulated the expression of *NFKBIA, NFKB2* and *RELB* in normal fibroblasts, but substantially less in fibroblasts from AD EDA-ID patients (Supplemental Fig. S2, B).

DISCUSSION

We establish a robust genotype-phenotype correlation in AD EDA-ID and demonstrate that IκBα point mutants are expressed at higher levels, impair more severely canonical and non-canonical NF-κB signaling, and result in more severe disease than IκBα truncation mutants. Non-canonical NF-κB dependent genes important for lymphorganogenesis were inducible in patients with IκBα truncation mutations, but absent in patients with IκBα point mutations, providing a potential explanation for their virtual lack of tonsils and LNs, and their poor immune reconstitution following HSCT.

Disease severity score was significantly higher in the six patients with IκBα point mutations compared to the three patients with IκBα N-terminal truncation mutations, demonstrating that IκBα point mutations are associated with more severe disease compared to IκBα truncation mutations in patients with AD EDA-ID. The outcome of HSCT was poor in all six patients with point mutations in IκBα despite excellent donor cell chimerism, suggesting that a defect in stromal cells may be at play.

Using fibroblasts from the patients, we were able to demonstrate lower expression of the truncated IκBα compared to WT IκBα. However, we were not able to determine the contribution of WT IκBα versus point mutant IκBα. Using HEK293T cells transfected with IκBα mutants, we observed that truncation and point mutants were expressed at significantly lower levels than WT IκBα, suggesting that both types of mutants, and more so the truncation mutants, are intrinsically unstable. We cannot, however, 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 IκBα mutants compared to WT IκBα was relatively lower in transfected cells than in fibroblasts from the patients.

Consistent with their higher expression levels, IκBα 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-κB pathway. Whereas pathways other than the canonical NF-κB pathway can be activated by LPS, LPS induction of IL-6 and p100 are strictly dependent on NF-κB.[56, 57] The impairment of IL-6 secretion exceeded what would have been expected from the baseline ratio of mutant to WT IκBα for both patients with point mutations and truncation mutations. The decrease in the level of WT IκBα 48 hours post LPS stimulation suggests that upregulation of WT κBα is outweighed by its degradation. The marked increase in the truncation mutant to WT IκBα ratio in LPS-stimulated cells explains the DN effect exerted by these mutants despite their lower expression compared to WT protein in unstimulated cells. We previously reported that the W11X mutant did not exert a DN effect on canonical NF-κB signaling when transiently co-transfected with WT IκBα and an NF-κB reporter into HEK293T cells, which were then stimulated with TNFα for 6 hours.[24] In that study, the short stimulation time, the low expression level of the unstable mutant protein and endogenous WT IκBα may have masked the potential of the mutant to exert a DN effect.

We show for the first time that the non-canonical NF-κB pathway is impaired in patients with AD EDA-ID. We demonstrate that upregulation of the expression of p100 and RelB, two key components of this pathway known to be transcriptionally regulated by the canonical NF-κB 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 non-canonical NF-κB pathway, was deficient in LPS+anti-LTβR mAb stimulated fibroblasts from these patients. The defect was more severe in patients with IκBα point mutations than in those with IκBα truncation mutations, a reflection of the relative level of p100 in these patients. It should be noted that defect in the non-canonical NF-κB pathway in the patients is an extrinsic one due to the decreased ability to generate p100, a critical substrate for IKKα. In fact, although the level of p52 following activation with LPS+anti-LTβR is lower in the patients, the p52:p100 ratio is higher, suggesting that the intrinsic ability to activate NIK and IKKα, 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 non-canonical NF-κB activation.

Defective signaling via the non-canonical NF-κB pathway in LPS+anti-LTβR mAb stimulated fibroblasts from patients with AD EDA-ID was associated with defective regulation of gene expression as demonstrated by RNA-Seq analysis. Importantly, three genes critical for lymphorganogesis, *CCL20, ICAM1 and VCAM1* were significantly upregulated in LPS+anti-LTβR mAb stimulated normal fibroblasts. Several other genes in human LTos are important for lymphorganogenesis. These include CCL19, CCL21, and CXCL13.[58] The failure to detect upregulation of genes other than *CCL20, ICAM1 and VCAM1* 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 IκBα point mutations. Notably, expression of these three genes in fibroblasts from patients with IκBα truncation mutations, albeit lower for *VCAM1* and *ICAM 1*, was not significantly different from healthy controls. This finding suggests that the residual NF-κB pathway signaling in patients with IκBα 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 AD EDA-ID as a possibility.

Severely defective activation of the non-canonical NF-κB pathway in stromal cells from patients with IκBα 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 following HSCT. Secondary lymphoid organs are critical for adaptive immunity because they provide a scaffold for the interactions of antigen-bearing dendritic cells derived from peripheral tissues, with re-circulating 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 IκBα point mutations may explain the more severe disease, the impaired development of secondary lymphoid organs, and the poor response of these patients to HSCT. Given the severe functional defect in the non-canonical pathway in patients with point mutations, strategies aimed at correcting the stromal cell niche essential for lymphorganogenesis need to be considered in these patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

AD EDA-ID	Autosomal dominant anhidrotic ectodermal dysplasia with immune deficiency
BAFF-R	B cell activating factor receptor
BM	Bone marrow
CCL	C-C motif chemokine ligand
CXCL	C-X-C motif chemokine ligand
DN	Dominant negative
HSCT	Hematopoietic stem cell transplantation
ICAM-1	Intercellular adhesion molecule 1
Ig	Immunoglobulin
IκBα	NF-κB inhibitor alpha
IKK	IκB kinase
IL-6	Interleukin-6
LN	Lymph node
LPS	Lipopolysaccacharide
LTβR	Lymphotoxin beta receptor
LTi	Lymphoid tissue inducer
LTo	Lymphoid tissue organizer
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NF-κB2	Nuclear factor kappa B subunit 2
NIK	NF-κB inducing kinase
qPCR	Quantitative polymerase chain reaction
RAG2	Recombination activating gene 2
RelB	RELB proto-oncogene, NF-κB subunit
RT-PCR	Reverse transcription polymerase chain reaction
SLO	Secondary lymphoid organ
TLR	Toll-like receptor

TNFα	Tumor necrosis factor alpha
VCAM-1	Vascular adhesion molecule 1
WT	Wild-type

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Key messages

- IκBα point mutations are associated with more severe disease compared to IκBα truncation mutations in patients with AD EDA-ID.

- IκBα point mutants are expressed at significantly higher levels in fibroblasts and transfectants compared to IκBα truncation mutants.

- IκBα point mutations are associated with greater impairment of canonical and non-canonical NF-κB activity, and severely defective expression of genes important for lymphorganogenesis, compared to IκBα truncation mutations.
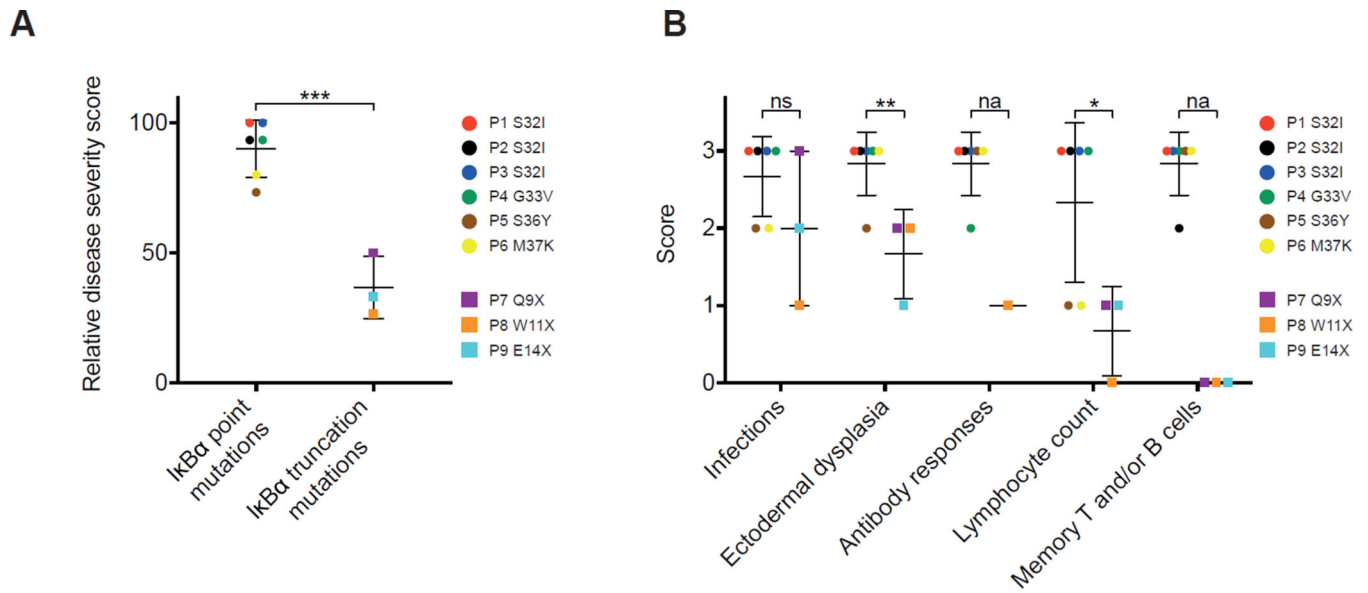
Figure 1. Relative disease severity score in AD EDA-ID patients

A. Cumulative disease severity score expressed as a percentage of the maximal score based on the evaluable disease criteria listed in Table 2. **B**. Individual disease category scores for patients with IκBα point mutations and patients with IκBα truncations. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, not significant; na, not applicable.
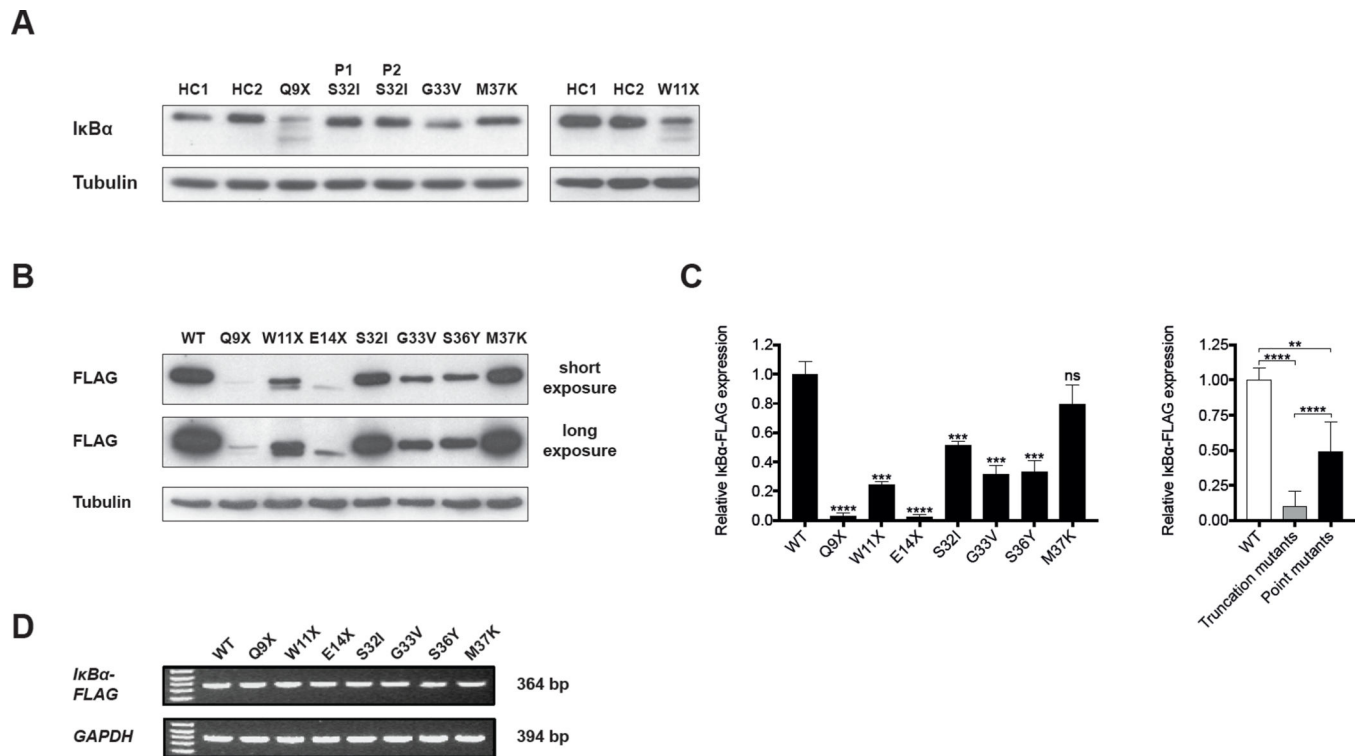
Figure 2. Expression of IκBα in fibroblasts and transfectants

A. Representative immunoblot of IκBα expression in cultured skin-derived fibroblasts from healthy controls (HC) and AD EDA-ID patients (P). P1 S32I and P2 S32I are patients 1 and 3 in Table I. **B, C**. Representative immunoblot (B) and quantitative analysis (C) of the expression of Flag-tagged WT and mutant IκBα in transfected HEK293T cells. Results in the left panel of C are expressed as the mean ratio of IκBα to tubulin of each individual mutant analyzed, relative to the mean of WT IκBα set at 1.0. Results in the right panel of 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. The amounts of cDNA used were in the linear range determined for mRNA encoding FLAG-tagged WT IκBα. Data is representative of three independent experiments. Columns and bars represent the mean±SD of at least three independent experiments. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.
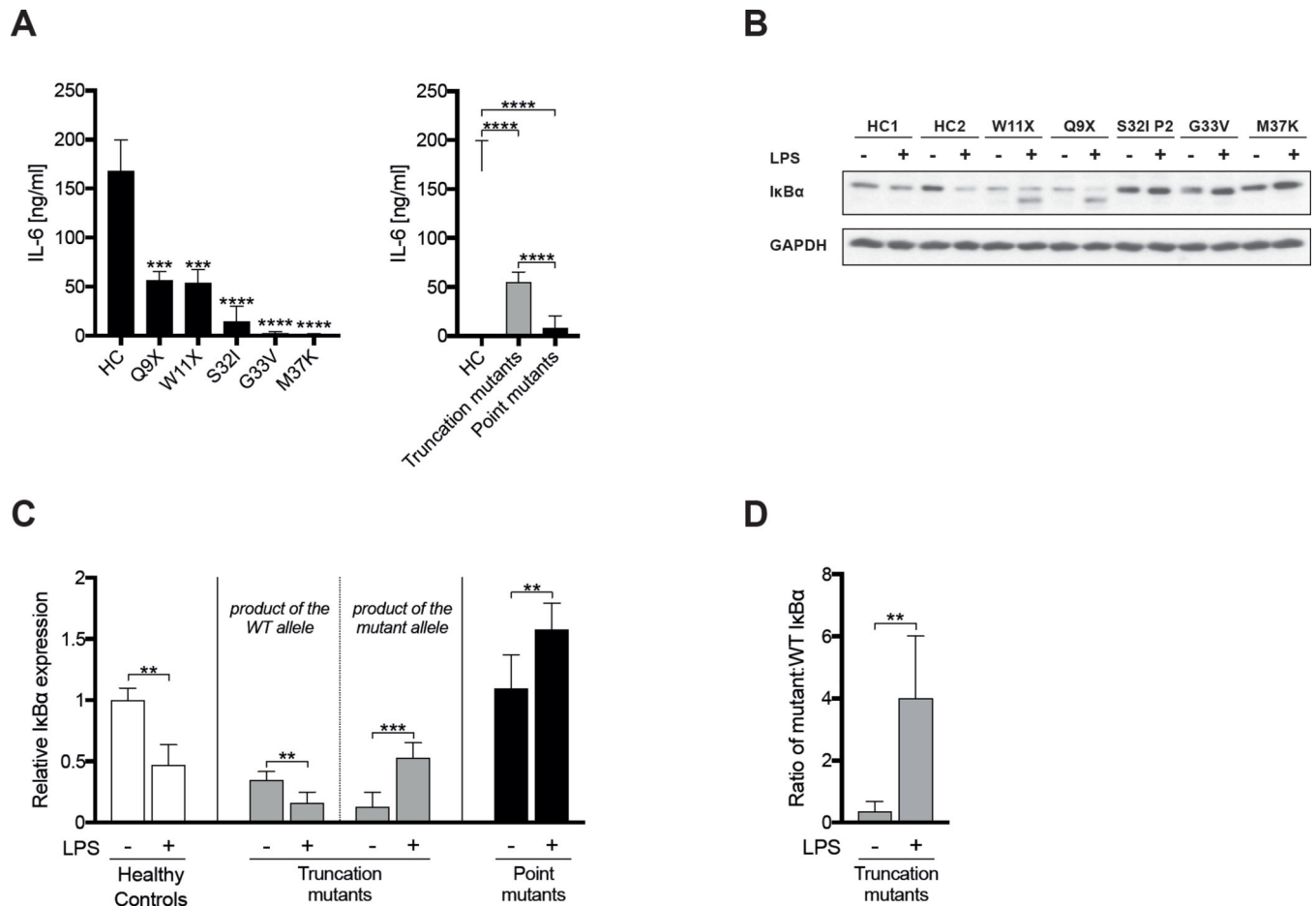
Figure 3. IκBα point mutants exert a stronger dominant negative effect on IL-6 secretion and accumulate to a greater extent following LPS stimulation than IκBα truncation mutants

A. LPS-driven IL-6 secretion by fibroblasts. Left panel: mean of three determinations on each of two healthy controls (HC), two patients with S32I, and individual patients with the other IκBα mutations. Right panel: Pooled results showing the mean IL-6 secretion by fibroblasts from HC (n=2), patients with IκBα truncation mutations (n=2) and patients with IκBα point mutations (n=4). **B, C**. Effect of LPS stimulation on IκBα levels: Representative (B) and quantitative (C) immunoblot analysis of IκBα expression in fibroblasts from two HC and AD EDA-ID patients before and after stimulation with LPS. Results from only one patient with S32I mutation are shown in B because of the limited number of lanes in the gel, but results from both patients with S32I mutation studied are included in the quantitative analysis, which includes 2 controls, 2 patients with IκBα truncation mutations and 4 patients with IκBα point mutations. Results in 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 AD EDA-ID patients with truncating mutations in IκBα. Columns and bars represent the mean±SD of three independent experiments. **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.
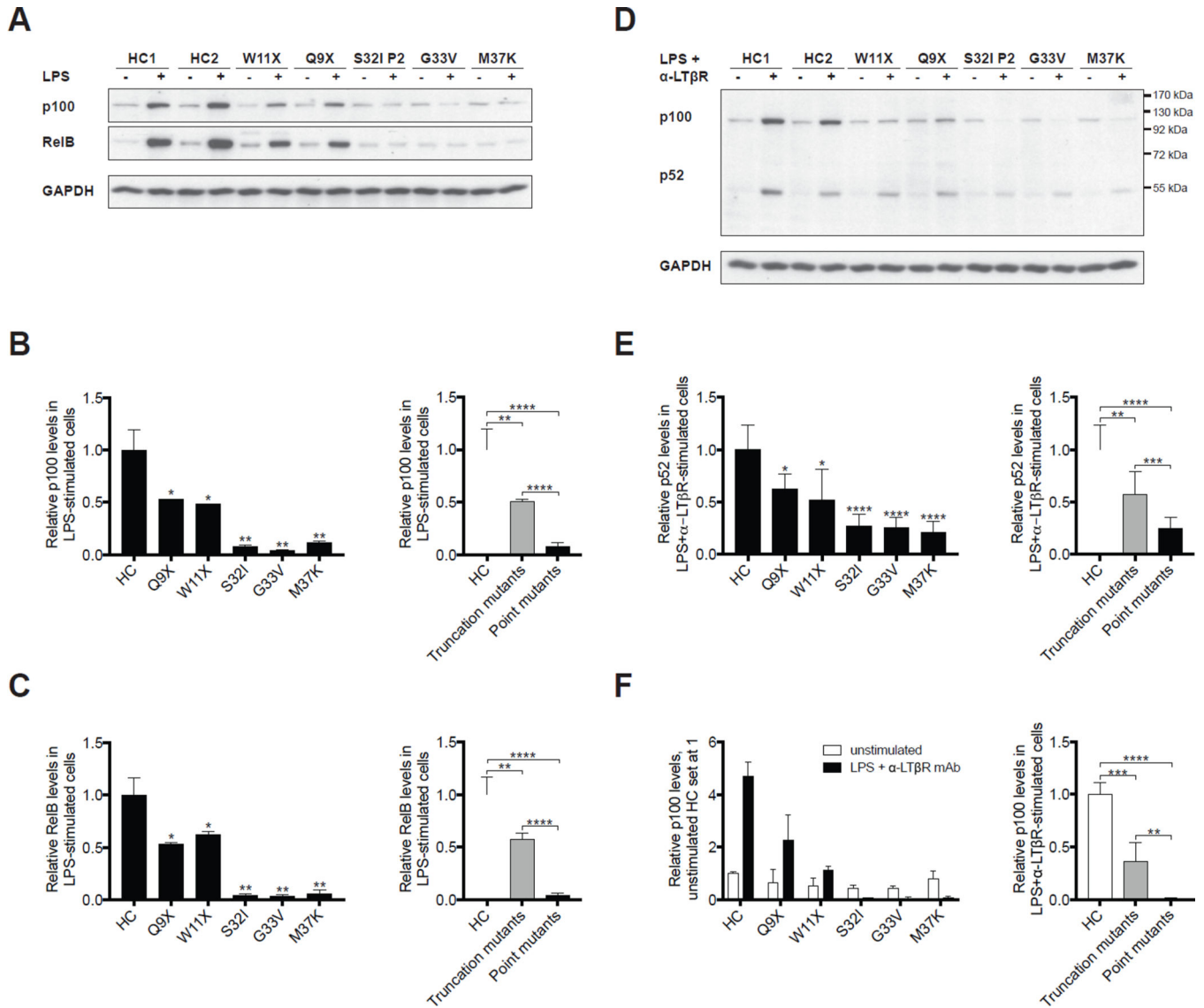
Figure 4. IκBα point mutants impair more severely LPS-driven expression of NF-κB2/p100 and RelB, and activation of the non-canonical NF-κB pathway than IκBα truncation mutants

A. Representative immunoblot analysis of p100 and RelB levels in fibroblasts from two healthy controls (HC) and AD EDA-ID patients (P) before and after stimulation with LPS for 48 hours. **B, C**. Quantitative analysis of expression of p100 (B) and RelB (C) in LPS-stimulated fibroblasts from two HC and individual patients (left panels), and pooled results representing the mean p100 and RelB expression levels in fibroblasts from patients with IκBα truncation mutations (n=2) and IκBα 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 two HC and AD EDA-ID patients before and after stimulation with LPS+anti-LTβR mAb for 48 hours. **E, F**. Quantitative analysis of expression of p52 (E) and p100 (F) in LPS+anti-LTβR mAb stimulated fibroblasts from healthy controls (HC) and individual patients (left panels) and pooled results representing the mean p52 and p100

expression levels in fibroblasts from HC (n=2), patients with IκBα truncation mutations (n=2) and IκBα point mutations (n=4) (right panels). Results are expressed as the ratio of p52 and p100 to GAPDH relative to the mean of LPS+anti-LTβR mAb stimulated (E) or unstimulated (F) HC set at 1.0. Columns and bars represent the mean±SD of at least three independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.
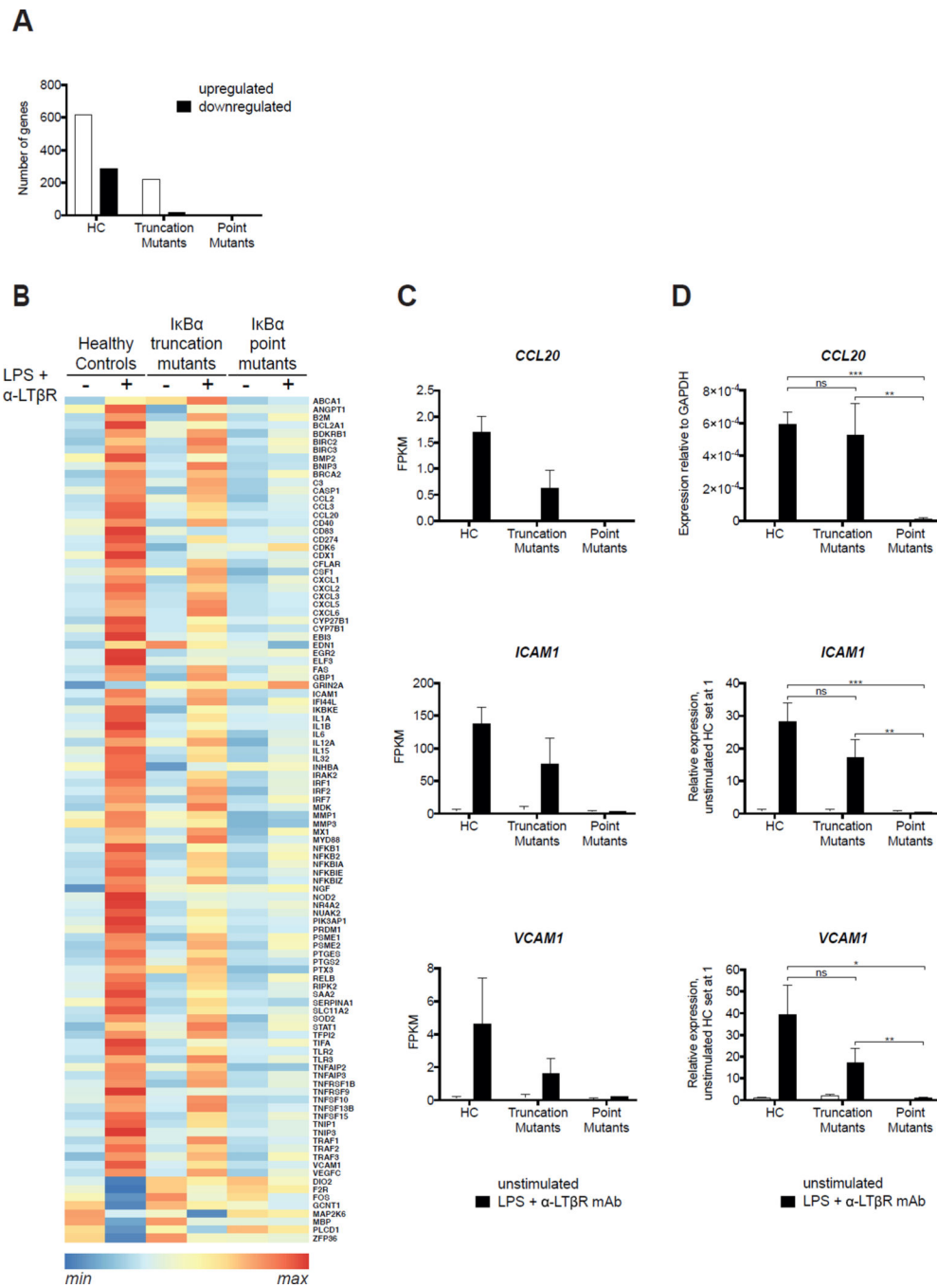
Figure 5. IκBα point mutants impair the induction of genes important for lymphorganogenesis more severely than IκBα truncation mutants

A. Number of genes significantly (*p*<0.05) upregulated or downregulated by two folds or more in LPS+anti-LTβR mAb stimulated fibroblasts from healthy controls (n=3) and patients with IκBα truncation (n=2) and point mutations (n=2). **B**. Heat map analysis of NF-κB regulated genes the expression of which was significantly altered by LPS+anti-LTβR stimulation of normal fibroblasts. **C**. RNA-Seq analysis of the expression of *CCL20, ICAM1* and *VCAM1* mRNA in unstimulated and LPS+anti-LTβR mAb stimulated fibroblasts from

healthy controls (n=3) and patients with IκBα truncation (n=2) and point mutations (n=2). Results are expressed as Fragments per Kilobase of Exon per Million (FPKM). Columns and bars represent the mean±SD for each of the three groups. **D**. q-PCR analysis of the expression of *CCL20, ICAM1* and *VCAM1* mRNA in unstimulated and LPS+anti-LTβR mAb stimulated fibroblasts from healthy controls (n=3) and patients with IκBα 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 the $2^{-\Delta\Delta Ct}$ 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 the $2^{-\Delta Ct}$ method. Columns and bars in D represent the mean±SD of three independent experiments. *, *p*<0.05; **, *p*<0.01; ***, *p*<0.001; ns, not significant.

TABLE I

Clinical characteristics.

Patient	IκBα mutation	Disease score[†]	Secondary lymphoid organs		HSCT (age)	Outcome	Reference
			Tonsils	Palpable lymph nodes			
1	S32I	15/15	Absent	Absent	Yes (1 year)	Recurrent infections, on IVIG; Alive 17 years post HSCT	Courtois *et al.*, 2003
2	S32I	14/15	Absent	Absent	Yes (7 years)	Recurrent infections, on IVIG; Alive 3 years post HSCT	unpublished
3	S32I	15/15	Absent	Absent	Yes (2.5 years)	Recurrent infections, on IVIG; Deceased 4 months post HSCT	Janssen *et al.*, 2004
4	G33V	14/15	Absent	Absent	Yes (2 years)	Recurrent infections, on IVIG; Alive 1 year post HSCT	unpublished
5	S36Y	11/15	Very small	Very small	Yes (6 years)	GvHD and autoimmune thrombocytopenia, Deceased 7 months post HSCT with sepsis	Yoshioka *et al.*, 2013
6	M37K	12/15	Absent	Absent	Yes (4.8 years)	Recurrent infections, on SCIG; Deceased 18 months post HSCT with sepsis	Schimke *et al.*, 2013
7	Q9X	6/12	Yes	Yes	Yes (15 months)	Healthy 5 years post HSCT, off IVIG	Ohnishi *et al.*, 2012
8	W11X	4/15	Yes	Yes	No	Healthy, on IVIG	McDonald *et al.*, 2007
9	E14X	4/12	Yes	Yes	Yes (8 months)	Deceased day 31 post HSCT with sepsis	Lopez-Granados *et al.*, 2008

HSCT, hematopoietic stem cell transplantation; *GvHD*, graft versus host disease; *IVIG*, intravenous immunoglobulin replacement therapy; *SCIG*, subcutaneous immunoglobulin replacement therapy.

[†] Disease scoring is based on five 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 was not available on one of the criteria, resulting in a maximum disease score of 12.

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Table II

Disease severity scoring system for AD EDA-ID.

Score	Infections	Ectodermal dysplasia: systems affected[†]	Antibody responses[‡]	Lymphocyte count/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	Two	Protein antigen-deficient	20,000–30,000	Severe decrease
3	Opportunistic	Three or more	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 a rise in antibody titers of less than four-fold post-immunization.

[*]Modest decrease: Values between 2 and 3 standard deviations (SD) below the mean for age. Severe decrease: Values more than 3 SD below the mean for age but above zero.