A recessive form of hyper-IgE syndrome by disruption of ZNF341-dependent STAT3 transcription and activity

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INTRODUCTION

Hyper–immunoglobulin E (IgE) syndrome (HIES) is a relatively common primary immunodeficiency (PID; Online Mendelian Inheritance in Man #147060), first described as Job’s syndrome by Wedgwood in 1966 (1) and renamed HIES by Buckley in 1972 (2). It was subsequently shown to typically display autosomal dominant (AD) inheritance, with variable expressivity (3). AD-HIES is characterized by bacterial infections, including, in particular, various staphylococcal diseases, and by fungal infections, such as chronic mucocutaneous candidiasis (CMC) in particular. In the course of infection, clinical and biological signs of inflammation are paradoxically weak in these patients. Patients also display cutaneous and systemic manifestations of allergy (in the broad sense of the term), along with high serum concentrations of total and allergen-specific IgE, and extrahematopoietic features, including facial dysmorphism, deciduous tooth retention, osteopenia, hyperextensibility, and vascular abnormalities (3, 4). They also have B cell and antibody (Ab) deficiencies (5). In 2007, Minegishi et al. (6) identified heterozygous, dominant-negative (DN) mutations of the gene encoding signal transducer and activator of transcription 3 (STAT3) as responsible for AD-HIES. Most, if not all, cases of AD-HIES are caused by STAT3 DN mutations (7–9).

Some nonhematopoietic features of AD-HIES were explained by the discovery of patients with overlapping phenotypes, carrying bi-allelic mutations of genes encoding leukaemia inhibitory factor receptor (LIFR), interleukin-11 receptor (IL-11R), and the IL-6ST/gp130 common subunit of the IL-6 receptor family, which signal via STAT3 in various extrahematopoietic cells (10–12). Myeloid cell development is essentially normal in AD-HIES, but lymphocyte development is severely affected, with low frequencies of CD4⁺ and CD8⁺ central memory T cells, T helper 17 (TH17) cells, T follicular helper (TFH) cells, mucosal-associated invariant T (MAIT) cells, natural killer (NK) cells, and memory B cells (5, 7, 13–17). Patients with inborn errors of receptors or cytokines upstream from STAT3 display overlapping syndromes. Memory B cell deficiency has been detected in IL-6ST–deficient patients and in IL-21R–deficient patients, who also have low frequencies of central memory CD8⁺ T cells, TFH cells, and NK cells (12, 13, 15, 17–19).

Some leukocyte functions are also abnormal in AD-HIES patients, as shown by studies in vitro. The patients’ naïve CD4⁺ T cells display impaired TH17 differentiation upon stimulation under TH17-polarizing conditions in vitro, providing a mechanism for the patients’ CMC, as seen in patients with inborn errors of IL-17 immunity (14, 20, 21). CD4⁺ T cells are also biased toward the TH2 lineage, accounting for some of the patients’ allergic manifestations (22), whereas the inability of their naïve B cells to differentiate into plasma cells upon stimulation with CD40L and IL-21 underlies Ab deficiency, which is also observed in patients with mutations in IL21 or IL21R (5, 18, 19). IL-10 does not inhibit the response of the patients’ myeloid cells to lipopolysaccharide (LPS) (6, 23). Nevertheless, these patients do not display the early-onset colitis observed in patients with IL-10, IL-10R1,
and IL-10R2 deficiencies (24). Last, poor responses of myeloid cells to IL-6 and related cytokines probably account for the patients’ low levels of inflammation, as inferred from the patient with IL-6ST deficiency (12).

In this context, we investigated patients with an autosomal recessive (AR) form of HIES—including CMC, staphylococcal infections, severe allergy, and high serum IgE levels—but apparently with stronger inflammatory responses and fewer extrahematopoietic manifestations than patients with AD-HIES. Their phenotype more closely resembled that of patients with STAT3 DN mutations than that of patients with other PIDs involving high serum IgE levels often referred to as AR forms of HIES, such as DOCK8 (dedicator of cytokinesis 8) deficiency (25–28) and PGM3 (phosphoglucomutase 3) deficiency (29, 30). Patients with DOCK8 deficiency present none of the extrahematopoietic features of AD-HIES but are highly vulnerable to skin-tropic viral infections. Likewise, patients with PGM3 deficiency display different extrahematopoietic manifestations, autoimmunity, and a broader susceptibility to infections. We thus tested the hypothesis that the patients studied suffered from a previously undescribed AR inborn error of immunity, closely related to the AD form of HIES. Given the clinical similarity of the AD and AR forms of HIES, we hypothesized that the disease-causing gene underlying the AR form would encode a protein physiologically related to STAT3.

**RESULTS**

The patients are homozygous for truncating mutations of ZNF341

We investigated eight patients from six unrelated families, of Moroccan (kindred A), Afro-Caribbean (kindred B), Iranian (kindred C), Turkish (kindreds D and E), and Lebanese (kindred F) descent (Fig. 1, A and B; fig. S1, A to J; tables S1 and S2; and the “Case reports” section). Four families were known to be consanguineous, whereas the other two families were shown to be consanguineous by whole-exome sequencing (WES), which revealed a high percentage of homozygosity in the patients (fig. S1K) (31). We performed genome-wide linkage analysis on the three living patients from kindreds A and B (P2, P3, and P4), testing the hypothesis of a shared AR trait with full penetrance (fig. S1, L and M). A single 16.8-Mb region on chromosome 20 provided a significant cumulative log of odds score of 4.8 (fig. SIM). We also performed WES for these three patients (fig. S1N). Within the linked region containing 162 protein-coding genes, only ZNF341, a gene of unknown function, displayed homozygosity for a rare variant (table S3). This variant (c.904C>T) was the same in both families tested and caused replacement of the Arg302 codon with a premature stop codon (R302X). By WES (P5, P6, P7, and P8), we showed that the other four unrelated patients from kindreds C to F also carried homozygous mutations of ZNF341. Kindred C displayed the same c.904C>T mutation (R302X), whereas kindred D had a frameshift deletion (c.1062delG) leading to a premature stop codon (K355fs), kindred E had a nonsense mutation (c.1626C>G) replacing the Tyr542 codon with a premature stop codon (Y542X), and kindred F had a nonsense mutation (c.583C>T) replacing the Gln195 codon with a premature stop codon (Q195X). Sanger sequencing confirmed all the mutations identified by WES. The segregation of the four mutant alleles of ZNF341 in the six families was consistent with a fully penetrant AR trait (Fig. 1A and fig. S1, O to T). The K355fs, Y542X, and Q195X mutations were private to kindreds D, E, and F, respectively. There were only two R302X heterozygotes in the ExAC database. Kindreds A, B, and C, which carried R302X, belonged to three different ethnic groups, as confirmed by principal components analysis (fig. S1U) (31). The mutation was recurrent due to a hotspot rather than a founder effect, because the haplotypes encompassing ZNF341 differed between the three families (Fig. 1C). The four ZNF341
mutations are located in four different exons scattered across the gene. They are the only known mutations of this gene predicted to be loss of function and found in the homozygous state in public and in-house databases. Moreover, these mutations also have the four highest damage prediction scores [combined annotation–dependent depletion (CADD)] of all the variants found to be homozygous (Fig. 1D) (32, 33). Together, these findings strongly suggest that the homozygous ZNF341 mutations identified in these patients are both deleterious and disease-causing.

The mutant ZNF341 alleles do not encode full-length isoforms

Nothing is known about the biology of ZNF341. The human gene has 15 exons and encodes two main transcripts, differing by 21 in-frame nucleotides due to alternative splicing involving different acceptor sites at the 3′ end of exon 6 (Fig. 1E), and yielding proteins of 847 (isoform 1) or 854 (isoform 2) amino acids. The stop codons created by Q195X and R302X are upstream from these 21 nucleotides, unlike those created by K355fs and Y542X. The encoded protein has 12 predicted DNA binding C2H2 zinc finger (ZNF) domains and two predicted nuclear localization sequences (NLSs), suggesting that it is a transcription factor (Fig. 1E) (34, 35). The stop codons created by Q195X and R302X are upstream from the two NLSs, whereas those created by K355fs and Y542X are located between them. We transfected human embryonic kidney (HEK) 293T cells with complementary DNAs (cDNAs) encoding wild-type (WT) ZNF341 (isoforms 1 and 2), Q195X or R302X (both of which encode a single isoform), K355fs (isoform 1), K362fs (isoform 2), Y542X (isoform 1), or Y549X (isoform 2) and analyzed the products by Western blotting with a polyclonal Ab (pAb) against the N-terminal segment of ZNF341 (HPA024607; Fig. 2A and fig. S2A). Isoforms encoded by the WT [predicted molecular weight (M_w) values of ~92 and 93 kDa], K355fs/K362fs (~40 and ~41 kDa), and Y542X/Y549X (~58 and ~59 kDa) cDNAs were detected in the nucleus, whereas those encoded by the Q195X (~20 kDa) and R302X (~31 kDa) cDNAs were retained in the cytoplasm. We transfected SV40-transformed fibroblasts from P4 (R302X/R302X) with cDNAs encoding C-terminally V5-tagged
 proteins. We then analyzed the subcellular distribution of these proteins by confocal microscopy with a V5-specific monoclonal Ab (mAb; Fig. 2B and fig. S2B). Both isoforms of the WT and the K355fs, K362fs, R302X (and by inference R195X) proteins were retained in the cytoplasm, whereas the Y542X, and Y549X proteins were localized in the nucleus, whereas the R302X (and by inference R195X) proteins were retained in the cytoplasm, whereas the Y542X, and Y549X proteins were localized in the nucleus, whereas the

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Western blots representative of three independent experiments are shown. Bar graphs show the mean and the SD. Dots represent the mean of technical duplicates. (**P < 0.01).
poorly, and two of the mutant proteins (Q195X and R302X) are retained in the cytoplasm.

**Full-length ZNF341 isoforms are not detected in the patients’ cells**

Public databases (FANTOM5, HPA, and GTEx) suggest that ZNF341 is ubiquitously transcribed. We studied the expression of endogenous ZNF341 in cell lines from healthy controls and patients. Polymerase chain reaction (PCR) and sequencing showed that both full-length transcripts were present in Epstein-Barr virus (EBV)–transformed B cells from P4 (fig. S2, F to I) and in herpes virus saimiri (HVS)–transformed T cells from P3 and P4 (fig. S2). Moreover, total ZNF341 mRNA levels, as determined by reverse transcription quantitative PCR (RT-qPCR), were significantly higher in EBV-B cells and slightly higher in HVS-T cells from all patients tested (P3, P4, P6, and P7) than in controls, suggesting that these mutations did not provoke nonsense-mediated mRNA decay in these cells and that WT ZNF341 down-regulates its own transcription (Fig. 2, D and E). We also detected ZNF341 mRNA in primary human umbilical vein endothelial cells (HUVECs), in three hematopoietic and nine nonhematopoietic cancer cell lines tested, and in control SV40-transformed fibroblasts and primary keratinocytes (fig. S2, K to M). We performed Western blotting with a ZNF341–specific pAb (HPA067108) or an in–house mAb (8B3.1), both raised against C-terminal residues 366 to 468 (downstream from Q195, R302, and K355 but upstream from Y542 in isoform 1). We detected ZNF341 (Mr~100 kDa) in the nuclei of all nine cancer cell lines, in primary HUVECs, primary and SV40–transformed fibroblasts, and primary keratinocytes from healthy donors (fig. S2, N to P). By contrast, ZNF341 was not detected in primary and SV40 fibroblasts or in keratinocytes from P2, P3, and P4 (fig. S2, O and P). Similarly, we detected full-length ZNF341 (Mr~100 kDa) in the nuclei of six hematopoietic cell lines (RAJI, Jurkat, K562, U937, HL-60, and clone 15 HL-60; fig. S2N) and EBV-B and HVS-T cells from healthy controls but not in any of the four patients tested (corresponding to three mutations; Fig. 2, F and G). A protein of lower Mr (~70 kDa) was observed in the nuclei of EBV-B cells from P7, whose Y542X mutation is predicted to preserve the 8B3.1 mAb epitope (Fig. 2F). Because the pAb (HPA024607) recognizing the N-terminal segment failed to detect endogenous ZNF341 in control cells, we cannot exclude the possibility that low levels of the truncated R302X (and Q195X) and K355fs proteins were also present in the cytoplasm and nucleus, respectively, of the corresponding patients’ cells. Last, production of the full-length ZNF341 protein was rescued in the EBV-B cells of P4 (R302X) and HVS-T cells of P3 (R302X) by stable transduction with WT ZNF341 (isoform 1; Fig. 2, F and G). Together, these data indicate that both full-length ZNF341 isoforms are absent from the nuclei of primary keratinocytes, primary and SV40 fibroblasts, EBV-B cells, and HVS-T cells derived from patients with various mutations of ZNF341.

**ZNF341 deficiency alters the development of lymphoid, but not myeloid, subsets**

We analyzed the pattern of ZNF341 expression in leukocyte subsets from healthy controls. ZNF341 transcripts for both isoforms were detected, in similar amounts, in monocytes, NK, B, CD4+, and CD8+ T cells, by RT-qPCR and RT-PCR (Fig. 2H and fig. S2Q). Accordingly, a single protein corresponding to either or both isoforms was detected in the nuclei of T, B, NK, monocytes (including both CD14+ and CD16+ subsets), basophils, and dendritic cells (including pDCs, cDC1, and cDC2; Fig. 2, I to L). ZNF341 was also detected in the HL-60 and clone 15 HL-60 cell lines (fig. S2N), two acute promyelocytic leukemia cell lines. These lines can be differentiated into neutrophil-like and eosinophil-like cells in vitro, respectively, suggesting that primary neutrophils and eosinophils may express ZNF341, like basophils (Fig. 2L). In this context, we analyzed the distribution of leukocyte subsets in the patients, by flow cytometry. The seven patients tested (P2 to P8) had normal counts of circulating neutrophils and basophils, monocytes, B cells, and T cells but had low counts of NK cells (table S2 and the “Case reports” section). Eosinophil counts were high in three patients (table S2 and the “Case reports” section). The proportions of myeloid (cDC1 and cDC2) and plasmacytoid (pDC) dendritic cells (fig. S3A) and of monocyte subsets (fig. S3B) were normal. CD56bright NK cells were more abundant among NK cells, but their maturation profile was otherwise normal (Fig. 3A). The proportions of innate lymphoid cells (ILCs) among peripheral blood mononuclear cells (PBMCs) were also low, particularly for ILC1 and ILC2 (Fig. 3B). The global proportion of circulating memory B cells was low (Fig. 3C), with low (IgM+, IgA+) and high (IgG+ ‘) proportions of Ig isotype–specific memory B cells (Fig. 3D), consistent with the patients’ high serum concentrations of IgG1 and IgG4 (table S2). The patients had normal or subnormal serum titers of antigen–specific Abs after infection with common pathogens (table S2), and P4 displayed a normal response to a vaccine booster injection (table S2). The patients’ T cells proliferated normally in response to mitogens and antigens in vitro (table S2). The patients had a higher proportion of naïve CD4+ T cells and lower proportions of central memory CD4+ and CD8+ T cells, and of MAIT cells, than controls, but had normal proportions of regulatory T (Treg) cells, γδ T cells, and invariant NKT (iNKT) cells (Fig. 3, E to G). This distribution of leukocyte subsets very closely resembles that of patients with HIES due to STAT3 DN mutations, who also have high frequencies of naïve CD4+ T cells and low frequencies of CD4+ and CD8+ central memory T cells, memory B cells, MAIT cells (5, 7, 13, 15–17), and ILC1 and ILC2 cells (Fig. 3B). However, ZNF341–deficient patients also have low NK cell counts (table S2 and Fig. 3A).

**ZNF341 is a transcription factor that binds the STAT3 promoter in T and B cells**

We performed chromatin immunoprecipitation sequencing (ChIP-seq) analysis with the anti-ZNF341 mAb 8B3.1 to determine whether ZNF341 bound DNA in vivo and to identify its binding sites throughout the genome. In EBV-B cells from P4 transduced with cDNAs encoding WT ZNF341 isoforms 1 and 2, we found 5842 and 6570 ZNF341–binding DNA regions, respectively, 5003 of which were common to both isoforms. We also analyzed healthy control T cells activated with plate-bound anti-CD3 and soluble anti-CD28 Abs: we identified 1457 binding regions, only 229 of which were common to EBV-B cells. Computational analysis revealed two top-ranked motifs: a ZNF-like binding motif, GGAAC/GA/GGC (P = 5 × 10^{-31}), and an Sp1-like binding motif, GGAGG (P = 3.7 × 10^{-44}, Fig. 4A). The ZNF-like and Sp1-like motifs have not been described before, and the closest known motifs, for ZNF263 and Sp1, respectively, are markedly different (fig. S4, A and B). The strongest ZNF341–binding site in both EBV-B cells and T cells was located in the STAT3 promoter (P = 10^{-310} being the most significant P value of all binding sites). Strong ZNF341 binding was also observed for the STAT1 promoter and ZNF341 intron 1 (P = 10^{-242} and 10^{-310},
respectively; Fig. 4B). Furthermore, in the 229 motifs common to both control T cells and patient EBV-B cells transduced with ZNF341, we also identified bipartite binding sites containing the Sp1-like motif GGGAGG upstream from the ZNF-like motif GGAAC/GA/GGC (P < 5.3 × 10^{-172}; Fig. 4A). We found that there was a preferential spacing of 13 to 14 nucleotides between the two motifs (Fig. 4, C and D), associated with a stronger binding intensity, as assessed by determining peak intensity (fig. S4C).

We then incubated nuclear extracts of HEK293T cells transfected with an empty vector, the C-terminal DDK-tagged WT, R302X, K355fs, or Y542X ZNF341 cDNA with a 5′-tagged fluorescent DNA probe containing the putative bipartite ZNF341-binding motif from the STAT3 promoter (Fig. 4E and fig. S4D). In electrophoretic mobility shift assays (EMSA), nuclear proteins from WT-transfected cells bound the probe, whereas complex formation was inhibited by an unlabeled specific probe [competitor probe (CP)], and super-shifting of the complex was observed with the specific anti-ZNF341 8B3.1 or anti-DDK mAb but not with an isotype control mAb. By contrast, nuclear proteins from R302X- and K355fs-transfected cells did not bind the probe, whereas Y542X-transfected cells displayed weak binding (Fig. 4E and fig. S4D). Incubation of the same nuclear extracts with a 5′-biotinylated DNA probe resulted in the pulldown, with streptavidin-coupled magnetic beads, of complexes consistent with the EMSA results (Fig. 4F). Pulldown experiments with nuclear extracts from healthy control resting primary CD3+ T cells or EBV-B cells showed that endogenous WT ZNF341 bound efficiently to the bipartite DNA binding motif (Fig. 4, G and H). By contrast, the truncated Y542X ZNF341 protein was not pulled down in this system, in experiments with nuclear extracts from P7 EBV-B...

ZNF-like and Sp1-like motifs cooperate to enhance ZNF341 DNA binding

We characterized the ZNF341-binding motif in more detail by testing WT ZNF341 in pulldown experiments with probes either corresponding to the bipartite DNA binding motif or containing various cell lines (Fig. 4H). Thus, WT ZNF341 specifically binds a bipartite consensus DNA motif that is present in the promoter of STAT3, whereas the three mutant ZNF341 proteins tested (and, by inference, Q195X), including K355fs and Y542X, which can translocate to the nucleus, do not.
systematic mutations (fig. S4, E and F). We deleted the Sp1-like (ΔSp1 probe) or the ZNF-like (ΔZNF probe) motif or introduced single-nucleotide ($n = 8$; probes #1 to #8) or multiple-nucleotide mutations ($n = 4$; #47, aA, aG, and aZ probes) at various positions in the ZNF-like motif. Nuclear extracts from HEK293T cells transfected with WT ZNF341 isoform 1 cDNA or an empty vector were used as positive and negative controls, respectively. The deletion of the Sp1-like (74%) or ZNF-like (94%) motif decreased ZNF341 binding relative to the consensus sequence (WT probe), and deletion of the ZNF-like motif had the strongest impact (fig. S4E). This result is consistent with our ChiP-seq data, showing that the ZNF-like motif is present in all 500 top peaks, whereas the Sp1-like motif is present in only 45% of these peaks. In a similar pulldown experiment, single-nucleotide mutagenesis of the ZNF-like motif (GGAAAGAGC) only modestly decreased ZNF341 binding relative to the WT consensus DNA sequence (fig. 4F). However, mutations of one of the most (#3) and one of the least conserved nucleotides (#5) within the ZNF-like motif were associated with the largest (82%) and smallest (40%) decreases in DNA binding, respectively. Replacements of multiple nucleotides within the ZNF-like motif (aG, aA, and aG probes) decreased DNA binding by about 94%, to levels similar to those observed with a probe lacking the ZNF-like motif (ΔZNF probe). Overall, although either motif within the bipartite sequence is sufficient for at least some detectable binding of ZNF341 to DNA, the ZNF-like motif is more important and acts in synergy with the Sp1-like motif to ensure strong binding of ZNF341 to DNA.

ZNF341 overexpression drives the induction of STAT3

We assessed the ability of ZNF341 to induce transcription from the STAT1 and STAT3 promoters (44 base pairs of each promoter containing the canonical bipartite motif) in a luciferase reporter assay in HEK293T’ cells (Fig. 4I and fig. S4, G and H). Both WT isoforms induced expression from the STAT1 and STAT3 promoters. Three of the five mutant isoforms tested induced no luciferase activity, confirming that the R302X and K355fs/K362fs mutant alleles (and, by inference, Q195X) were loss of function. By contrast, the Y542X/Y549X mutant, which bound the canonical motif on EMSA and in pulldown experiments in the overexpression system, but not in EBV-B cells from P7, yielded intermediate levels of luciferase activity, suggesting that it is hypomorphic, at least when overexpressed. The luciferase STAT1 and STAT3 constructs containing the Sp1-like or the ZNF-like motif alone failed to induce luciferase activity in the presence of WT ZNF341, demonstrating the requirement of both motifs for ZNF341 activity (fig. S4, G and H). Cotransfection with the WT ZNF341 cDNA together with an Sp1 cDNA did not further enhance luciferase activity from STAT3 or STAT1 bipartite canonical sequences over that observed for WT ZNF341 alone (fig. S4I). Consistently, after the overexpression of Sp1 and ZNF341 in HEK293T cells, no interaction was detected between these two proteins in immunoprecipitation experiments (fig. S4J). In conclusion, ZNF341 induces the transcription of STAT1 and STAT3 by binding to the bipartite consensus sites in their promoters. Two of the three mutant alleles tested are loss of function (R302X and K355fs), the third being at least severely hypomorphic (Y542X), and the fourth is predicted to be loss of function (Q195X). In light of the clinical and immunological similarities between patients with ZNF341 and STAT3 mutations, these findings strongly suggest that ZNF341 may be essential for the transcription of STAT3, at least in cells expressing ZNF341, the pattern of expression of which is apparently as broad as that of STAT3.

STAT3 function is normal in ZNF341-deficient immortalized cell lines

We performed total RNA sequencing (RNA-seq) to determine whether ZNF341 controlled the transcription of target genes, including STAT3 in particular, in immortalized lymphoid cell lines. ZNF341 overexpression had very little impact on the transcriptome of P4 EBV-B cells (Fig. 4I). We found that, after transduction with WT ZNF341 isoform 1 or 2, mRNA levels differed significantly ($P < 0.01$) from those in cells transfected with an empty vector for only 28 and 52 mRNAs (tables S4 and S5), respectively, including STAT1 [fold change (FC) = 3.0 and 3.3], but not STAT3 (FC = 1.4 and 1.6; Fig. 4K). Thus, the binding of ZNF341 to the promoters of STAT3 and other genes does not strongly drive their transcription in EBV-B lines. We then analyzed STAT1 and STAT3 mRNA and protein levels in cell lines from patients and controls (fig. S4, K to O). We found that STAT1 mRNA levels were in the lower part of the control range in EBV-B cells, HVS-T cells, and SV40-immortalized fibroblasts from patients, as determined by RT-qPCR (Fig. 4K). STAT1 protein levels were also lower (about two- to threefold) in the patients’ EBV-B cells, HVS-T cells, and SV40 fibroblasts than in controls, as shown by Western blotting and flow cytometry (fig. S4, L to O). The complementation of HVS-T cells from P3 and EBV-B cells from P4 with WT ZNF341 increased STAT1 mRNA and protein levels, as shown by comparison with cells transfected with an empty vector (fig. S4, K to O). By contrast, STAT3 mRNA and protein levels were within or near the normal range in EBV-B cells from the patients (fig. S4, K, L, and O). These levels were slightly lower in patient HVS-T cells and SV40 fibroblasts (fig. S4, K and M to O). The transduction of HVS-T cells from P3 and EBV-B cells from P4 with WT ZNF341 increased STAT3 mRNA and protein levels relative to those in cells transfected with an empty vector (fig. S4, K to M and O). Last, the amounts of phosphorylated STAT1 and STAT3 were within the normal range in EBV-B cells stimulated with interferon-α (IFN-α) and IL-21, respectively (fig. S4P). Accordingly, the induction of target genes (CXCL10 and SOCS3) in patients’ EBV-B cells stimulated with IFN-α or IL-21 was normal (fig. S4Q), as was that in patients’ SV40 fibroblasts stimulated with IFN-α or IL-6/IL-6Rα (fig. S4R). Overall, immortalized cell lines from the patients displayed no major STAT3 phenotype, with the possible exception of slightly low levels of STAT3 mRNA and protein in HVS-T cells and SV40 fibroblasts.

STAT3 function is impaired in ZNF341-deficient primary fibroblasts

Some ZNF341-deficient patients displayed nonhematopoietic features seen in AD-HIES patients, such as facial dysmorphism (P6 and P7), joint hyperextensibility (P6 and P8), recurrent bone fractures (P7), jaw and tooth abnormalities (P3 and P5), and a high palate (P4, P5, P6, and P7). We therefore analyzed the impact of ZNF341 deficiency in primary fibroblast cell lines. We evaluated STAT3 mRNA levels by RT-qPCR and protein levels by flow cytometry and Western blotting. We found that STAT3 levels were about 50% lower in the patients’ fibroblasts ($n = 3$) than in fibroblasts from healthy controls (Fig. 5, A and B, and fig. S5A). We then assessed the impact of the STAT3-activating cytokine IL-6. After 30 min of costimulation with IL-6 and soluble IL-6Rα (hereafter referred to as IL-6/IL-6Rα), STAT3 phosphorylation was ~60% lower in ZNF341-deficient fibroblasts than in WT fibroblasts (Fig. 5C). Low levels of STAT3 phosphorylation were associated with weaker ($P = 0.06$) SOCS3 mRNA induction after 2 hours of stimulation (Fig. 5D). The activation of STAT3

Fig. 5. STAT3 production and function in primary cells. (A to D) STAT3 mRNA levels (A) and protein expression (B) in primary fibroblasts. (C) SOCS3 mRNA levels, as evaluated by RT-qPCR after RNA extraction from the primary fibroblasts of three controls and three patients (P2 to P4). (D) Data are displayed as $2^{-\Delta\Delta CT}$ after normalization relative to GUS (endogenous control) expression ($\Delta GUS$). Data representative of two independent experiments are shown. (E) STAT3 levels, as evaluated by flow cytometry, in primary fibroblasts. (Left) Representative image of STAT3 expression in fibroblasts from P3 and a healthy control, as well as the isotypic control. (Right) Recapitulative graph showing the mean fluorescence intensity (MFI) of STAT3, as measured by flow cytometry, in three controls and three patients (P2 and P4). Data representative of two independent experiments are shown. (F) Recapitulative graph of the MFI of pY705-STAT3, as evaluated by flow cytometry, in primary fibroblasts from three controls and three patients (P2 and P4), left unstimulated or after 30 min of stimulation with IL6/IL-6Rα, IL6/IL-6Rα, and P4, with or without 2 hours of stimulation with IL6/IL-6Rα. Data are displayed as $2^{-\Delta\Delta CT}$ after normalization relative to GUS (endogenous control) expression ($\Delta GUS$). Data representative of two independent experiments are shown. (G) Recapitulative graph of the MFI of pY705-STAT3, as evaluated by flow cytometry, in primary monocytes of three controls, P4, and P5, left unstimulated or after 30 min of stimulation with IL-10. Representative image for two patients tested. (H) SOCS3 mRNA levels, as evaluated by RT-qPCR after RNA extraction from the primary monocytes of three controls, P4, and P5. Data are displayed as $2^{-\Delta\Delta CT}$ after normalization relative to GUS (endogenous control) expression ($\Delta GUS$). (I) STAT3 expression, as measured by flow cytometry in primary monocytes. Recapitulative graph of the MFI of STAT3, as measured by flow cytometry, in three controls, P4, and P5. (J) Graph of the MFI of STAT3 measured in four controls and P4, as evaluated by flow cytometry, in primary CD56 dim NK cells from four controls and P4, left unstimulated or after 30 min of stimulation with IL-21. (K) Flow cytometry quantification of STAT3 levels in primary B cells. Recapitulative graph of the MFI of STAT3 measured in six controls, P4, P6, and P8, as evaluated by flow cytometry, in primary CD56 dim NK cells from one healthy individual, one STAT3-DN patient, and six ZNF341-deficient patients (P8). (L) Flow cytometry quantification of STAT3 levels in primary B cells. Recapitulative graph of the MFI of STAT3 measured in six controls, P4, and P6, as evaluated by flow cytometry, in nonstimulated cells of the control ($\Delta GUS$) and nonstimulated cells of the control ($\Delta GUS$). (M) Sorted naïve B cells from controls ($n = 9$), one STAT3-DN patient, and six ZNF341-deficient patients (P8). (N) Flow cytometry quantification of STAT3 levels in primary B cells. Recapitulative graph of the MFI of STAT3 measured in six controls, P4, P6, and P8, as evaluated by flow cytometry, in primary CD56 dim NK cells from one healthy individual, one STAT3-DN patient, and one ZNF341-deficient patient (P8) after 0, 4, 48, and 96 hours of stimulation with the indicated combinations of CD40L and IL-21. Data are displayed as $2^{-\Delta\Delta CT}$ after normalization relative to GUS (endogenous control) expression ($\Delta GUS$) and nonstimulated cells of the control ($\Delta GUS$). (Q) Sorted naive B cells from controls ($n = 14$), one STAT3-DN patient, and six ZNF341-deficient patients (P2 to P4, P6, P7, and P8) were cultured in the presence of CD40L (200 ng/ml), with or without IL-21 (50 ng/ml), for 7 days. The production (pg/ml) of IgM, IgG, and IgA was then assessed by Ig heavy chain–specific ELISA on cell culture supernatants. (R) Sorted memory B cells from controls ($n = 12$) and four ZNF341-deficient patients (P2 to P4, and P8) were cultured in the presence of CD40L (200 ng/ml), with or without IL-21 (50 ng/ml), for 7 days. The production (pg/ml) of IgM, IgG, and IgA was then assessed by Ig heavy chain–specific ELISA on cell culture supernatants.
by IL-6 in murine cells or the human HepG2 cell line up-regulates expression of the STAT3 gene itself (36, 37). After IL-6/IL-6R treatment, we observed a weak induction of STAT3 mRNA in patients’ cells relative to control cells (fig. S5B). These data indicate that ZNF341 deficiency results in low baseline levels of STAT3 mRNA and protein, together with impaired STAT3 phosphorylation and transcriptional activity, in primary fibroblasts and, by inference, in other nonhematopoietic cell types. These experiments provide a mechanism of action of ZNF341, and a mechanism of disease in the studied AR-HIES patients, by functionally connecting the ZNF341 deficit with STAT3, which is mutated in patients with AD-HIES. These data also explain the extrahematopoietic features observed in some patients. The somewhat rarer and milder nonhematopoietic features of ZNF341-deficient patients than of patients with DN STAT3 mutations may reflect a certain level of redundancy of ZNF341 in some cell types.

**STAT3 function is normal in ZNF341-deficient monocytes**

Another particular feature of AR ZNF341 deficiency, differentiating this condition from AD STAT3 deficiency, is the apparently stronger inflammatory response during infection. Because monocytes from healthy donors contain the ZNF341 protein (Fig. 2J), we analyzed the impact of ZNF341 deficiency in primary monocytes. We found low levels of STAT3 mRNA and protein in the monocytes of ZNF341-deficient patients, as shown by RT-qPCR and flow cytometry (Fig. 5, E and F). Moreover, STAT3 phosphorylation levels were ~50% lower in ZNF341-deficient monocytes after stimulation with IL-10, a STAT3-activating cytokine, than in control cells (Fig. 5G). Monocyte IL-10 signaling is impaired in STAT3-DN patients (6). We therefore measured SOCS3 mRNA induction in monocytes from five controls, P4, and P5 after 2 hours of IL-10 stimulation. Levels of SOCS3 mRNA induction were found to be normal in the patients (Fig. 5H). We then studied tumor necrosis factor (TNF) production by fresh monocytes from healthy controls (n = 9), STAT3-DN patients (n = 3), and P4 in response to LPS stimulation, alone or in the presence of IL-10 (Fig. 5I). As expected, IL-10 inhibited TNF production upon LPS activation in monocytes from controls but not in monocytes from the STAT3-DN patients. By contrast, the IL-10–mediated inhibition of TNF production in P4 monocytes upon LPS stimulation was reproducibly intact, suggesting that ZNF341 deficiency had no effect on STAT3-dependent responses to IL-10, and, by inference, to other cytokines, in these cells, as shown for IL-6 (fig. S5C). These findings indicate that low baseline levels of STAT3 do not necessarily lead to low levels of STAT3 activity in ZNF341-deficient cells, although monocytes and fibroblasts differ in this respect. This experiment also provided an explanation for the stronger inflammation observed in ZNF341-deficient patients than in STAT3-DN patients.

**STAT3 production and activation are impaired in ZNF341-deficient cytotoxic lymphocytes**

ZNF341-deficient patients have low NK cell counts and low frequencies of central memory CD8+ T cells. Both these cell types expressed ZNF341 in healthy controls (Fig. 2I). We analyzed the impact of ZNF341 deficiency in the remaining primary NK cells and in naïve CD8+ T cells, analyzing the expression and activation of STAT3 (Fig. 5, J to M, and fig. S5, D to F). We tested STAT3 expression by flow cytometry in CD56dim NK cells and in naïve CD8+ T cells and found that STAT3 expression was decreased in the patients by about 50% in both subsets, when compared with healthy controls (Fig. 5, J to L). After IL-21 stimulation, STAT3 phosphorylation was decreased by ~40 and 60% in ZNF341-deficient CD56dim NK cells and naïve CD8+ T cells, respectively, relative to controls (Fig. 5, K and M). Normal NK cell numbers in STAT3-DN patients suggests that low STAT3 expression and function in ZNF341-deficient NK cells is unlikely to explain their NK cell lymphopenia. In contrast, the similarly low frequency of central memory CD8+ T cells in ZNF341-deficient patients and patients with DN STAT3 mutations suggests that low STAT3 expression and function is responsible for impaired central memory CD8+ T cell differentiation in ZNF341-deficient patients. Collectively, these findings showed that the impact of ZNF341 deficiency, in terms of STAT3 baseline expression and inducible activation, extended beyond fibroblasts and monocytes to cytotoxic NK and CD8+ T cells. However, we did not test STAT3 activity in these two cell types.

**STAT3 activity is impaired in ZNF341-deficient B cells**

We next analyzed the impact of ZNF341 deficiency in primary naïve and memory B lymphocytes. Compared with controls, STAT3 expression was decreased in patients’ primary naïve B cell subsets by about 50%, as determined by flow cytometry (Fig. 5N and fig. S5G). After IL-21 stimulation, STAT3 phosphorylation was decreased by ~50% in naïve B cells from ZNF341-deficient patients, when compared with controls (Fig. 5O and fig. S5H). We assessed STAT3 induction in naïve B cells from controls, a STAT3-DN patient, and a ZNF341-deficient patient (P8), upon stimulation with CD40L with or without IL-21 (Fig. 5P). Stimulation for 4 hours with CD40L alone induced a transient tripling of STAT3 mRNA levels in controls and patients. Incubation with CD40L plus IL-21 induced a ~10-fold increase in STAT3 mRNA levels in naïve B cells from controls after 4 hours of stimulation, demonstrating synergy between the CD40L and IL-21 pathways. In contrast, naïve B cells from a ZNF341-deficient patient displayed no such induction after stimulation with CD40L and IL-21. Normal induction was detected with STAT3-DN naïve B cells, suggesting that the residual STAT3 activity in these cells was sufficient to induce STAT3 mRNA up-regulation upon costimulation with CD40L and IL-21. Because ZNF341-deficient patients have selectively high serum IgG and IgE levels and high levels of allergen-specific IgE (fig. S5I), together with memory B cell deficiency, we investigated the ability of their B cells to secrete Ig in various culture conditions requiring STAT3 activation. We sorted naïve and memory B cells from controls and patients and cultured them for 7 days in the presence of CD40L, alone or together with IL-21 (Fig. 5, Q and R). The induction of IgM, IgG, and IgA secretion by naïve B cells from the patients in response to stimulation with CD40L and IL-21 was severely impaired (<0.1 to 10% of normal levels; Fig. 5Q). By contrast, the levels of IgM and IgA secretion by ZNF341-deficient memory B cells were 10 to 30% those in controls, whereas the secretion of IgG was unaffected (Fig. 5R). Naïve and memory B cells from the patients produced normal levels of IgM in response to CD40L/CpG/BCR engagement (used as a control; fig. S5J). These data closely resemble published findings for patients with AD-HIES due to DN STAT3 mutations (5, 18). It strongly suggests that ZNF341 deficiency in circulating naïve B cells affects their function by preventing these cells from responding to IL-21 via STAT3. These findings may explain the B cell phenotype observed in both ZNF341- and STAT3-mutated patients with HIES. They also suggest that impairment of the autoinduction of STAT3 (i.e., the induction of STAT3 mRNA and protein by phosphorylated STAT3 dimers) is a critical mechanism underlying the pathogenesis of ZNF341 deficiency.
STAT3 autoinduction is impaired in ZNF341-deficient naïve CD4+ T cells

We then assessed the impact of ZNF341 deficiency in primary CD4+ T lymphocytes. Steady-state endogenous levels of STAT3 mRNA and protein were about 50% lower in the patients’ (P2, P3, and P4) naïve CD4+ T cells than in those of healthy controls (Fig. 6, A and B). These low levels of STAT3 were associated with lower levels of IL-6/IL-6Rα–induced STAT3 phosphorylation (~65%; Fig. 6C). The autoinduction of Stat3 in mice is observed in T cells, in which it enhances cell proliferation and survival upon CD3 and IL-6 costimulation (37). Furthermore, impaired autoinduction of Stat3 was reported in mouse protein kinase C-θ (PKC-θ)–deficient naïve CD4+ T cells upon T cell receptor (TCR) stimulation with TGF-β1–transforming growth factor–β (TGF-β), IL-1β, IL-6, IL-21, and IL-23; Fig. 6D and fig. S6A). Stimulation for 2 or 4 hours with IL-6/IL-6Rα, anti-CD2/CD3/CD28 mAb-coated beads, or TGF-β1–polarizing cytokines alone induced a transient twoto sixfold increase in STAT3 mRNA levels, peaking after 2 hours of stimulation in controls (Fig. 6D and fig. S6A). Incubation with anti-CD2/CD3/CD28 mAb-coated beads plus IL-6/IL-6Rα or TGF-β1–polarizing cytokines induced a 10- to 20-fold increase in STAT3 mRNA levels in naïve CD4+ T cells from controls after 2 hours of stimulation, demonstrating a potent synergy between the CD2/CD3/CD28 and IL-6R pathways (Fig. 6D and fig. S6A). By contrast to the four STAT3-activating cytokines tested—IL-6 and IL-21 (STAT3) and IFN-α and IFN-γ (STAT1 and STAT3)—the IL-2, IL-4, and IL-12 cytokines, which activate STAT5, STAT16, and STAT4, respectively, induced no such synergy (fig. S6B). Naïve CD4+ T cells from ZNF341-deficient patients displayed no such synergy after stimulation with anti-CD2/CD3/CD28 mAb-coated beads plus IL-6/IL-6Rα or TGF-β1–polarizing cytokines (Fig. 6D and fig. S6A). Normal synergy was detected with STAT3-DN naïve CD4+ T cells, suggesting that the residual STAT3 activity in these cells was sufficient to induce STAT3 mRNA up-regulation upon costimulation (Fig. 6D and fig. S6A). We compared the transcriptomes of naïve CD4+ T cells from four controls and two patients (P4 and P7) on

Fig. 6. Impaired STAT3 production and function in naïve CD4+ cells from ZNF341-deficient patients. (A) STAT3 mRNA levels, as evaluated by RT-qPCR after RNA extraction from the naïve CD4+ T cells of five controls, P4, P5, and P7. Data are displayed as 2−ΔΔCt after normalization relative to GUS (endogenous control) expression (ΔCt). (B) STAT3 protein levels, as evaluated by flow cytometry, in naïve CD4+ T cells. (Left) Representative histogram of STAT3 levels in P4, one healthy control, and the isotypic control. (Right) Recapitulative graph of the MFI of STAT3, as evaluated by flow cytometry, in naïve primary CD4+ T cells from five controls, P2, P3, and P4, left unstimulated or after 30, 120, and 240 min of stimulation with IL-6/IL-6Rα. (C) Flow cytometry quantification of the phosphorylation of STAT3 (pY705) in naïve CD4+ T cells after stimulation with IL-6/IL-6Rα. (Left) Representative flow cytometry histogram for STAT3 (pY705) in the naïve CD4+ T cells of P4 and in those of one healthy control, left unstimulated or after 30 min of stimulation with IL-6/IL-6Rα. (Right) Recapitulative graph of the MFI of STAT3 (pY705) for five controls, P2, P3, and P4 after 0, 30, 120, and 240 min of IL-6/IL-6Rα stimulation. Dots and error bars show the means and SDs, respectively. (D) Levels of STAT3 mRNA in thawed naïve CD4+ T cells from healthy individuals (n = 5), two STAT3 DN (S3DN) patients, and three ZNF341-deficient patients (P4, P5, and P7) after 2 hours of stimulation with the indicated combinations of IL-6/IL-6Rα and beads (anti-CD2/CD3/CD28 mAb-coated beads). Data are displayed as 2−ΔΔCt after normalization relative to GUS (endogenous control) expression (ΔCt). (E) Western blot of cytoplasmic and nuclear protein extracts of naïve CD4+ T cells from healthy individuals (n = 5), two STAT3 DN patients (S3DN), and two (P4 and P7) or three (P4, P5, and P7) ZNF341-deficient patients after 2 hours of stimulation with the indicated combinations of IL-6/IL-6Rα and beads (anti-CD2/CD3/CD28 mAb-coated beads). Data are displayed as 2−ΔΔCt after normalization relative to GUS (endogenous control) expression (ΔCt). Bar graphs and error bars represent the mean and SD.

microarrays after 2 hours of costimulation with anti-CD2/CD3/CD28 mAb-coated beads and IL-6/IL-6Ra. Relevant cytokine receptor mRNAs (i.e., IL2RG, IL6R, IL6ST, IL21R, and IL12R) were present at normal levels in the patients’ cells, suggesting that the impairment of STAT3 induction was not due to impaired cytokine receptor expression. Only 3 and 65 transcripts showed a fold change in expression (mean mRNA level of patients/mean mRNA level of controls) of less than 0.4 or more than 2.5, respectively (table S6). Of the three genes underexpressed in patients, STAT3 mRNA induction in the patients’ cells was the largest decrease in expression (FC = 0.34), demonstrating that the lack of ZNF341 resulted primarily in a decrease in STAT3 mRNA induction in the patients’ cells. Conversely, of the 65 genes less strongly expressed in controls than in patients after costimulation, the largest difference in mRNA levels was observed for IFNG (FC = 11.6). This finding is consistent with the higher levels of IFN-γ expression observed in naïve CD4 T cells from STAT3-DN patients than in control cells, after stimulation with anti-CD2/CD3/CD28 mAb-coated beads and Th1-polarizing cytokines (22). We further investigated STAT3 autoregulation in naïve CD4 T cells by assessing the impact of 4 hours of stimulation with anti-CD2/CD3/CD28 mAb-coated beads, IL-6, or both on STAT3 protein production, phosphorylation, and localization (Fig. 6E). STAT3 and phosphorylated STAT3 levels were higher in the nucleus of control naïve CD4 T cells after 4 hours of costimulation than after stimulation with IL-6/IL-6Ra or anti-CD2/CD3/CD28 mAb-coated beads alone. This increase was affected by cycloheximide treatment, suggesting that STAT3 mRNA autoinduction is associated with the de novo synthesis of STAT3 protein (Fig. 6E). Moreover, costimulation with anti-CD2/CD3/CD28 mAb-coated beads and IL-6/IL-6Ra resulted in long-lasting SOCS3 mRNA induction in naïve control CD4 T cells (Fig. 6F). Accordingly, the induction of SOCS3 mRNA was impaired in ZNF341-deficient patients after 2 and 4 hours of costimulation (Fig. 6G and fig. S6C). Last, we found that basal ZNF341 mRNA levels were normal in STAT3-DN naïve CD4 T cells but were high in ZNF341-deficient cells (Fig. 6H), further suggesting that ZNF341 autoregulates its own expression (Fig. 2D). Consistent with this finding, we detected a strong ZNF341-binding site in the first intron of ZNF341 by ChIP-seq (Fig. 4B). Two hours of IL-6/IL-6Ra stimulation in ZNF341-deficient naïve CD4 T cells restored normal ZNF341 mRNA levels, suggesting that STAT3 may also regulate ZNF341 expression (Fig. 6H). Overall, these data show that ZNF341 is required for baseline STAT3 production, autoinduction, activation, and sustained activity in primary human T cells.

**T**h17 development is impaired in ZNF341-deficient patients

We investigated the mechanism underlying CMC in ZNF341-deficient patients by analyzing the development of IL-17+ CD4 T cells. Inborn errors of IL-17 immunity underlie all known forms of isolated or syndromic CMC (21, 39–45), including HIES due to DN STAT3 mutations (14, 20, 46, 47). The proportions of Th1 subsets among the circulating memory CD4 T cells of ZNF341-deficient patients were

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**Fig. 7.** Impaired Th17 differentiation in ZNF341-deficient patients. (A) Frequency of Th1 subsets within the CD4 memory compartments of controls (n = 49) and patients (n = 5). Subsets were defined as follows: Th1 (CXCR5+, TCF71 (CXCR5-CXCR3-CCR4-CCR6-), Th2 (CXCR5-CXCR3-CCR4+CXCR6+), Th17 (CXCR5-CXCR3+CXCR4+CXCR6+), and Th22 (CXCR5-CXCR3+CXCR4-CXCR6+). Mann-Whitney tests were used for comparisons. (B) Secretion (pg/ml) of IFN-γ and TNF, IL-5, IL-13, and IL-17 cytokines by memory CD4 T cells after 5 days of culture under Th0 conditions (anti-CD2/CD3/CD28 mAb-coated beads). Mann-Whitney tests were used for comparisons. (C) Secretion (pg/ml) of IL-17 (IL-17A and IL-17F) cytokines by naïve CD4 T cells after 5 days of culture under Th0 conditions (anti-CD2/CD3/CD28 mAb-coated beads) and Th17-polarizing conditions (anti-CD2/CD3/CD28 mAb-coated beads together with IL-1, IL-6, IL-21, IL-23, and TGF-β). Mann-Whitney tests were used for comparisons. (D) Expression of RORC, TBX21, and GATA3 by naïve CD4 T cells after 5 days of culture under Th0, Th1, Th17-polarizing conditions (anti-CD2/CD3/CD28 mAb-coated beads together with IL-1, IL-6, IL-21, IL-23, and TGF-β). Mann-Whitney tests were used for comparisons. (E) Expression of RORC, TBX21, and GATA3 by naïve CD4 T cells after 5 days of culture under Th0, Th1, Th17-polarizing conditions (anti-CD2/CD3/CD28 mAb-coated beads together with IL-1, IL-6, IL-21, IL-23, and TGF-β). Mann-Whitney tests were used for comparisons. (F) Secretion (pg/ml) of Th17 (IL-17A and IL-17F) cytokines by naïve CD4 T cells after 5 days of culture under Th0-polarizing conditions (anti-CD2/CD3/CD28 mAb-coated beads) and Th17-polarizing conditions (anti-CD2/CD3/CD28 mAb-coated beads together with IL-1, IL-6, IL-21, IL-23, and TGF-β). Mann-Whitney tests were used for comparisons.

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normal for T_{H}1 cells, high for T_{H}2 cells, and low for T_{H}17, T_{H}1*, and T_{H}17 cells, as shown by the expression of specific surface markers (Fig. 7A), as in STAT3 DN patients (48, 49). We stimulated memory CD4^+ T cells with anti-CD2/CD3/CD28 mAb-coated beads (T_{T}_{H}0) for 5 days and measured cytokine production by enzyme-linked immunosorbent assay (ELISA), cytometric bead array, and intracellular staining methods (Fig. 7B and fig. S7, A to C). The production of T_{H}1 cytokines (TNF and IFN-γ) was low to normal (Fig. 7B and fig. S7A) and that of IL-6, IL-10, and IL-21 was normal (fig. S7, B and C), whereas that of T_{H}2 cytokines (IL-4, IL-5, and IL-13) was normal or higher than normal (Fig. 7B and fig. S7A). In marked contrast, the production of T_{H}17 cytokines (IL-17A, IL-17F, and IL-22) by ZNF341-deficient memory CD4^+ T cells was much weaker than that by control cells (Fig. 7B and fig. S7A). We then purified naive CD4^+ T cells from the patients and cultured them in vitro under T_{H}1- or T_{H}17-polarizing conditions. T_{H}1 polarization induced normal and high levels of TNF and IFN-γ, respectively, in the patients’ cells (fig. S7D), consistent with the microarray data (table S6) and similar to the results obtained from STAT3-DN patients (22). By contrast, T_{H}17 polarization did not lead to the production of detectable amounts of IL-17A and IL-17F (Fig. 7C). Accordingly, RORC expression tended to be weaker, although this difference was not statistically significant, in memory CD4^+ T cells and naive CD4^+ T cells from patients cultured for 5 days under T_{H}17 conditions in vitro (Fig. 7D). These data are consistent with the ChIP-seq data for T_{H} cells, which show no ZNF341-binding site was detected in the RORC gene. By contrast, the expression of TBX21 and GATA3 was normal or slightly higher than normal in memory CD4^+ T cells and naive CD4^+ T cells from patients cultured under T_{H}1- and T_{H}17-polarizing conditions, respectively (Fig. 7D). Mutations of ZNF341 may have had a global effect on T cell responses, impairing not only responses elicited through cytokine receptors but also those elicited through the TCR.

We tested this hypothesis by measuring the calcium flux in naive CD4^+ T cells induced by TCR engagement (fig. S7E). The kinetics and magnitude of the TCR-mediated activation of naive CD4^+ T cells from two ZNF341-deficient patients were similar to those of corresponding cells from healthy donors, suggesting that TCR signaling was intact in the absence of ZNF341. Furthermore, ZNF341-deficient naive and memory CD4^+ T cells underwent rounds of normal proliferation in vitro in response to T_{T}_{H}0 or polarizing conditions (fig. S7, F and G). The IL-17 phenotype observed in ZNF341-deficient patients was not, therefore, due to impaired cell division and TCR-induced calcium signaling.

**Patients’ T_{H}1 cells show an abnormal transcriptome profile**

The patients’ CD4^+ T cells were further studied globally by gene expression analysis (fig. S7H). Consistent with the skewing toward T_{H}2 cells, ZNF341-deficient memory CD4^+ T cells had higher levels of mRNAs encoding not only GATA3, IL-4, IL-5, and IL-13 but also IL-5R, IL-31 (produced by T_{H}12 cells), and IL-18BP (inhibiting IL-18-induced IFN-γ production), thereby relieving the inhibitory effect of IFN-γ on T_{H}2 responses). ZNF341-deficient memory CD4^+ T cells continued to produce large amounts of GATA3, IL4, IL13, and IL31 under T_{H}17-polarizing conditions. Moreover, ZNF341-deficient memory CD4^+ T cells had lower basal levels of IL17A, IL17F, IL22, and CCR6 expression under T_{T}_{H}0 conditions than control memory CD4^+ T cells, and the expression of IL17A, IL17F, and CCR6 was not increased by culturing ZNF341-deficient memory CD4^+ T cells under T_{H}17 conditions (fig. S7H). Impaired expression was also observed for other genes typically associated with T_{H}17 cells, including RORA (encoding ROR-α); IL26 and CCL20 (both expressed by human T_{H}17 cells); and IL23A, IL1A, IL1R1, IL1R2, IL6R, and IL23R (which encode proteins through which IL-1β, IL-6, and IL-23 signal to induce T_{H}17 cells). The increase in RORC, CCL20, IL1A, IL1R1, IL1R02, and IL23A expression observed in normal memory CD4^+ T cells after culture in T_{H}17-polarizing conditions was abolished by ZNF341 mutations, highlighting the key role of this transcription factor in T_{H}17 differentiation. Consistently, total RNA sequencing in isolated CD3^+ T cells from P4 and a control after 24 and 48 hours of stimulation with anti-CD3 and anti-CD28 mAbs revealed that 6 of the 10 genes for which the most significant decreases in expression were recorded in patients were key markers of IL-17 immunity (IL17F, IL26, RORC, and CCR6; table S7). In summary, ZNF341-deficient CD4^+ T cells can differentiate in vivo and in vitro into some types of effectors cells, but their ability to generate T_{H}17 cells is selectively and severely impaired.

As in patients with DN STAT3 mutations, this defect accounts for the CMC observed in the patients, as fibroblasts and keratinocytes from P2 to P4 responded normally to IL-17A (fig. S7, I to K) (21, 43–45). The lack of ZNF341 prevents T_{H}1 cells from producing sufficient amounts of functional STAT3, and thereby of ROR-γ/ROR-γT, during T_{H}17 development (14, 41).

**Allergy and hyper-IgE are associated with enhanced T_{H}2 responses**

Unlike the CMC observed in patients and our findings for IL-17, the pathogenesis of severe allergy and hyper-IgE in these patients is more difficult to decipher, because monogenic causes of isolated allergy and hyper-IgE affecting specific cytokine signaling pathways are only just beginning to emerge (50, 51). However, the microarray analysis of CD4^+ T cells was instructive, revealing aberrant expression of potential mediators of inflammation in ZNF341-deficient patients (fig. S7H). We detected high levels of expression of the inflammatory chemokine genes CCL1, CCL3, and CCL5 and the inflammatory chemokine receptor gene CXCR3, which is coexpressed by T_{H}1 cells. This finding is consistent with the detection of high levels of expression for IFNG and EOMES, encoding a transcription factor required for the generation of pathogenic proinflammatory IFN-γ-producing CD4^+ T cells. The persistently higher levels of expression of many of these inflammatory or T_{H}2-type genes in ZNF341-deficient than in control memory CD4^+ T cells under conditions of T_{H}17 polarization also highlight the role of ZNF341 in repressing the expression of gene signatures characteristic of alternative fates of effector CD4^+ T cells. The patients thus displayed an enhanced T_{H}2 bias in multiple assays (Fig. 7B and fig. S7A), with a higher frequency of T_{H}2 cells and higher levels of GATA3 mRNA in memory CD4^+ T cells (Fig. 7, A and D). Last, we tested the hypothesis that the patients’ CD8^+ T cells also contributed to their allergic phenotype (52, 53). Genome-wide transcriptome analysis showed that up to 12 mRNAs were up-regulated in the patients’ CD8^+ HVS-T cells, to levels at least 2.5 times those in CD8^+ HVS-T cells from healthy controls (fig. S7L). Two of these genes encoded cytokines known to play a key role in the allergy process: IL-5, which drives the development of eosinophils (54), and IL-9, which drives the development of basophils (55). These data suggest that the allergic features of ZNF341-deficient patients were due to the enhanced production of some, but not all, T_{H}2-like cytokines, including IL-5 and IL-9, in particular, by both CD4^+ and CD8^+ T cells. These T cell abnormalities are similar to those seen in HIES patients with DN mutations of STAT3 (22, 49).
DISCUSSION

We report ZNF341 deficiency in eight patients from six unrelated kindreds displaying an AR form of HIES, with stronger inflammatory responses and fewer nonhematopoietic manifestations than reported for AD-HIES caused by DN STAT3 mutations. This experiment of nature indicates that human ZNF341 is required for protective mucocutaneous immunity to Candida albicans. The impaired development of IL-17+ CD4+ T cells accounts for the development of CMC in these patients (7, 14, 20, 39–42, 46, 47). The cellular mechanisms by which ZNF341 regulates allergy are less clear, although several Th2 cytokines (e.g., IL-5 and IL-9) are produced in excess by the patients’ T cells. The molecular mechanism by which ZNF341 operates in T cells involves STAT3. No haploinsufficiency has been observed at the human STAT3 locus; the HIES-causing mutations studied to date are all DN STAT3. Consequently, STAT3-dependent genes, such as RORC, are poorly induced. Consistent with these findings, the overexpression of a hyperactive form of STAT3 in naïve mouse CD4+ T cells involves STAT3. The mechanism by which ZNF341 deficiency is pathogenic in T cells is based on a 50% decrease in constitutive levels of STAT3 and a >60% decrease in levels of STAT3 phosphorylation upon stimulation with STAT3-activating cytokines. ZNF341 deficiency prevents the induction of STAT3 transcription by cytokines that activate STAT3 itself (autoinduction). Together, low baseline STAT3 mRNA and protein levels, and the impaired autoinduction of STAT3 itself, result in lower levels of STAT3 activation (phosphorylation) and transcriptional activity, as low as those in patients with DN STAT3 mutations. Consequently, STAT3-dependent genes, such as RORC, IL17A, and IL17F, are poorly induced. Consistent with these findings, the overexpression of a hyperactive form of STAT3 in naive mouse CD4+ T cells and STAT3 gain-of-function mutations in humans were shown to increase Th17 cell differentiation (58, 59). The same mechanism probably operates in B cells, accounting for the B cell phenotypes of ZNF341-deficient patients being identical to those of HIES patients with DN STAT3 mutations (5, 18). Given that patients with the various forms of inherited STAT1 deficiency suffer from mycobacterial and/or viral diseases (60) and that individuals heterozygous for loss-of-function STAT1 alleles that are not DN are asymptomatic, the lower levels of STAT1 observed in ZNF341-deficient patients are unlikely to account for or even contribute to their immunological or clinical phenotypes.

Patients with ZNF341 deficiency appear to develop fewer and milder nonhematopoietic developmental phenotypes than those with STAT3 DN mutations. A possible explanation for this is that IL-11R, LIFR, IL-6ST, and similar receptors in cells responsible for the tooth, skeletal, joint, and vascular phenotypes of AD-HIES patients may not necessarily require as much STAT3 to function properly as the cytokine receptors tested in primary fibroblasts (10–12). However, these patients do develop such somatic features, consistent with the STAT3 phenotype detected in the fibroblasts of ZNF341-deficient patients. One of the more distinctive features of ZNF341 deficiency is the somewhat stronger inflammatory response in the course of infection. We detected ZNF341 in all leukocytes tested, including basophils, monocytes, and dendritic cells. However, the expression of this protein in monocytes seems to be functionally redundant for STAT3 activation, in contrast to the situation in STAT3-DN patients, possibly explaining the normal biological and clinical inflammation seen in ZNF341-deficient patients. Conversely, the NK lymphopenia seen in ZNF341-deficient but not STAT3-DN patients suggests that ZNF341 may be essential for at least one STAT3-independent function. However, an alternative interpretation is that STAT3 activation is more profoundly impaired in NK progenitors lacking ZNF341 than in those carrying a DN STAT3 mutation. Further studies will be required to compare the expression patterns of ZNF341 and STAT3 proteins across human tissues and cell types. Studies of Zfp341, the mouse ortholog of human ZNF341, may also facilitate analysis of the STAT3-dependent and STAT3-independent roles of this transcription factor in different cell types.

The identification of ZNF341 highlights the power of human genetics for discovering previously unknown biochemical and immunological pathways of biological and medical relevance (61–63) and defining the level of redundancy of human genes for host defense in natural conditions (64). Much is already known about the structure and function of STAT proteins, but this study reveals the importance of the transcriptional regulation of STAT genes (65–68). Previous studies had hinted at the importance of STAT transcriptional regulation (36–38). Unlike STAT3, which resides in the cytosol and only accumulates in the nucleus upon activation, ZNF341 is constitutively expressed in the nucleus, where it is required for optimal baseline and inducible STAT3 transcription, and particularly for autoinduction, the lack of which prevents the production of sufficient amounts of STAT3 protein and the sustained activity of this protein during cell stimulation. Further studies are required to identify potential partners of ZNF341 during STAT3 autoinduction. In T and B cells, we have further shown that ZNF341 protein deficiency is associated with higher levels of ZNF341 mRNA. Both ZNF341 and STAT3 are potent inducers of STAT3 transcription, and they may act in concert to decrease ZNF341 transcription. This would prevent excessive STAT3-dependent responses that would be deleterious to the host, as observed in patients with heterozygous gain-of-function mutations of STAT3 (69–71).

MATERIALS AND METHODS

Study design

We studied eight patients from five kindreds suffering from HIES. Using WES, we found that all living patients carry homozygous truncating mutation in the ZNF341 gene. No predicted homozygous loss-of-function mutation of ZNF341 has been described in public databases. ZNF341 function is unknown in mouse and human. The main objective of this study was to characterize in-depth ZNF341 expression and function in human, and the molecular, cellular, and immunological impacts of its disruption in human.

Case reports

Detailed case reports of all eight patients can be found in the Supplementary Materials.

Experimental methods

Information about reagents used and experimental procedures can be found in the Supplementary Materials.

Statistics

Two-tailed Mann-Whitney tests or unpaired t tests were used for single comparisons of independent groups. Paired t tests were used for single comparisons of paired groups. In the relevant figures, “ns” indicates “not significant.” ***P < 0.001, **P < 0.01, and *P < 0.05. Analyses were performed with GraphPad software.

SUPPLEMENTARY MATERIALS

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MATERIALS AND METHODS

Fig. S1. Clinical features, histology, and genetics.


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A recessive form of hyper-IgE syndrome by disruption of ZNF341-dependent STAT3 transcription and activity


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Fingers on the trigger

Hyper–immunoglobulin E syndromes (HIESs) are rare genetic immunodeficiency diseases characterized by bacterial infections, chronic mucocutaneous candidiasis, allergies, and skeletal abnormalities that are associated with excessive Th17 responses and impaired TH17 immunity. Béziat et al. and Frey-Jakobs et al. have studied patients with an autosomal recessive form of HIES and identified mutations in the zinc finger transcription factor ZNF341 as the culprit. Loss-of-function mutations encoding truncated forms of ZNF341 interfered with its ability to recognize a bipartite binding site located in the promoter of STAT3, the transcription factor mutated in most cases of autosomal dominant HIES. ZNF341-supported transcription of STAT3 is a key upstream regulatory step needed to trigger the normal induction of the TH17 differentiation pathway. These findings reveal a previously unappreciated layer of transcriptional regulation controlling JAK-STAT signaling.