

ST2, and they confirm the important role of ST2 signaling in asthma.

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Aspergillosis, eosinophilic esophagitis, and allergic rhinitis in signal transducer and activator of transcription 3 haploinsufficiency



To the Editor:

Dominant-negative mutations in human signal transducer and activator of transcription 3 (*STAT3*) result in approximately 75% loss of *STAT3* function and cause hyper-IgE syndrome (HIES), also known as Job's syndrome.¹⁻³ Patients with HIES manifest with elevated serum IgE level, atopy, recurrent pulmonary and skin bacterial infections, chronic mucocutaneous candidiasis (CMC), locally invasive pulmonary aspergillosis, and connective tissue abnormalities.¹⁻³ However, *STAT3* mutations resulting in haploinsufficiency and their associated phenotypes have not

been well characterized to date. Herein, we describe a patient with a *de novo* *STAT3* splice-site mutation resulting in *STAT3* haploinsufficiency who presented with elevated IgE level, allergic rhinitis, eosinophilic esophagitis, and fatal invasive aspergillosis.

Our patient was a 37-year-old man with a 1-year history of eosinophilic esophagitis and allergic rhinitis, which was treated with occasional topical steroids. The patient developed subacute symptoms of headache and sinus pain that did not respond to several courses of antibacterial antibiotics, and progressed to develop facial numbness, diplopia, and visual loss over the course of 4 weeks. He was found to have a locally invasive sino-orbital infection with cavernous sinus involvement (Fig 1, A-D). Sinus tissue biopsy showed chronic inflammation and invasive fungal acute-angle branching hyphae (Fig 1, E and F). Sinus cultures grew *Aspergillus fumigatus* as identified by morphology and the sequences of beta-tubulin and DNA replication licensing factor *Mcm7*. Minimum inhibitory concentrations (in µg/mL) for the isolate were as follows: micafungin, 0.015 or less; amphotericin, 2; posaconazole, 0.25; voriconazole, 0.25; and terbinafine, 0.06. The patient underwent multiple surgical debridements with removal of infected necrotic tissue, including right orbital exenteration, and was treated over time with various antifungal drug combinations (amphotericin B, micafungin, voriconazole, posaconazole, terbinafine), adjunct immunotherapy (IFN-γ, GM-CSF), and hyperbaric oxygen. Despite these efforts, the infection progressed into the right middle cranial fossa and meninges (Fig 1, A-D) and the patient died 1 year after his sino-orbital symptoms began. Of note, the patient's infection did not involve the lungs or other distal anatomical sites, nor did he have any underlying lung disease. He had no family history of atopy or unusual infections. The patient had normal blood lymphocyte, monocyte, and neutrophil counts. At presentation, his eosinophil count was transiently mildly elevated (range, 100-1090/µL). His serum IgE level was elevated at 1003 IU/mL, whereas all other immunoglobulin levels were within normal range. A dihydrorhodamine test was normal. Other unremarkable laboratory tests included antineutrophil cytoplasmic antibodies, antinuclear antibody, complement CH50, and HIV antibody. Pneumococcal antibody titers were positive in 3 out of 14 serotypes tested. We ruled out an acquired immunodeficiency state caused by autoantibodies against IFN-γ, IL-17A, GM-CSF, or IL-12 (data not shown).

Before his death, the patient was evaluated at the National Institutes of Health Clinical Center and he was enrolled on an institutional review board–approved research protocol. Informed consent was obtained from the patient and his family members. Whole-exome sequencing was performed using DNA isolated from whole blood from the patient and his unaffected sister and nonconsanguineous parents (Fig 2, A). No mutations were found in *STAT1*, *CARD9*, other genes encoding members of the C-type lectin receptor signaling pathway, or the 5 nicotinamide adenine dinucleotide phosphate oxidase subunits, all of which are critical for systemic antifungal host defense.⁴ A novel, *de novo* *STAT3* splice-site mutation was found before exon 13 (c.1140-3C>G) on the maternal haplotype, which was confirmed by Sanger sequencing using DNA extracted from whole blood and from formalin-fixed sinus tissue biopsy (data not shown). Sequencing of this locus in the patient's son did not reveal this mutation (Fig 2, A). This mutation was not observed in the 1000 Genomes, Exome Sequencing Project, or gnomAD databases (February 2018 access), which include exome data from more than

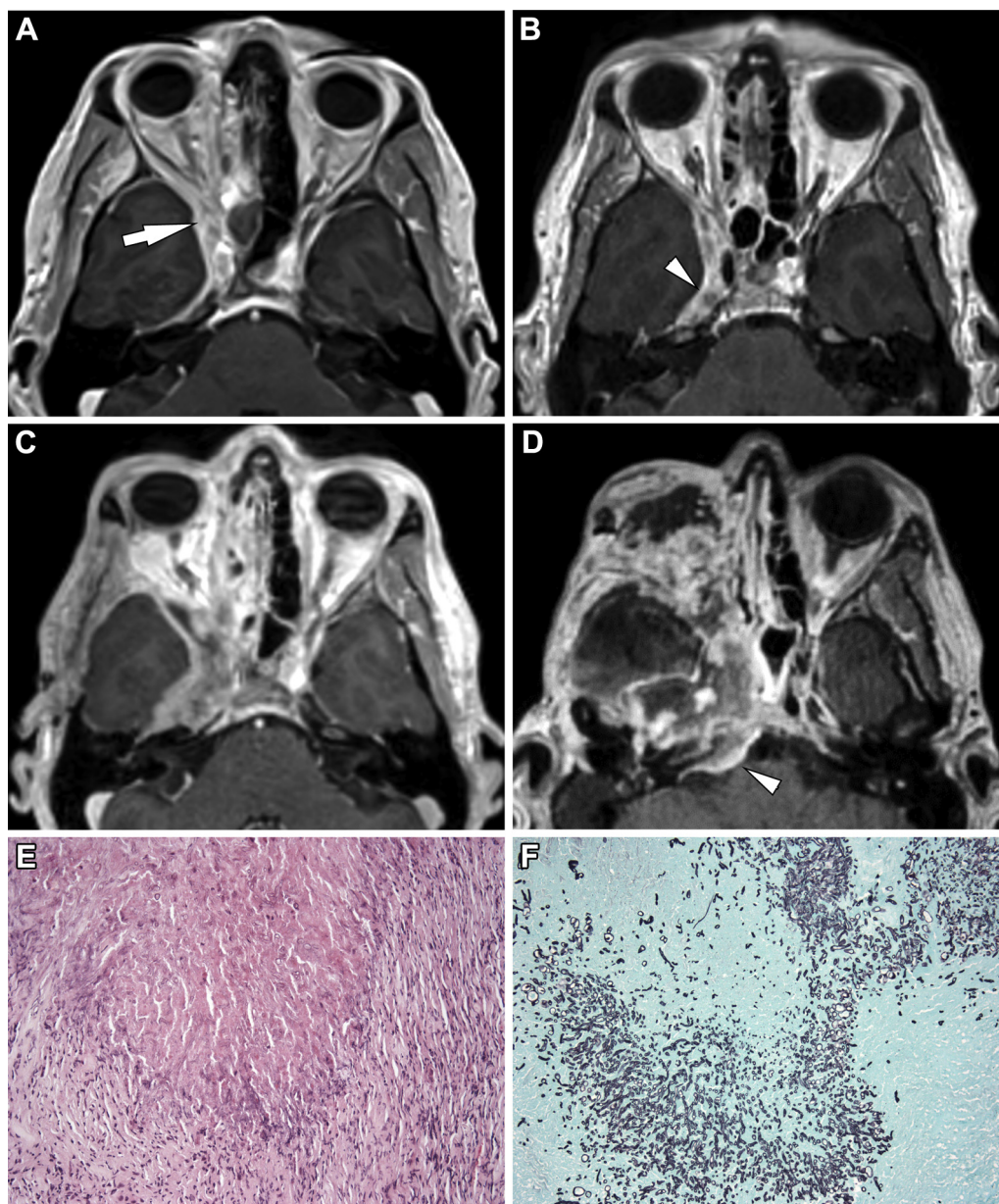


FIG 1. Radiographic and histological features of the invasive sino-orbital-cerebral *Aspergillus* infection. **A-D**, Representative images of magnetic resonance imaging brain (T1 postgadolinium) showing the progression of the infection despite aggressive antifungal treatment. **A**, Initial infection diagnosis with early cavernous sinus involvement (arrow). **B**, One month after infection diagnosis, with involvement of Meckel's cave (arrowhead) and of the right orbit. **C**, Four months after infection diagnosis, with involvement of the middle cranial fossa and worsening of right orbital disease. **D**, Ten months after infection diagnosis, status-post right orbital exenteration with involvement of the posterior fossa (arrowhead). **E** and **F**, Representative histological images from *Aspergillus*-infected sinus tissue demonstrating necrosis, chronic inflammation, and invasive fungal hyphae by H&E (Fig 1, **E**) and GMS (Fig 1, **F**) stains. H&E, Hematoxylin and eosin; GMS, Grocott's methenamine silver. Magnification 20 $\times$.

246,000 alleles. Of note, there were no other rare variants (maximum allele frequency, <0.1%) using either a dominant *de novo*, an autosomal-recessive, an X-linked recessive, or a compound heterozygous inheritance model. The identified *STAT3* mutation is predicted to disrupt splicing (Human splicing finder v3.1) and lead to a premature stop codon. Initial cDNA sequencing of the *STAT3* gene did not reveal any splicing defects. However, to investigate the possibility of nonsense-mediated

decay (NMD) of the affected allele, we sequenced a heterozygous single nucleotide polymorphism in the 3' untranslated region of *STAT3* (c.*1671C>T, rs1053004), which resides on the paternal haplotype. This cDNA sequencing revealed expression of only the paternal allele (Fig 2, **B**), consistent with NMD of the allele containing the splice-site mutation. To definitely demonstrate that our patient's mutation leads to abnormal splicing and NMD, we treated PBMCs with an NMD inhibitor

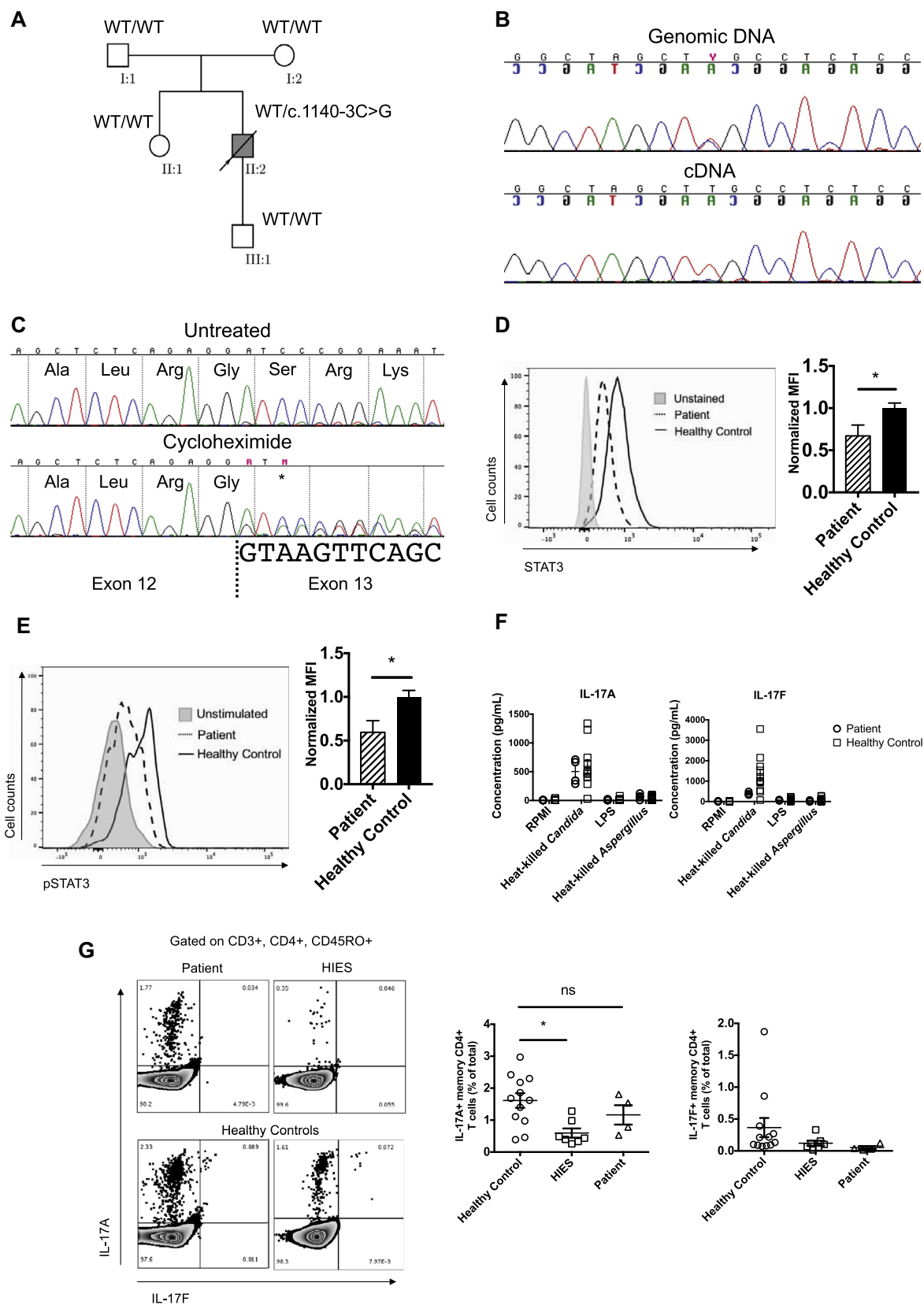


FIG 2. A *de novo* *STAT3* splice-site mutation results in NMD, *STAT3* haploinsufficiency, and decreased *STAT3* levels and phosphorylation without impairing T_H17 responses. **A**, Pedigree of the patient. The patient is indicated in gray and by an arrow. The genotype of *STAT3* alleles is indicated for each family member. **B**, Sequencing chromatograms of *STAT3* from genomic DNA (upper panel) and cDNA (lower panel) at position c.*1671 in the 3' untranslated region demonstrating loss of heterozygosity in the cDNA sequence, consistent with NMD. **C**, Sequencing chromatograms of *STAT3* cDNA at the exon

(cycloheximide), isolated cDNA, and sequenced the exon 12/13 junction. This sequencing showed that the cDNA from cycloheximide-treated cells had abnormal splicing after exon 12 in one allele with the retention of intron 12 and the introduction of a premature stop codon (p.S381*), which would lead to NMD (Fig 2, C). To determine whether the identified *STAT3* mutation results in haploinsufficiency, we first evaluated the effect of this mutation on *STAT3* expression by measuring total *STAT3* levels in cultured T cells by flow cytometry and found a significant reduction in *STAT3* protein in the patient compared with healthy controls (Fig 2, D). To further determine the functional consequence of reduced total *STAT3* in the patient's T cells, we measured phosphorylated *STAT3* (p*STAT3*) after IL-6 stimulation in PBMCs by flow cytometry and found a significant reduction of p*STAT3* in memory T cells (Fig 2, E). Together these data indicate that the identified *de novo STAT3* mutation results in *STAT3* haploinsufficiency. Because our patient did not present with CMC, we next examined the impact of *STAT3* haploinsufficiency in our patient's cells on the production of IL-17A and IL-17F, which are critical molecules for anti-*Candida* mucosal host defense.^{4,5} Consistent with the absence of CMC in our patient, he did not exhibit a defect in peripheral T_H17 cell numbers or in the production of IL-17A or IL-17F by PBMCs upon fungal stimulation (Fig 2, F and G). These data suggest that *STAT3* haploinsufficiency, as opposed to dominant-negative *STAT3* mutations that underlie HIES,⁶ is not sufficient to impair T_H17 cell development (Fig 2, G). This finding of intact T_H17 cells is similar to patients described with somatic mosaicism in which *STAT3* is mutated in a fraction of cells.⁷ In addition, these data indicate that the patient's susceptibility to invasive aspergillosis is mediated via a non-IL-17 pathway-dependent mechanism. The latter observation is in keeping with the absence of reports of aspergillosis in patients with inborn errors of IL-17 immunity.^{4,5}

Interestingly, although our patient did not present with the full scope of manifestations of HIES, he had several features that are seen in patients with HIES, such as an elevated serum IgE level, atopy manifesting as eosinophilic esophagitis, which occurs in 65% of patients with HIES,⁸ and locally invasive aspergillosis, which occurs in approximately 30% of adult patients with HIES.⁴ However, he did not manifest with recurrent bacterial infections, the connective tissue abnormalities of HIES, or CMC. Another notable distinct feature of our patient is the rhino-orbital-cerebral involvement by *Aspergillus* infection as compared with HIES, which typically presents as pulmonary infection in patients with underlying structural lung disease (see Table E1 in this article's Online Repository at

www.jacionline.org). The differences between our patient's phenotype and that of patients with HIES may be explained by the different *STAT3* genetics, as a deeper decline in residual functional *STAT3* is seen in patients with HIES who have dominant-negative *STAT3* mutations. Alternatively, and/or in parallel, additional mutations in coding or noncoding genetic elements in our patient may have contributed to the anatomical site-specific variation in aspergillosis and to the incomplete penetrance of the HIES phenotype. Interestingly, Anolik et al⁹ reported a patient with a similar *STAT3* mutation to ours (c.1140-2A>C), but with a more typical HIES clinical presentation. However, because the effect of the c.1140-2A>C mutation on *STAT3* splicing, protein levels, and T_H17 response was not reported, it is difficult to comment on the relationship between that variant, its functional consequences, and the patient's clinical phenotype.

In summary, we describe a patient with elevated IgE level, atopy, and fatal invasive aspergillosis associated with a *de novo STAT3* splice-site mutation that results in NMD, *STAT3* haploinsufficiency, and reduced *STAT3* phosphorylation. The identification and phenotypic characterization of additional patients with *STAT3* mutations that result in *STAT3* haploinsufficiency will help in better understanding the full spectrum of illness associated with various *STAT3* mutations.

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12/13 junction (thick dotted line) from untreated cells (upper panel) and cells treated with an NMD inhibitor (cycloheximide; lower panel) demonstrating abnormal splicing after exon 12 in 1 allele and the introduction of a premature stop codon (*). The introduced sequence from intron 12 is listed below. **D**, Reduced total *STAT3* protein expression by flow cytometry in cultured T cells compared with healthy controls. Representative histogram (left panel) and summary data of geometric mean fluorescence intensity (MFI) (right panel) from 3 independent experiments (n = 3 for our patient, 6 different healthy donors). **E**, Reduced p*STAT3* levels in memory T cells upon IL-6 stimulation. Representative histogram (left panel) and summary data of MFI (right panel) from 3 independent experiments (n = 3 for our patient, 6 different healthy donors). **F**, PBMCs were stimulated with the listed stimuli or were left unstimulated in RPMI medium as control and IL-17A (left panel) and IL-17F (right panel) protein levels were measured in the supernatant by Luminex assay. Results are from 2 independent experiments (n = 4 for our patient, n = 10 for healthy controls). **G**, IL-17A and IL-17F-expressing memory T cells were measured by flow cytometry. Representative flow cytometric plots (left panels) and summary data (right panels) of the percent of IL-17A and IL-17F-expressing cells from 3 independent experiments (n = 4 for our patient, 7 patients with HIES, 12 different healthy donors). ns, Not significant; UTR, untranslated region. *P < .05.

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Deconstructive somatic cell nuclear transfer reveals novel regulatory T-cell subsets



To the Editor:

Regulatory T (Treg) cells have been subject of many debates and controversies. Discrepancies exist in regard to Treg-cell development, their recognition of antigen, specificity of immune regulation, requirement for T-cell receptor (TCR) activation, and regulatory mechanisms.^{1,2} We previously used somatic cell nuclear transfer (SCNT) to generate a naturally occurring Treg (nTreg)-cell model, T138.³ Particularly, we found that the TCR strength of T138 contradicted findings in wild-type (WT) mice, prompting us to hypothesize that Treg cells are heterogeneous with distinctive subsets. With our unbiased SCNT approach, we generated another Treg-cell model (T143) using FoxP3^{GFP}-expressing CD4⁺ T cells from the spleen of NOD-FoxP3^{GFP}-Rag^{+/-} mice as described previously.³⁻⁵

We identified the V(D)J rearrangements underlying T143 as Trv5-4-J40 and Trbv15-J2-3-C2 (see Fig E1, A, in this article's Online Repository at www.jacionline.org). Thymic development of T143-Rag1^{-/-} revealed a significant increase in CD4 single-positive (CD4SP) cells (~12% WT vs ~39% T143) and a significant decrease in CD4-CD8 double-positive (DP) cells when compared with WT NOD mice (~77% WT vs ~34% T143) (Fig 1, A). Interestingly, CD4 expression levels in DP cells were lower in T143 than in WT. Strikingly, we identified thymic FoxP3⁺ T cells in CD4SP cells of T143-Rag1^{-/-} (Fig 1, B; ~6% WT vs 0.3% T143). In the spleen, we found approximately 10% FoxP3⁺ CD4⁺ T cells in T143-Rag1^{-/-} compared with approximately 17% in WT (Fig E1, B). Given that we had previously identified a fate-determining role for nTreg cell-derived TCRβ, we tested whether TCRβ of T143 contributes to lineage choice. We found that 2 copies of T143 TCRβ skewed T-cell development similarly as seen in T143-Rag1^{-/-} (lower frequency of DP, higher frequency of CD4SP, and lower CD4 levels on DP), while a single copy had negligible impact on lineage choice (Fig E1, C).

By determining expression levels of Nur77 and CD5, we previously showed that FoxP3⁺ nTreg and FoxP3⁻ pre-nTreg cells possess comparable TCR strength, thus arguing against agonist-dependent development.^{3,6} We determined TCR strength in T143, and found significantly higher Nur77 and CD5 levels in FoxP3⁺ CD4⁺ T cells than in their FoxP3⁻ counterpart, suggesting higher TCR strength (Fig 1, C and D). Hence, we refer to T143 as agonist-induced Treg (aTreg) cell.

We previously showed that FoxP3⁻ CD4⁺ pre-nTreg cells carrying the same TCR as nTreg cells were poised toward FoxP3 expression and away from T_H1 differentiation. Thus, we determined whether FoxP3⁻ CD4⁺ T cells carrying the same TCR as aTreg cells were poised similarly. FoxP3⁻ CD4⁺ T cells from T143 were significantly better in differentiating into FoxP3⁺ T cells than from WT (~62% T143 vs 52% WT) (Fig 1, E). However, we observed no significant difference in their ability to differentiate into T_H1 (Fig 1, F; ~48% WT vs ~52% T143). For consistency, we refer to FoxP3⁻ CD4⁺ T cells from aTreg cells as pre-aTreg cells.

We previously found that CD8⁺ T cells expressing nTreg cell-derived TCRβ had reduced capabilities to express FoxP3 upon induction. We observed similar results for CD8⁺ T cells expressing aTreg cell-derived TCRβ (see Fig E2, A, in this article's Online Repository at www.jacionline.org; ~48% WT vs ~8% T143), whereas we found no difference in their capability to differentiate into IFN-γ-secreting cells (~76% WT vs ~79% T143, Fig E2, B).

The development, frequencies, and plasticity of T143 were quite distinct from those in our previously reported T138, and urged us to compare transcriptional programs of both. We performed RNA-Seq on FoxP3⁺ CD4⁺ T cells from T143, T138, and WT. Genome-wide hierarchical clustering revealed differentially expressed genes in FoxP3⁺ T cells from T138, T143, and the bulk from WT (Fig 2, A). We identified a gene cluster that was specifically activated (eg, Ccr1), and one that was specifically repressed in aTreg cells (eg, Ctla4) (Fig 2, A and B). Next, we used Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) to determine specific protein-protein interaction network in these differentially expressing gene clusters.⁷ Ccr1 forms an interconnected gene network with many aTreg cell-specific active genes, whereas Ctla4 forms a

METHODS

Study participants

The patient, his relatives, and the patients with AD-HIES were enrolled in protocols approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board committee; healthy blood donors were enrolled in a protocol approved by the National Institutes of Health Clinical Center Institutional Review Board committee. All subjects provided written informed consent for participation in the study. This study was conducted in accordance with the Declaration of Helsinki.

Exome sequencing

We isolated DNA from whole blood using the salting-out method (Qiagen, Valencia, Calif), followed by phenol chloroform extraction. We prepared whole-genome libraries according to the KAPA HTP Library Construction Kit protocol (Kapa Biosystems, Wilmington, Mass), performed exome capture according to the Nimblegen's SeqCap EZ Library + UTR protocol, and sequenced with Illumina HiSeq 2500. We mapped reads for all samples to human GRCh37 reference with BWA^{E1} v0.7.13 using the default settings and marked duplicate reads with Picard (<http://broadinstitute.github.io/picard>). Next, we applied GATK^{E2} v3.5 base quality score recalibration, indel realignment, and performed SNP and INDEL discovery and genotyping across all samples simultaneously according to GATK Best Practices recommendations.^{E3,E4} We normalized and decomposed variants with vt^{E5} v0.577 and annotated variants with variant effect predictor^{E6} v84. We filtered variants using GEMINI^{E7} v0.19.1 to obtain rare or novel variants with allele frequency of less than 0.1% according to 1000 Genomes, Exome Sequencing Project, and ExAC.

Sanger sequencing

Complementary DNA (cDNA) was prepared from PBMCs from the affected patient using the RNeasy Mini kit (Qiagen, Germantown, Md) and the SuperScript IV First-Strand Synthesis System (Invitrogen, Waltham, Mass). Primers 172F (5'-CGCCCGTCCCGGCACACG-3') and 2926R (5'-CCGCAGGCACAGGAGGCACTTGCT-3') were used to amplify the STAT3 coding sequence and primer 1057F (5'-CAGACCCGTCAACAAATTAAGAACTGGAG-3') was used to sequence the exon 13 splice site. Primers 2002F (5'-AAGGAGCGGGAGCGGGCCATCTTG-3') and 4682R (5'-ACAGGGCTCAGCTCCTCTCAGAACTTTGCTACAA-3') were used to amplify the STAT3 3'-untranslated region from the cDNA and primers 3962F (5'-CAGCGACGTGTCTGGTTGAGCTCAGGAATA-3') and 4682R were used to amplify the STAT3 3'-untranslated region from the genomic DNA. Primer 4682R was used to sequence the SNP c.*1671C>T from genomic DNA and cDNA.

Cycloheximide treatment

PBMCs from the affected patient were thawed and cultured in the presence of cycloheximide (100 ng/mL) for 4 hours. Isolation and sequencing of cDNA were as described above. The use of cycloheximide as an inhibitor of NMD has been reported previously.^{E8}

Measurement of total STAT3 levels

PBMCs from the affected patient and healthy blood donors (obtained from NIH Blood Bank) were activated with 1 µg/mL anti-CD3 (clone HIT3α; BD Biosciences, Franklin Lakes, NJ) and 1 µg/mL anti-CD28 (clone CD28.2; BD Biosciences). After 3 days, activated T cells were washed and then cultured in complete RPMI medium supplemented with 100 U/mL recombinant human IL-2 (rIL-2; R&D Systems, Minneapolis, Minn) for 4 to 18 days. Cells were then fixed with 4% paraformaldehyde (PFA), permeabilized with methanol overnight, washed 3 times with fluorescence-activated cell sorting (FACS) buffer, and then stained with allophycocyanin-conjugated CD4 antibody (clone RPA-T4; BD Biosciences) and phycoerythrin (PE)-conjugated total STAT3 antibody (clone M59-50; BD Biosciences). After staining, the cells were washed 3 times with FACS buffer and acquired on a 5-laser BD LSRFortessa, equipped with BD FACS Diva software (BD Biosciences). Data were analyzed using FlowJo (FlowJo LLC, Ashland, Ore).

pSTAT3 measurement

Frozen PBMCs from the affected patient and healthy controls were thawed and then washed with warm RPMI media containing 10% heat-inactivated FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1 U/mL DNase (Roche, Basel, Switzerland) (R10 media). Cells were rested for 2 hours in R10 media at 37°C in a 5% CO₂ incubator, spun, and resuspended in RPMI media without serum (R0 media). Cells were then rested in R0 media for 1 hour, filtered through a 40-µm strainer, counted, and then diluted to 3 × 10⁵ cells/mL with R0 media. Cells were then stimulated with IL-6 (37.5 ng/mL; Peprotech, Rocky Hill, NJ) for 20 minutes at 37°C in a 5% CO₂ incubator and then fixed with 1.6% PFA (final concentration). Cells were then spun, the PFA was decanted, and then the cells were permeabilized with cold methanol overnight. The cells were then washed with FACS buffer twice and stained with the following conjugated antibodies (BD Biosciences): AF700-conjugated CD3 (clone UCHT1), BUV395-conjugated CD4 (clone RPA-T4), BV711-conjugated CD45RA (clone HI100), and Pacific blue-conjugated pSTAT3 p705 (clone 4/P-STAT3). After staining, the cells were washed with FACS buffer and acquired on a 5-laser BD LSRFortessa, equipped with BD FACS Diva software (BD Biosciences). Data were analyzed using FlowJo. Memory T cells were gated as CD3+, CD4+, CD45RA- cells.

T_H17 cell measurement

PBMCs were prepared as described above for pSTAT3 analysis. Instead of stimulation with IL-6, cells were stimulated with phorbol 12-myristate 13-acetate (20 ng/mL) and ionomycin (1 µM) in the presence of GolgiStop (BD Biosciences) for 4 hours at 37°C. Thirty minutes before harvesting cells, they were stained with Live/Dead fluorescent dye (Invitrogen). After staining, cells were washed once with FACS buffer, fixed with PFA, and then permeabilized with cold methanol overnight. Cells were washed 3 times with FACS buffer and then stained with the following conjugated antibodies (BD Biosciences, except where noted otherwise): AF700-conjugated CD3 (clone UCHT1), BUV395-conjugated CD4 (clone RPA-T4), BV510-conjugated CD8 (clone SK1), PE-Texas Red-conjugated CD45RO (clone UCHL1; Beckman Coulter, Indianapolis, Ind), PE-conjugated IL-17A (clone ebio64cap17; eBioscience, Waltham, Mass), and fluorescein isothiocyanate-conjugated IL-17F (clone poly5166; BioLegend, San Diego, Calif). The cells were acquired on a 5-laser BD LSRFortessa, equipped with BD FACS Diva software (BD Biosciences). Data were analyzed using FlowJo. Memory T cells were gated as live/dead-, CD3+, CD4+, CD8-, CD45RO+ cells.

Cytokine measurement by Luminex

Fresh PBMCs from the affected patient and healthy controls were stimulated and cytokine levels from the supernatant were measured by the Luminex array as previously described.^{E9} In brief, 5 × 10⁵ PBMCs were stimulated with either RPMI media (as negative control), LPS (100 ng/mL), heat-killed *Candida* (1 × 10⁶/mL), or heat-killed *Aspergillus* (1 × 10⁸/mL) for 48 hours at 37°C in a 5% CO₂ incubator. The supernatant was collected and stored at -80°C until analysis by the Luminex array.

Anticytokine autoantibody measurement

The patient's serum was screened for anticytokine autoantibodies using a particle-based technology as previously described.^{E10}

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TABLE E1. Comparison of clinical features observed in our patient with *STAT3* haploinsufficiency and in patients with dominant-negative *STAT3* mutations that result in HIES

Clinical feature	<i>STAT3</i> haploinsufficiency	HIES
Noninfectious manifestations		
Elevated serum IgE level	+	++
Allergic rhinitis	+	+
Eosinophilic esophagitis	+	+
Connective tissue abnormalities	—	+
Infectious manifestations		
Pulmonary aspergillosis	—	+
Sino-orbital aspergillosis	+	—
Bacterial skin infections	—	+
CMC	—	+