A deep intronic splice mutation of *STAT3* underlies hyper IgE syndrome by negative dominance

Joëlle Khourieh, Geetha Rao, Tanwir Habib, Danielle T. Avery, Alain Lefèvre-Utile, Marie-Olivia Chandesris, Aziz Belkadi, Maya Chrabieh, Hanan Alwaseem, Virginie Grandin, Françoise Sarrot-Reynauld, Agathe Sénéchal, Olivier Lortholary, Xiao-Fei Kong, Stéphanie Boisson-Dupuis, Capucine Picard, Anne Puel, Vivien Béziat, Qian Zhang, Laurent Abel, Henrik Molina, Nico Marr, Stuart G. Tangye, Jean-Laurent Casanova and Bertrand Boisson

Supplementary methods

Methods

Patient case reports. The patients from kindred A consulted at various French hospitals and were identified through the CEREDIH (*Centre de Référence des Déficits Immunitaires Héréditaires*) by the Study Center for Primary Immunodeficiencies (CEDI) at Necker Enfants Malades Hospital, Paris, France. The physicians treating the patients completed a detailed questionnaire on which clinical and biological data were collected (**Table 1**). The institutional review board of Necker Hospital approved the study and informed consent was obtained from all patients or their families (for minors), in accordance with the Helsinki Declaration (CNIL authorization: no. 908256, October 14th 2008). We used the National Institutes of Health (NIH) scoring system. Each feature was scored separately, and all the scores obtained were then summed to obtain a total score (scores over 40 points indicate that the subject probably has an AD HIES phenotype, scores of 20 to 40 points are inconclusive concerning the presence of AD HIES and scores below 20 points indicate that the subject is unlikely to have AD HIES) (1). The definition of *Aspergillus* spp.-related pulmonary invasive fungal disease (IFD) was based on the European Organization for the Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) consensus definitions (2). IFD was considered proven if the presence of the fungus was systematically demonstrated in diseased tissues, probable if a host factor, clinical features and mycological evidence were present and possible if there were appropriate host factors and sufficient clinical evidence consistent with IFD, but no mycological confirmation. Immunological investigations (**Table 1**) were based on those described in previous studies and all antibody determinations were performed before or several months after the end of IgG treatment (2).

Genetic analysis. Genomic DNA was prepared from blood samples from patients and controls by the standard phenol-chloroform extraction method. Exons 2 to 24 of *STAT3* and their flanking intron sequences were amplified by PCR with specific oligonucleotide primers (available on request) and sequenced with the Big Dye Terminator kit v1.1 (Applied Biosystems #4337455) and an ABI Prism 3500xl Genetic Analyzer (Applied Biosystems). WES was performed for P1, P2, and P3. Exome capture was performed with the SureSelect Human All Exon 71 Mb kit (Agilent Technologies). Paired-end sequencing was performed on a HiSeq 2500 sequencing system (Illumina) generating 100-base reads. We aligned the sequences with the GRCh37 reference build of the human genome with the Burrows-Wheeler aligner (3). Downstream processing and variant calling were performed with the Genome Analysis Toolkit (4), SAMtools (5), and Picard. Substitution and InDel calls were made with the GATK Unified Genotyper. All variants were annotated with SnpEff annotation software (6-8).

Genome-wide analysis Genome-wide linkage analysis was performed by combining genome-wide array data. Ten family members were genotyped with the Genome-Wide Human SNP Array 6.0. Genotype calling was achieved with the Affymetrix Power Tools Software Package (http://www.affymetrix.com/estore/partners_programs/programs/developer/tools/powertools.affx) with population-based filters (9), resulting in the use of 905,420 SNPs for linkage analysis. WES was performed as described in the corresponding section, for three family members, P1, P2 and P3. Parametric multipoint linkage analysis was performed with the Merlin program (10), using the combined set of 960,267 variants. We assumed an AD mode of inheritance, with a frequency of the deleterious allele of 10^{-5} and a complete penetrance model. Data for the family and for Europeans from the 1000 Genomes project were used to estimate allele frequencies and to define linkage clusters, with an r^2 threshold of 0.4.

Cell culture, cDNA synthesis and sequencing. EBV-immortalized B cells (EBV-B cells) from the patients were cultured in complete RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS). HEK293T cells, derived from the human embryonic kidney 293 cell line, which expresses a mutant version of the SV40 large T antigen, were cultured in complete DMEM supplemented with 10% FBS. *STAT3*$^{-/-}$ A4 cells, derived from human DLD1 colon cancer cells by homologous recombination (11) and kindly provided by JE Darnell, were cultured in McCoy's 5A medium containing 10% heat-inactivated fetal bovine serum (FBS). Cells were incubated at 37°C under an atmosphere containing 5% CO_2. Total RNA was extracted with the RNeasy plus Micro kit (Qiagen). RNA was then reverse-transcribed directly with oligo dT primers and SuperScript® II Reverse Transcriptase (Invitrogen). We then amplified the cDNA by PCR with specific oligonucleotide primers (available on request) and sequenced it as described for genomic DNA.

Exon trapping. *STAT3* genomic DNA was amplified from nucleotides chr17:40481719 to chr17:40478099 (GRCh37 reference), with the following primers: forward, 5′-ATAAGAATgcggccgc- (NotI)-ACTTGGTCACCTACATAGTTGATTG-3′; reverse, 5′-GCGgatcc- (BamHI)-TGTACGTAGCCTCTCACCGATTCTG -3′. It was then inserted into the pSPL3 plasmid (Life Technologies #18449-017), and Sanger sequencing was performed to check the sequence of the insert. COS-7 cells were transfected with the plasmids, with the X-tremeGENE™ 9 DNA Transfection Reagent kit (Sigma-Aldrich), according to the manufacturer's instructions. Cells were harvested 24 hours after transfection, and total RNA was extracted as previously described. cDNA was generated by reverse transcription, and amplified by PCR with the dUSD2 (CUACUACUACUAGTGAACTGCACTGTGACAAGCTGC) and dUSA4 (CUACUACUACUACACCTGAGGAGTGAATTGGTCG) primers for pSPL3 plasmids. The amplified cDNAs were inserted into the pGEM®-T Easy plasmid. The dUSD2 and dUSA4 primers were used for amplifications for the splicing analysis (12).

Expression vectors and transfection experiments. The mutated c.1282-89C>T *STAT3* allele was generated by amplifying the full-length cDNA from P1 EBV-B cells and inserting the amplicon into a TOPO cloning plasmid (pCR®2.1-TOPO® vector; Invitrogen). Other STAT3 mutants (R518*, D698Tfs*9, R382W, Y705F) were generated by directed mutagenesis. The WT, or a mutated c.1282-89C>T STAT3, R382W, or Y705F allele was introduced into the PCMV6- Myc-DDK vector (Origene). N-terminal V5-tagged WT STAT3 vector and DDK tagged D427ins17 STAT3 vector were also generated. *STAT3*$^{-/-}$ A4 cells were transfected with a WT allele-containing, mutant allele-containing or PCMV6 empty vector, with the X-tremeGENE™ 9 DNA Transfection Reagent (Sigma-Aldrich), according to the manufacturer's instructions.

Western blotting. EBV-B cells from controls and patients were stimulated by incubation with 100 ng/mL IL-6/IL-6Rα (R&D #8954-SR), and 100 ng/mL IL-21 (R&D #8879-IL-010), for 20 minutes at 37°C, under an atmosphere containing 5% CO_2. *STAT3*$^{-/-}$ A4 cells non-transfected or transfected with the empty pCMV6 vector, wild-type STAT3 or mutant STAT3 constructs were stimulated by incubation with 50 ng/mL IL-6 for 20 minutes at 37°C, under an atmosphere containing 5% CO_2. For whole-cell extracts, the cells were lysed by incubation in the following buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100), supplemented with a mixture of protease inhibitors, for 30 minutes at 4°C. The lysates were then centrifuged at 18,000 x *g* for 20 minutes at 4°C. The supernatants were processed directly for western blotting. We used the NE-PER™ kit (Thermo Fisher Scientific #78833) for the preparation of cytoplasmic and nuclear extracts. Western blotting was carried out as previously described (13), on total extract (30 µg protein), or cytoplasmic and nuclear extracts (5 µg protein) from transfected EBV-B cells or *STAT3*$^{-/-}$ A4 cells, with antibodies against the C-terminal (Cell Signaling Technology #9139) or N-terminal (Santa Cruz Biothechnology #sc-8019) part of the STAT3 protein and antibodies specific for Tyr705-phosphorylated STAT3 (Cell Signaling Technology #9145)

Immunoprecipitation and tandem mass spectrometry

Total protein extracts were obtained by incubating a million cells in the following buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 1% Triton X-100) supplemented with a mixture of protease inhibitors, for 30 minutes at 4°C. The resulting lysates were then centrifuged at 18,000 x *g* for 20 minutes at 4°C. The supernatants were processed directly for immunoprecipitation (IP). In total, 500 µg of protein lysate per sample was incubated with the STAT3 C-terminal mAb conjugated with protein G agarose beads by overnight incubation at 4°C. The beads were washed with lysis buffer and processed for western blotting. The immunoprecipitated proteins were then separated by SDS-PAGE. Bands were selected for analysis on the basis of a western blot performed in parallel. Gel bands were digested with trypsin (Promega) (14) and analyzed by reverse-phase nano-LC-MS/MS (EasyLC 1200, Fusion Lumos, Thermo Fisher Scientific). Peptides were separated with a gradient in which the proportion of solvent B increased from 2% to 90% over 71 minutes. MS and MS/MS data were recorded at resolutions of 60.000 and 15.000 with AGC's of 100,000 and 80,000, respectively. Parallel reaction monitoring (PRM) (15) was used to target peptides unique to the STAT3 mutant (WSFAVLLR [2+] and LVSNSWAQASLIVTEELHLITFETEVYHQGLK [3+/4+]) and STAT3 WT (ANCDASLIVTEELHLITFETEVYHQGLK [3+/4+]) peptides and peptides common to both the WT and mutant forms (GLSIEQLTTLAEK [2+], SAFVVER [2+], FPELNYQLK [2+], DSGDVAALR [2+], SIVSELAGLLSAMEYVQK [2+]).

The data were used to search a UniProt human database (March 2016) concatenated with the mutated STAT3 sequence and common contaminants (16), with ProteomeDiscoverer v. 1.4.0.288 (Thermo Fisher Scientific) / Mascot v. 2.5 (Matrix Science). Methionine oxidation and N-terminal acetylation were allowed as variable modifications and all cysteines were considered to be carbamidomethylated. Peptide matches were filtered with a Percolator (17)-calculated false discovery rate of 5%. PRM experiment data were analyzed with SkyLine v.4.2 (18). Samples were analyzed in the following order: control, patient, overexpressed mutant *STAT3* and overexpressed WT *STAT3*.

Electromobility shift assay (EMSA). EMSAs were performed as previously described (19). EBV-B cells were stimulated with 100 ng/mL IL-21 (R&D #8879-IL), and *STAT3*$^{-/-}$ A4 cells were stimulated with 100 ng/mL IL-6/IL-6Rα (R&D #8954-SR). We used the NE-PER™ kit to prepare cytoplasmic and nuclear extracts (Thermo Fisher Scientific #78833). Nuclear extract (5 µg protein) was incubated with IRD700-labeled m67SIE probe (5′-CATTTCCCGTAAATCGTCGA-3′) (20) and subjected to electrophoresis in a 5% polyacrylamide gel in TBE. For supershift experiments, extracts were incubated with no

antibody as a control, with an isotype control antibody (Santa Cruz Biotechnology #sc-3878) to demonstrate the specificity of the complex, and with a specific anti-STAT3 antibody (Cell Signaling Technology #9139) to detect DNA-protein complex supershift. All EMSA results were visualized with the Odyssey® CLx Imaging System and quantified with the ImageStudio software.

RNA-seq. EBV-B cells from healthy controls (*n*=3), D427ins17 patients' (P1, P2, P3), and AD HIES patients' (*n*=3) heterozygous for *STAT3* mutations (R382W, V463del, T708N), were starved by incubation in pure RPMI for two hours, and then stimulated by incubation for two hours with 100 ng/mL IL-6/IL-6Rα (R&D #8954-SR), 100 ng/mL IL-21 (R&D #8879-IL), 100 ng/mL IL-23 (R&D #1290-IL), 50 ng/mL IL-10 (R&D #217-IL), or 10^4 IU/mL IFNα (IntronA MSD). RNA was then extracted with the RNeasy plus micro kit (Qiagen #74034). RNA quality was assessed with the Agilent 2200 TapeStation nucleic acids system. RNA-seq was carried out as previously described (21). In brief, samples were mapped onto the human genome sequence (hg38 assembly) with STAR (22) and counts for the aligned reads for each gene were calculated with HTSeq-count (23). Downstream analysis and heatmap generation were performed with an in-house script in R (24), with DESEq2 (25), gplots, and RColorBrewer.

In vitro *differentiation and stimulation* of ex vivo *naïve and effector/memory CD4$^+$ T cells.* $CD4^+$ T cells were isolated as previously described (26, 27). Briefly, PBMCs were labeled with anti-CD4, anti-CD45RA, anti-CCR7, anti-CD127 and anti-CD25 antibodies. Naïve ($CD45RA^+CCR7^+$) T cells or effector/memory ($CD45RA^-CCR7^{\pm}$) $CD4^+$ T cells were isolated by cell sorting. Purified naïve or effector/memory $CD4^+$ cells were labeled with CFSE and cultured with T-cell activation and expansion beads (anti-CD2/CD3/CD28) alone or under Th1, Th2 or Th17 polarizing conditions for five days. For cytokine expression, activated $CD4^+$ T cells were restimulated with phorbol 12-myristate 13-acetate (PMA; 100 ng/mL)/ionomycin (750 ng/mL) for 6 hours. After incubation the cells and culture supernatants were harvested and used for assessments of proliferation based on CFSE dilution, and of the secretion of the indicated cytokines in cytometric bead array assays or by ELISA.

In vitro differentiation of B cells. Sorted naïve and memory B cells were cultured in the presence of CD40L, with or without IL-21 or BCR agonist and CpG, for seven days. The production of IgM, IgG and IgA was assessed by Ig heavy chain-specific ELISA on the culture supernatant (26, 27).

Luciferase reporter assay. The reporter vector pGL4.47 (Promega #E4041) contains five copies of a STAT3-responsive element, the sis-inducible element (SIE), linked to the luciferase reporter gene *luc2P*. *STAT3$^{-/-}$* A4 and HEK293T cells were transfected with the pCMV6 vector containing the wild-type or mutant *STAT3* cDNA, the reporter construct pGL4.47, and an expression vector for *Renilla* luciferase. After 24 hours, the transfected cells were stimulated with 100 ng/mL IL-6 for 24 hours. Relative luciferase activity was then determined by normalizing the values obtained against the *Renilla* luciferase signal.

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Supplementary table

Table S1: Summary of the mechanisms of action of the mutations associated with AD HIES

Mutation	STAT3 domain	Expression	Dimerization	Phosphorylation	Translocation	Binding	Transcriptional activity	Negative dominance	References
H332Y	DBD	normal	normal	normal	normal	no	nd	nd	(56)
G342D	DBD	normal	nd	normal	nd	nd	nd	nd	(37)
R382W	DBD	normal	normal	normal	low	no	no	yes	(56, 57, 88)
R382Q	DBD	normal	normal	normal	normal	no	no	yes	(13, 56)
F384S	DBD	normal	normal	normal	normal	no	nd	nd	(56)
T389I	DBD	normal	normal	normal	nd	no	no	yes	(13)
N395Y	DBD	nd	nd	low	nd	no	nd	nd	(55)
R423Q	DBD	normal	normal	normal	normal	no	nd	nd	(56, 88)
N425Y	DBD	nd	nd	low	nd	no	nd	nd	(55)
H437P	DBD	low	normal	low	low	low	nd	nd	(57)
H437Y	DBD	normal	normal	normal	nd	no	no	yes	(13)
Vdel463	DBD	normal	normal	normal	nd	no	no	yes	(13, 88)
S465F	DBD	normal	normal	normal	normal	low	nd	nd	(57)
S611N	SH2	normal	no	no	no	no	nd	nd	(56, 88)
F621V	SH2	normal	no	no	no	no	nd	nd	(56)
V637M	SH2	low	nd	low	nd	no	nd	nd	(54, 55, 88)
Q644P	SH2	nd	low	low	nd	low	nd	nd	(57)
Y657C	SH2	nd	nd	low	nd	no	nd	nd	(55, 88)
Y657N	SH2	low	low	low	low	low	nd	nd	(57, 89)
L706M	TA	low	low	low	low	low	nd	nd	(57)

nd: not determined; DBD: DNA-binding domain; SH2: Src homology 2 domain; TA: transactivation domain

Supplementary figures

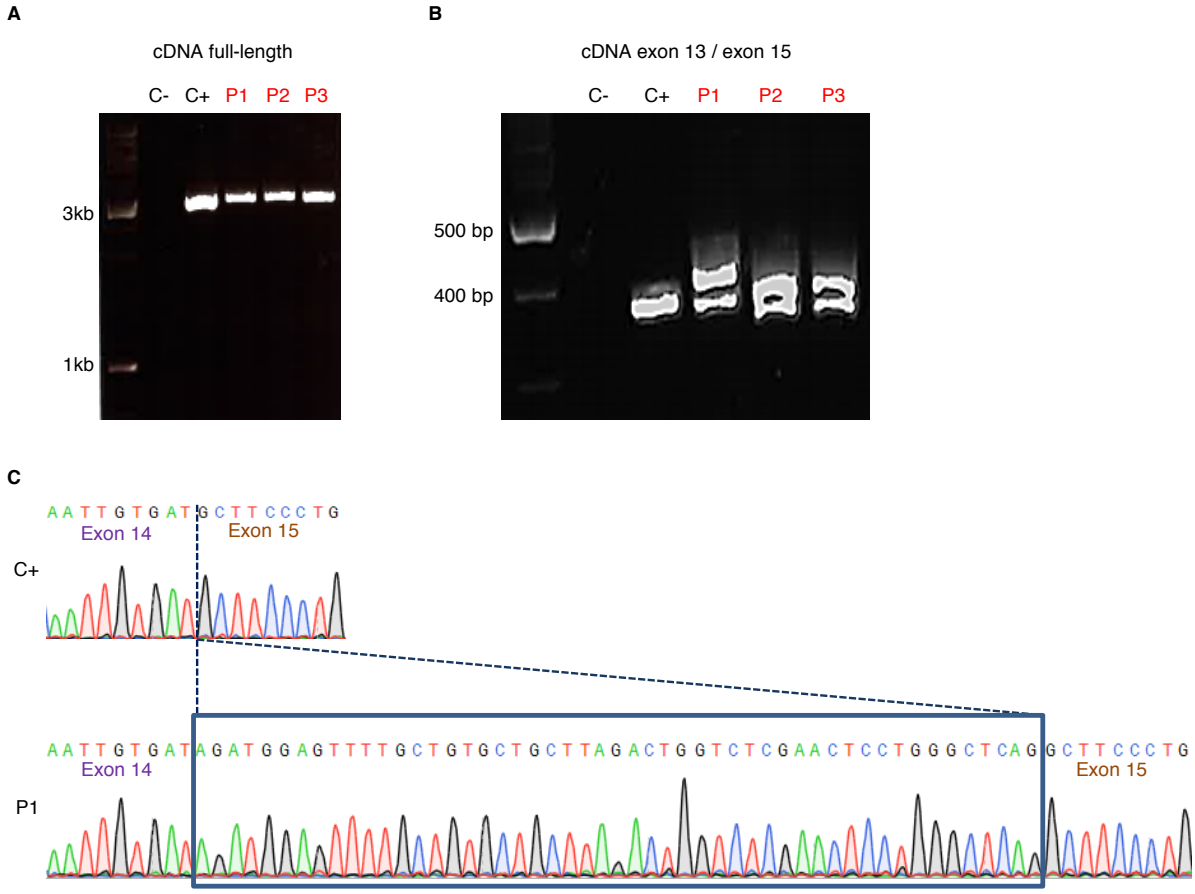

Figure S1: Study of the *STAT3* mRNAs
(**A**) Full-length *STAT3* cDNA amplification showing one *STAT3* transcript from healthy control, P1, P2 and P3 cells. (**B**) PCR of exons 12 to 15 of the *STAT3* cDNA showing the amplification of a single *STAT3* transcript from healthy control cells, and two different *STAT3* transcripts from the cells of P1, P2 and P3. (**C**) Sequencing chromatograms showing the result of the subcloning of PCR products. An insertion of 51 nucleotides of intron 14 of the STAT3 gene is observed in about 50% of the clones, between exons 14 and 15 of the *STAT3* cDNA.

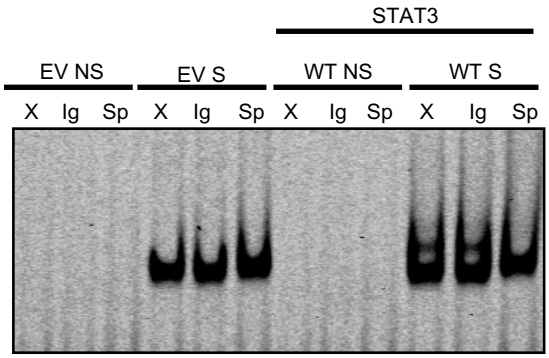

Figure S2: STAT3 supershift experiments
EMSA with the m67SIE probe on *STAT3*$^{-/-}$ A4 cells transfected with EV or the WT vector with (S) and without (NS) stimulation with 100 ng/mL IL-6/IL-6Rα for 30 min. Extracts were incubated with no antibody (X) as a control, with an isotype control antibody (Ig) to demonstrate the specificity of the complex, and with a specific anti-STAT3 antibody (Sp) to detect the supershift of the DNA-protein complex.

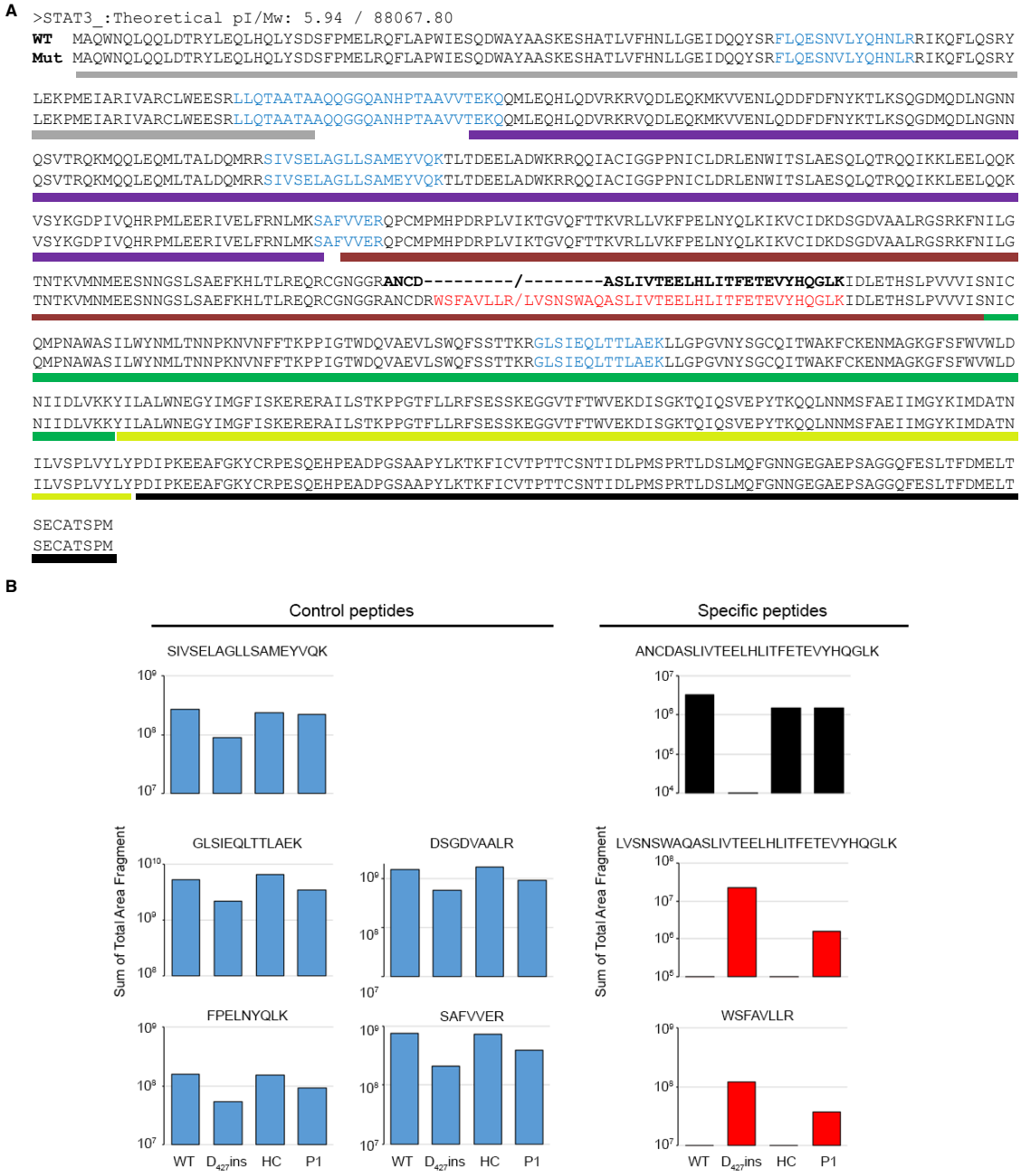

Figure S3: Detection of peptides specific to the D427ins17 mutant protein by LC/MS-MS. (**A**) Alignment of the STAT3 WT and D427ins17 mutant proteins. The STAT3 protein domains are indicated by colored bars: the NT domain (gray), C-C domain (purple), DBD (dark red), linker domain (green), SH2 domain (yellow), and TA domain (black). The five peptides common to the WT and mutant STAT3 proteins are shown in blue, whereas the two peptides specific to D427ins17 mutant STAT3 are shown in red and the peptide specific to WT STAT3 is shown in bold. (**B**) The levels of each peptide can be determined by summing peptide area (crude value). The five common peptides are shown in blue, the WT-specific peptide in black, and the two mutant-specific peptides in red. WT indicates *STAT3*$^{-/-}$ A4 cells transfected with the WT *STAT3* allele, mutant indicates *STAT3*$^{-/-}$ A4 cells transfected with the D427ins17 *STAT3* allele, control indicates EBV-B cells from a healthy control, and patient indicates EBV-B cells from P1.

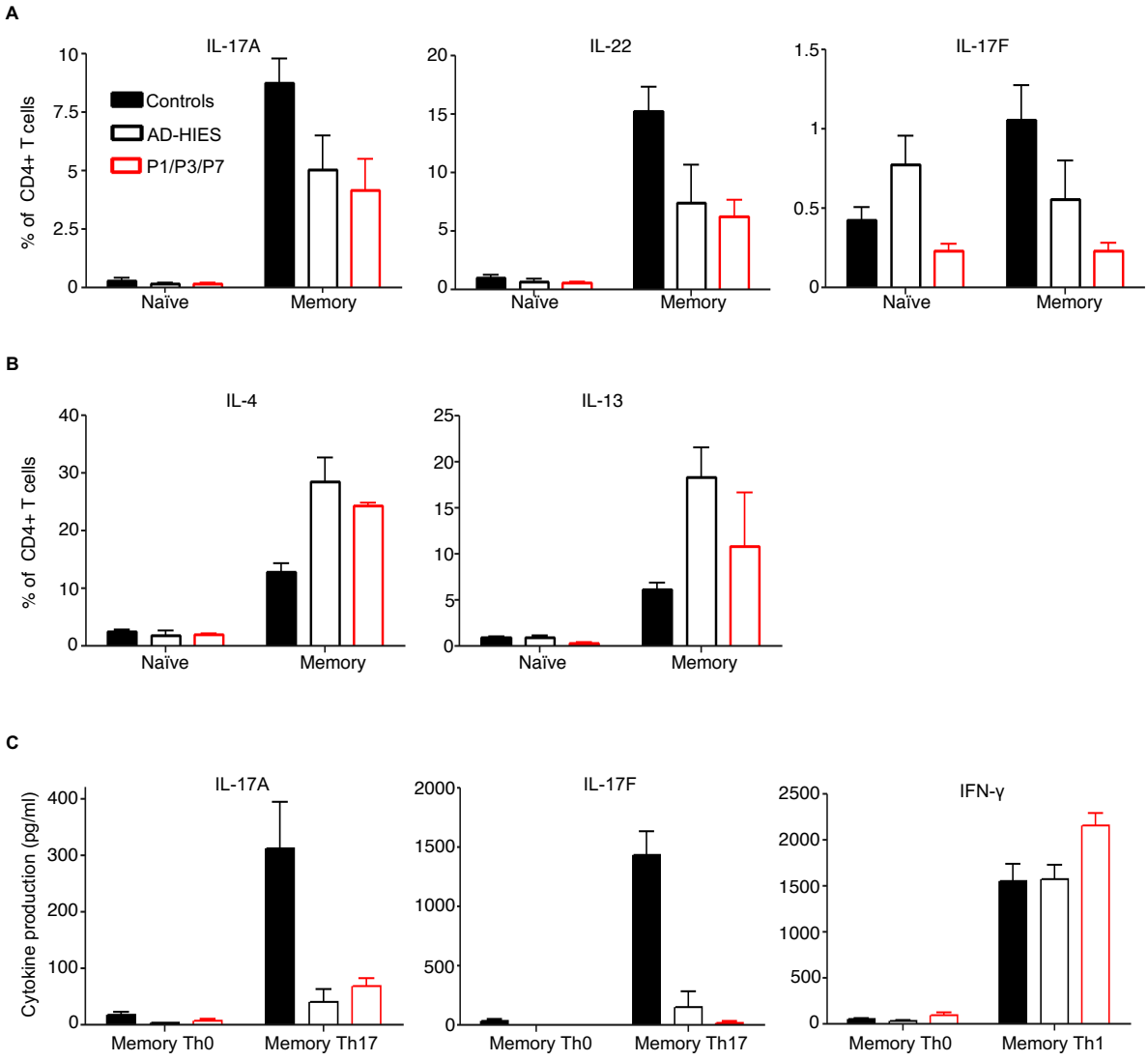

Figure S4: CD4$^+$ T-cell subpopulation immunophenotyping

(**A, B**) Functional Th17 and Th2 cells were quantified by determining the proportions of memory CD4$^+$ T cells expressing (**A**) IL-17A, IL-22, and IL-17F, or (**B**) IL-4 or IL-13. The data were obtained in experiments performed with memory CD4$^+$ T cells from 11 healthy donors, seven patients with typical AD HIES, and patients P1, P3, P7 with the D427ins17 mutation. (**C**) Memory CD4$^+$ T cells were purified by sorting from healthy donors (n=12), patients with *STAT3* mutations and AD HIES (n=7) and P1, P3 and P7. They were cultured for five days under Th0, Th17 or Th1 polarizing conditions. The secretion (pg/mL) of IL-17A, IL-17F, and IFN-γ was then determined. The data shown are means ± SEM.

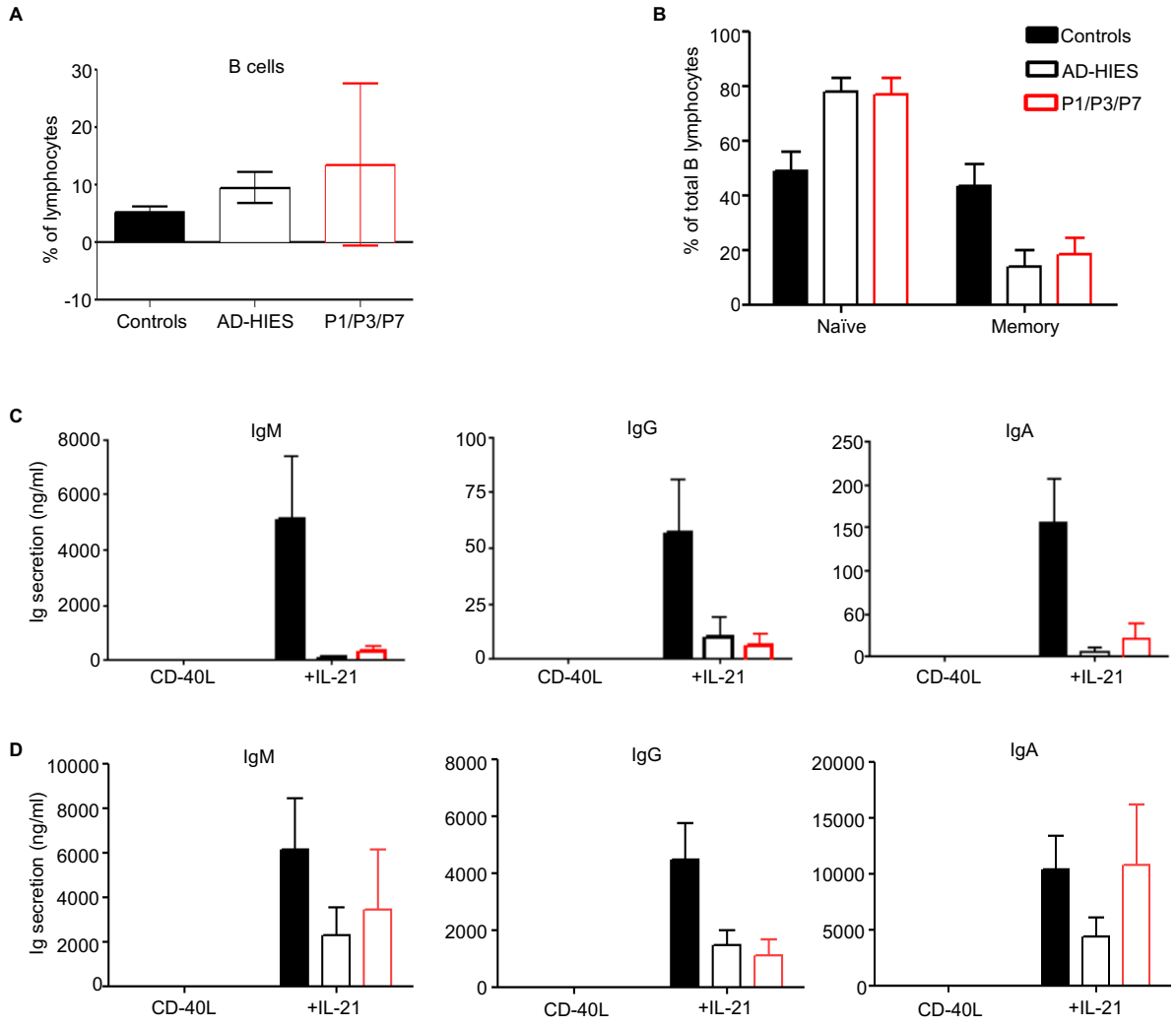

Figure S5: B-cell immunophenotyping and function

(A, B) Frequencies (%) of **(A)** total and **(B)** naïve and memory B cells in the peripheral blood of healthy donors, AD HIES patients and patients P1, P3, P7 with the D427ins17 mutation. (**C**) Naïve (**D**) and memory B cells sorted from the peripheral blood of healthy donors (*n*=11), AD HIES patients (*n*=7), and patients with the D427ins17 mutation (P1, P3, P7), were cultured with CD40L (200 ng/mL) alone or together with IL-21 (50 ng/mL), for 7 days. The production of IgA, IgG and IgM (ng/mL) was then assessed by Ig heavy chain specific-ELISA on cell culture supernatants. Dots and error bars represent the mean and standard deviation, respectively.

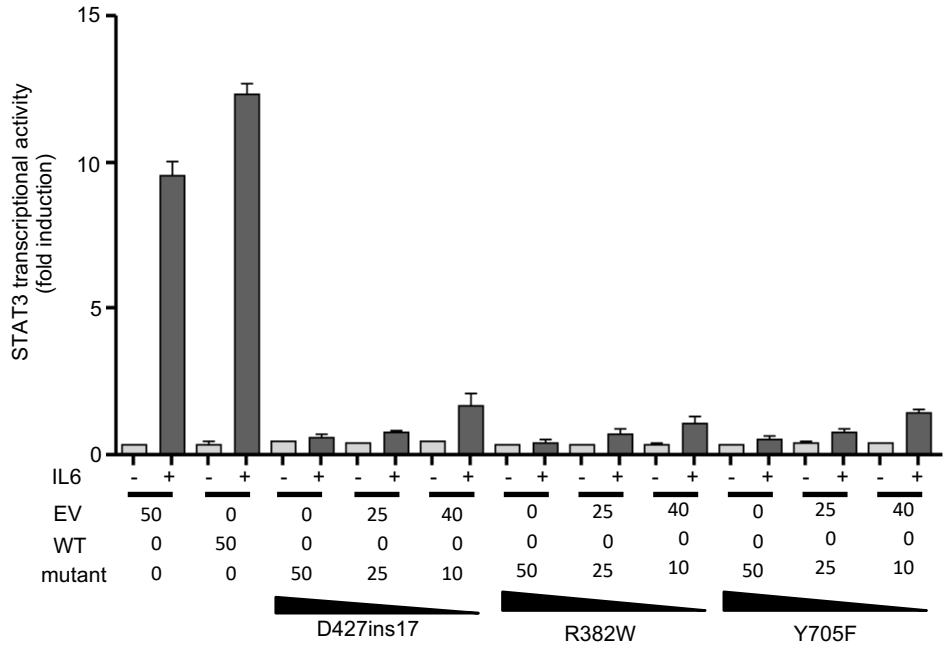

Figure S6: Dominant-negative effect of the D427ins17 allele on endogenous STAT3 levels in HEK293T cells

Luciferase assay on HEK293T cells transfected with empty vector (EV) or with the WT *STAT3* plasmid, or transfected with various amounts of *STAT3* mutants (D427ins17; R382W; Y705F), together with the pGL4.47 reporter construct, and an expression vector for *Renilla* luciferase. After 24 hours, the transfected cells were stimulated with 100 ng/mL IL-6 for 24 hours. The transcriptional activity of the STAT3 promoter normalized relative to unstimulated conditions in cells transfected with EV activity is shown on the *y* axis and the alleles used for transfection are indicated on the *x* axis.