Gain-of-function human \textit{STAT1} mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis


Abbreviations used: AD, autosomal dominant; AR, autosomal recessive; CMC, chronic mucocutaneous candidiasis; CMCD, CMC disease; EMSA, electrophoretic mobility shift assay; GAS, γ-activated sequence; ISKE, IFN-stimulated response element; MSMD, Mendelian susceptibility to mycobacterial disease; WB, Western blotting.

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Chronic mucocutaneous candidiasis disease (CMCD) may be caused by autosomal dominant (AD) IL-17F deficiency or autosomal recessive (AR) IL-17RA deficiency. Here, using whole-exome sequencing, we identified heterozygous germline mutations in STAT1 in 47 patients from 20 kindreds with AD CMCD. Previously described heterozygous STAT1 mutant alleles are loss-of-function and cause AD predisposition to mycobacterial disease caused by impaired STAT1-dependent cellular responses to IFN-γ. Other loss-of-function STAT1 alleles cause AR predisposition to intracellular bacterial and viral diseases, caused by impaired STAT1–dependent responses to IFN-α/β, IFN-γ, IFN-λ, and IL–27. In contrast, the 12 AD CMCD–inducing STAT1 mutant alleles described here are gain–of–function and increase STAT1–dependent cellular responses to these cytokines, and to cytokines that predominantly activate STAT3, such as IL–6 and IL–21. All of these mutations affect the coiled–coil domain and impair the nuclear dephosphorylation of activated STAT1, accounting for their gain–of–function and dominance. Stronger cellular responses to the STAT1–dependent IL–17 inducers IL–6 and IL–21, hinder the development of T cells producing IL–17A, IL–17F, and IL–22. Gain–of–function STAT1 alleles therefore cause AD CMCD by impairing IL–17 immunity.

Chronic mucocutaneous candidiasis (CMC) is characterized by persistent or recurrent disease of the nails, skin, oral, or genital mucosa caused by Candida albicans (Puel et al., 2010b). CMC may be caused by various inborn errors of immunity. CMC is one of a multitude of infectious diseases observed in patients with broad and profound T cell deficiencies. In contrast, patients with the autosomal dominant (AD) hyper IgE syndrome, caused by dominant–negative mutations of STAT3, are susceptible principally to CMC and staphylococcal diseases of the lungs and skin (Minegishi, 2009). These patients have very low proportions of circulating IL–17A– and IL–22–producing T cells, probably because of impaired responses to IL–6, IL–21, and/or IL–23 (de Beaucoudrey et al., 2008; Ma et al., 2008; Milner et al., 2008; Renner et al., 2008; Minegishi et al., 2009). Patients with autosomal recessive (AR) IL–12p40 or IL–12Rβ1 deficiency suffer from Mendelian susceptibility to mycobacterial disease (MSMD) and occasionally develop mild CMC (Filipe–Santos et al., 2006; de Beaucoudrey et al., 2010). Some have low proportions of IL–17A– and IL–22–producing T cells, presumably because of the abolition of IL–23 responses (de Beaucoudrey et al., 2008, 2010). The proportion of IL–17A–producing T cells was also found to be low in a family with AR CARD9 deficiency, dermatophytosis, invasive candidiasis, and CMC (Glocke et al., 2009). Finally, CMC is the only infection in patients with autoimmune polyendocrinopathy syndrome type 1, who have high titers of neutralizing autoantibodies against IL–17A, IL–17F, and IL–22 (Kisand et al., 2010; Puel et al., 2010a). Thus, regardless of the underlying illness, CMC pathogenesis apparently involves the impairment of IL–17A, IL–17F, and IL–22 immunity (Puel et al., 2010b).

The pathogenesis of CMC was eventually deciphered through investigations of patients with CMC disease (CMCD), in which CMC is isolated, with no other infectious or autoimmune signs (Kirkpatrick, 2001; Puel et al., 2010b). The definition of CMCD is not absolute, as illustrated in some patients by cutaneous staphylococcal disease, which is milder than that in patients with AD hyper IgE syndrome (Herrod, 1990), or by autoimmune features affecting the thyroid in particular, although fewer such features are observed than in patients with autoimmune polyendocrinopathy syndrome type 1 (Atkinson et al., 2001). It is unclear whether CMCD, with these or other manifestations (Shama and Kirkpatrick, 1980; Bentur et al., 1991; Germain et al., 1994), is immunologically and genetically related to pure CMCD. Low proportions of IL–17A–producing T cells have been documented in five patients with CMCD (Eyerich et al., 2008). Moreover, a candidate gene approach centered on IL–17 immunity recently revealed the first genetic etiologies of pure CMCD. In a consanguineous family from Morocco, a child with CMCD was found to display AR complete IL–17A deficiency (Puel et al., 2011). His leukocytes and fibroblasts did not respond to IL–17A or IL–17F homodimers, or to IL–17A/F heterodimers. Four patients from an Argentinean family were shown to harbor dominant–negative mutations in the IL17F gene (Puel et al., 2011). Mutated IL–17F–containing homodimers and heterodimers were produced in normal amounts but were not biologically active, as they were unable to bind to the IL–17 receptor. Morbid mutations in IL17RA and IL17F demonstrated that CMCD could be caused by inborn errors of IL–17 immunity. However, no genetic etiology has yet been identified for most patients with CMCD. We set out to identify new genetic etiologies of CMCD through a recently developed genome–wide approach based on whole–exome sequencing (Alcaïs et al., 2010; Bolze et al., 2010; Byun et al., 2010; Ng et al., 2010).

RESULTS

We investigated one sporadic case and the probands from five multiplex kindreds with AD CMCD, by whole–exome sequencing. The annotated data were analyzed with sequence analysis software that had been developed in–house and made it possible to analyze and compare several exome sequences simultaneously. A hierarchy of candidate variations was generated by filtering out known polymorphisms reported in dbSNP and 1,000–genome databases. We also used our own database of 250 exomes to filter out unreported polymorphisms (Table S1). The only relevant gene displaying heterozygous variations in at least four of the six unrelated patients with AD CMCD was STAT1 (Fig. 1, A and B, Kindreds A, B, G, and L; Table I; and Table S2). Three different STAT1 mutations were found in four patients; they were confirmed by Sanger
sequencing and shown to be missense mutations. All these mutations affected the coiled-coil domain, which plays a key role in unphosphorylated STAT1 dimerization and STAT1 nuclear dephosphorylation (Fig. 1, A and C; Chen et al., 1998; Levy and Darnell, 2002; Braunstein et al., 2003; Zhong et al., 2005; Hoshino et al., 2006; Mertens et al., 2006). We therefore sequenced the corresponding coding region of STAT1 (exons 6 to 10) in another 106 patients, including 57 with sporadic CMCD and 49 from 22 multiplex kindreds with AD CMCD. 29 patients from 16 kindreds were heterozygous for a STAT1 missense mutation (Fig. 1, A and B, Kindreds C-F, H-K, and M-T; Fig. 1 C; and Table I; Table S3). In total, 36 patients from 20 kindreds were heterozygous for 1 of the 12 missense mutations identified that affected the coiled-coil domain of STAT1. 11 other CMCD patients in these kindreds were therefore predicted to affect protein structure and function. Moreover, most of the affected residues were found to have been conserved throughout evolution in the species in which STAT1 had been sequenced (Table S3). Accordingly, POLYphen II predicted that all but one of these mutations would be possibly or probably damaging (Adzhubei et al., 2010; unpublished data). Finally, the mutations were found to have occurred de novo in at least four kindreds, which is consistent with a high clinical penetrance of these alleles. The mutations were not found in the National Center for Biotechnology Information, Ensembl, and dbSNP databases. They were also absent from 1,052 controls from 52 ethnic groups in the Centre d’Etude du Poly-morphisme Humain and Human Genome Diversity panels, suggesting that they were rare, CMCD-inducing variants rather than irrelevant polymorphisms.

The 12 missense mutations were not conservative and were therefore predicted to affect protein structure and function. Moreover, most of the affected residues were found to be phosphorylated in response to IFN-γ, STAT3 was not constitutively activated, and STAT3 phosphorylation levels were similar for the two alleles (Fig. 2 B). In contrast, IFN-γ, IFN-α, or IL-27, cells transfected with the R274Q allele responded two to three times more strongly than those transfected with the WT allele, as shown by measurement of the induction of γ-activated sequence (GAS)–dependent reporter gene transcription activity, with mock- and L706S-transfected cells serving as negative controls (Fig. 2 A and Fig. S1 A). All STAT1 alleles were expressed at an equal strength, as shown by Western blotting (WB; Fig. 2 B). Higher levels of STAT1 phosphorylation were observed for the R274Q allele than for the WT allele after stimulation with IFN-γ, IFN-α, and IL-27, whereas STAT3 phosphorylation levels were similar for the two alleles (Fig. 2 B). In contrast, the induction of IFN-stimulated response element (ISRE)–dependent transcription activity by IFN-α was normal (Fig. S1, B and C). In the same experimental conditions, the other 10 CMCD–associated STAT1 alleles tested were also gain-of-function, unlike the K201N and K211R alleles (Fig. S1 D). Upon stimulation with IFN-γ, IFN-α, or IL-27, an increase in GAS-binding activity was detected in cells transfected with the R274Q allele (Fig. S1 E). Accordingly, the transcription of the CXCL9 and CXCL10 target genes was enhanced (Fig. 2, C and D). Overall, these data indicate that at least 11 of the 12 CMCD-linked STAT1 missense alleles are intrinsically gain-of-function.

The mechanism involved an increase in STAT1 tyrosine 701 residue phosphorylation, as shown for R274Q by WB after stimulation with IFN-α, IFN-γ, and IL-27 (Fig. 2 B). STAT1 was not constitutively activated, and STAT3 was normally activated in R274Q–transfected cells (Fig. 2 B and not depicted). Almost all the mutant STAT1 molecules, which were phosphorylated in response to IFN-γ, translocated to and accumulated in the nucleus, as shown by immunofluorescence (Fig. S1 F). WB showed R274Q STAT1 to be more
Figure 1. Heterozygous missense mutations affecting the STAT1 coiled-coil domain in kindreds with AD CMCD. (A) The human STAT1 isoform is shown, with its known pathogenic mutations. Coding exons are numbered with roman numerals and delimited by a vertical bar. Regions corresponding to the coiled-coil domain (CC), DNA-binding domain (DNA-B), linker domain (L), SH2 domain (SH2), tail segment domain (TS), and transactivator domain (TA) are indicated, together with their amino-acid boundaries, and are delimited by bold lines. Tyr701 (pY) and Ser727 (pS) are indicated. Mutations in green are dominant and associated with partial STAT1 deficiency and MSMD. Mutations in brown are recessive and associated with complete STAT1 deficiency and intracellular bacterial and viral disease. Mutations in blue are recessive and associated with partial STAT1 deficiency and intracellular bacterial and/or viral disease. Mutations in red are dominant and associated with a gain-of-function of STAT1 and CMCD. (B) Pedigrees of 20 families with AD “gain-of-function” STAT1 mutations. Each kindred is designated by a letter (A to T), each generation is designated by a roman numeral (I-II-III-IV), and each individual is designated by an Arabic numeral (each individual studied is identified by a code of this type, organized from left to right). Black indicates CMCD patients. The probands are indicated by arrows. When tested, the genotype for STAT1 is indicated below each individual. (C) Three-dimensional structure of phosphorylated STAT1 in complex with DNA. Connolly surface representation, with the following amino acids highlighted: red, amino acids mutated in patients with CMCD; blue, amino acids located in the coiled-coil domain and mutated in patients with MSMD and viral diseases; yellow, amino acids identified in vitro as affecting the dephosphorylation process.
Table I. Summary of the clinical and genetic data for the patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at presentation</th>
<th>Origin</th>
<th>Clinical features of CMC</th>
<th>Cause of death (age/yr)</th>
<th>Autoimmunity</th>
<th>Genotype</th>
</tr>
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<tbody>
<tr>
<td>A-I-1</td>
<td>-</td>
<td>France</td>
<td>Nails</td>
<td>Not related to the disease (old age)</td>
<td>None</td>
<td>-</td>
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<tr>
<td>A-II-1</td>
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<td>France</td>
<td>Nails</td>
<td>Not related to the disease (old age)</td>
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<td>-</td>
</tr>
<tr>
<td>A-III-1</td>
<td>1 mo</td>
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<td>Nails, oral cavity, oropharynx</td>
<td>None</td>
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<td>B-II-1</td>
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<td>None</td>
<td>WT/K286I</td>
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<td>B-IV-2</td>
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<td>France &amp; Congo</td>
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<td>-</td>
<td>Turkey</td>
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<td>Thyroid autoimmunity</td>
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<td>Nails, oral cavity</td>
<td>None</td>
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<td>D-II-1</td>
<td>7 yr</td>
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<td>1 mo</td>
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<td>None</td>
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<td>Thyroid autoimmunity</td>
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<td>Thyroid autoimmunity</td>
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<td>9 mo</td>
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<td>Thyroid autoimmunity</td>
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<td>F-III-2</td>
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<td>WT/R274W</td>
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<td>Argentina</td>
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<td>-</td>
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<td>F-IV-3</td>
<td>6 mo</td>
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<td>-</td>
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<td>G-II-1</td>
<td>3 mo</td>
<td>Ukrainian</td>
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<td>H-I-2</td>
<td>1 yr</td>
<td>Japan</td>
<td>Skin, oropharynx, esophagus</td>
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<td>-</td>
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<td>H-II-2</td>
<td>5 yr</td>
<td>Japan</td>
<td>Oral cavity, oropharynx</td>
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<td>-</td>
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<tr>
<td>I-II-3</td>
<td>9 mo</td>
<td>Mexico</td>
<td>Skin, nails, oral cavity, genital mucosa</td>
<td>None</td>
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<td>J-I-2</td>
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<td>Oral cavity, oropharynx</td>
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<td>WT/T288A</td>
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<td>J-II-2</td>
<td>3 mo</td>
<td>Switzerland</td>
<td>Oral cavity, oropharynx</td>
<td>None</td>
<td>WT/T288A</td>
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<td>Thyroid autoimmunity</td>
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<td>France</td>
<td>Skin, nails, oropharynx, esophagus</td>
<td>Thyroid autoimmunity</td>
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<td>L-II-1</td>
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<td>France</td>
<td>Skin, nails, oropharynx, esophagus</td>
<td>Thyroid autoimmunity</td>
<td>WT/R274Q</td>
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<td>M-II-2</td>
<td>6 mo</td>
<td>Germany</td>
<td>Skin, nails, oropharynx, genital mucosa</td>
<td>Thyroid autoimmunity</td>
<td>WT/D165H</td>
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</table>
We investigated the dominance of the STAT1 alleles at the cellular level by testing EBV-B–transformed (EBV-B) cells and SV-40–transformed dermal fibroblasts from a CMCD patient heterozygous for the STAT1 R274Q allele. We observed enhanced IFN-α/β-, IFN-γ-, and IL-27–dependent STAT1 phosphorylation in EBV-B cells from a patient heterozygous for the STAT1 R274Q allele, as shown by WB (Fig. 3, B and D). Phospho–STAT1 accumulated in the nucleus of R274Q heterozygous SV-40 fibroblasts upon IFN-γ stimulation, as well as in EBV-B cells (Fig. 3 I and Fig. S3 D). Moreover, the IFN-α/β–, IFN-γ–, and IL-27–induced DNA-binding activity of GAF was stronger in cells from the CMCD patient than in those from a healthy control or from a MSMD patient carrying the L706S mutant allele, as shown by electrophoretic mobility shift assay (EMSA; Fig. 3, A and C). In contrast, the DNA-binding activity of ISGF-3 seemed to be normal in cells from the patient stimulated with IFN-α/β (Fig. S3 A). These data strongly suggest that the heterozygous R274Q allele is dominant for STAT1-dependent responses and gain-of-function for GAF-dependent cellular responses to key STAT1-activating cytokines, such as IFN-α/β, IFN-γ, and IL-27. The mutation may also affect IFN-λ responses.

We then tested cytokines that predominantly activate STAT3, rather than STAT1, such as IL-6, IL-21, IL-22, and IL-23 (Hunter, 2005; Kishimoto, 2005; Kastelijn et al., 2007; Spolski and Leonard, 2008; Donnelly et al., 2010; Sabat, 2010; Ouyang et al., 2011). Peripheral T cell blasts from a patient displayed normal STAT3 activation in response to IL-23, as shown by WB (Fig. S3 B). No increase in STAT1 phosphorylation was detected in cells from a patient or controls upon IL-23 stimulation. Furthermore, fibroblasts from a patient displayed normal activation of STAT3 in response to IL-22 (Fig. S3 C). In the same conditions, no STAT1 phosphorylation was detected in cells from the patient or controls (unpublished data). In contrast, the levels of STAT1 phosphorylation in response to IL-6 and IL-21 were higher in the patient's EBV-B cells than in cells from healthy controls and from a patient with MSMD heterozygous for the L706S allele, whereas STAT3 activation was normal as shown by WB (Fig. 3, F and H). Consistent with these findings, stronger GAS activity was observed in cells from the patient in response to IL-6 and IL-21 stimulation (Fig. 3, E and G). These data suggest that heterozygous missense mutations in the coiled-coil domain of STAT1 are dominant and gain-of-function for GAF-dependent cellular responses for cytokines that predominantly activate STAT3, such as IL-6 and IL-21. Overall, these data suggest that the STAT1 alleles are truly responsible for CMCD in these kindreds and raise questions about the immunological basis of CMCD.

Table I. Summary of the clinical and genetic data for the patients (Continued)

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<tr>
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<th>Clinical features of CMC</th>
<th>Cause of death (age/yr)</th>
<th>Autoimmunity</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-II-2</td>
<td>1 yr</td>
<td>Germany</td>
<td>Skin, nails, oropharynx</td>
<td>Squamous cell carcinoma (54)</td>
<td>None</td>
<td>WT/R274W</td>
</tr>
<tr>
<td>O-II-1</td>
<td>18 mo</td>
<td>Germany</td>
<td>Oral cavity, oropharynx</td>
<td>Not related to the disease (46)</td>
<td>None</td>
<td>WT/M202I</td>
</tr>
<tr>
<td>P-I-1</td>
<td>1 yr</td>
<td>Israel</td>
<td>Oropharynx, genital mucosa</td>
<td>Not related to the disease (46)</td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td>P-II-1</td>
<td>&lt;2 yr</td>
<td>Israel</td>
<td>Skin, nails, oropharynx</td>
<td>Not related to the disease (46)</td>
<td>None</td>
<td>WT/A267V</td>
</tr>
<tr>
<td>P-II-2</td>
<td>&lt;2 yr</td>
<td>Israel</td>
<td>Skin, nails, oropharynx</td>
<td>Not related to the disease (46)</td>
<td>None</td>
<td>WT/A267V</td>
</tr>
<tr>
<td>Q-II-1</td>
<td>1 mo</td>
<td>France</td>
<td>Skin, oral cavity, oropharynx, genital mucosa</td>
<td>Squamous cell carcinoma (55)</td>
<td>None</td>
<td>WT/R274W</td>
</tr>
<tr>
<td>R-I-1</td>
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<td>France</td>
<td>Skin, nails, oropharynx</td>
<td>Squamous cell carcinoma (55)</td>
<td>None</td>
<td>-</td>
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<tr>
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<td>18 mo</td>
<td>France</td>
<td>Lips, oropharynx</td>
<td>Systemic lupus erythematosus</td>
<td>None</td>
<td>WT/M202V</td>
</tr>
<tr>
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<td>France</td>
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<td>Systemic lupus erythematosus</td>
<td>None</td>
<td>WT/M202I</td>
</tr>
<tr>
<td>S-II-2</td>
<td>1 yr</td>
<td>France</td>
<td>Nails</td>
<td>None</td>
<td>None</td>
<td>WT/M202I</td>
</tr>
<tr>
<td>S-II-3</td>
<td>1 mo</td>
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<td>Skin, oropharynx</td>
<td>None</td>
<td>None</td>
<td>WT/M202I</td>
</tr>
<tr>
<td>T-II-3</td>
<td>1 yr</td>
<td>Germany</td>
<td>Skin, nails, oropharynx</td>
<td>Squamous cell carcinoma (41)</td>
<td>None</td>
<td>WT/Q271P</td>
</tr>
</tbody>
</table>

None of the patients displays autoantibodies against IL-17A, IL-17F, and IL-22. - , unknown.

strongly phosphorylated than the WT protein in both cytoplasmic and nuclear extracts (Fig. S1 G). The mechanism underlying the gain of R274Q phosphorylation was explored with the tyrosine kinase inhibitor staurosporine and the phosphatase inhibitor pervanadate. The dephosphorylation of IFN-γ–activated R274Q STAT1 was impaired by staurosporine, but less than that of the known dephosphorylation mutant F77A (Fig. 2 E; Zhong et al., 2005). In contrast, pervanadate normalized the phosphorylation of R274Q to WT levels (Fig. 2 F). Another CMCD-linked mutation, D165G (Fig. 1, A–C), also resulted in impaired dephosphorylation that could be normalized by adding pervanadate (Fig. 2 F and Fig. S1 H). Thus, at least two CMCD-linked STAT1 missense alleles (R274Q and D165G) are gain-of-function caused by the impairment of nuclear dephosphorylation. These alleles may therefore enhance cellular responses to cytokines activating STAT1 predominantly and STAT3 to a lesser extent, such as IFN-α/β, IFN-γ, IFN-λ, and IL-27, and possibly also responses to cytokines activating STAT3 predominantly and STAT1 to a lesser extent, such as IL-6, IL-21, IL-22, and IL-23 (Fig. S2).

We investigated the dominance of the STAT1 alleles at the cellular level by testing EBV-B–transformed (EBV-B) cells and SV-40–transformed dermal fibroblasts from a CMCD patient heterozygous for the STAT1 R274Q allele. We observed enhanced IFN-α/β–, IFN-γ–, and IL-27–dependent STAT1 phosphorylation in EBV-B cells from a patient heterozygous for the STAT1 R274Q allele, as shown by WB (Fig. 3, B and D). Phospho–STAT1 accumulated in the nucleus of R274Q heterozygous SV-40 fibroblasts upon IFN-γ stimulation, as well as in EBV-B cells (Fig. 3 I and Fig. S3 D). Moreover, the IFN-α/β–, IFN-γ–, and IL-27–induced DNA-binding activity of GAF was stronger in cells from the CMCD patient than in those from a healthy control or from a MSMD patient carrying the L706S mutant allele, as shown by electrophoretic mobility

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Moreover, mouse IFN-γ, IFN-α (Feng et al., 2008; Tanaka et al., 2008; Villarino et al., 2010) and human IFN-α/β (Chen et al., 2009; Ramgolam et al., 2009) have been shown to antagonize the development of IL-17–producing T cells via STAT1. In addition, IL-6, IL-21, and IL-23 are prominent inducers of IL-17–producing T cells, via a mechanism dependent on STAT3 and antagonized by STAT1 (Hirahara et al., 2010). Finally, we recently showed that inborn errors of IL-17F or IL-17RA were genetic etiologies of CMCD (Puel et al., 2010b, 2011). We thus determined the proportion of IL-17A– and IL-22–producing T cells by flow cytometry in patients with heterozygous STAT1 mutations and AD CMCD. The 18 CMCD patients carrying gain-of-function mutations in STAT1 that were tested had lower proportions of circulating IL-17A– and IL-22–producing T cells ex vivo than 28 healthy controls (P < 10^{-4}) and six patients bearing loss-of-function STAT1 alleles (P < 2.10^{-3}; Fig. 4, A and B; and Fig. S4 G). In contrast, they displayed normal proportions of IFN-γ–producing T cells (Fig. S4 F).

Villarino et al., 2010). Moreover, mouse IFN-γ (Feng et al., 2008; Tanaka et al., 2008; Villarino et al., 2010) and human IFN-α/β (Chen et al., 2009; Ramgolam et al., 2009) have been shown to antagonize the development of IL-17–producing T cells via STAT1. In addition, IL-6, IL-21, and IL-23 are prominent inducers of IL-17–producing T cells, via a mechanism dependent on STAT3 and antagonized by STAT1 (Hirahara et al., 2010). Finally, we recently showed that inborn errors of IL-17F or IL-17RA were genetic etiologies of CMCD (Puel et al., 2010b, 2011). We thus determined the proportion of IL-17A– and IL-22–producing T cells by flow cytometry in patients with heterozygous STAT1 mutations and AD CMCD. The 18 CMCD patients carrying gain-of-function mutations in STAT1 that were tested had lower proportions of circulating IL-17A– and IL-22–producing T cells ex vivo than 28 healthy controls (P < 10^{-4}) and six patients bearing loss-of-function STAT1 alleles (P < 2.10^{-3}; Fig. 4, A and B; and Fig. S4 G). In contrast, they displayed normal proportions of IFN-γ–producing T cells (Fig. S4 F).
Moreover, only very small amounts of IL-17A, IL-17F, and IL-22 were secreted by freshly prepared leukocytes after ex vivo stimulation with PMA and ionomycin ($P < 8 \times 10^{-3}$), as shown by ELISA (Fig. 4, C–E). In contrast, the amounts of secreted IL-17A, IL-17F, and IL-22 were normal in patients heterozygous or homozygous for loss-of-function or hypomorphic STAT1 mutations (Fig. 4, C–E). Interestingly, in all assays, the proportions of IL-17A– and IL-22–producing T cells and the amounts of IL-17A, IL-17F, and IL-22 secreted were smallest for the four patients with the most apparently severe clinical phenotype (Fig. 4, A–E and not depicted).

After the culture of PBMCs in vitro in the presence of various cytokines, including IL-6, TGF-$
\beta$, IL-1$\beta$, and IL-23, the proportion of IL-17A– and IL-22–producing T cell blasts remained significantly lower ($P < 10^{-4}$) in CMCD patients carrying STAT1 mutations than in controls (Fig. S4, A and B; and not depicted). In contrast, the proportions of IL-17A– and IL-22–producing T cell blasts were normal in patients with loss-of-function STAT1 mutations (Fig. S4, A and B; and not depicted).

Finally, levels of IL-12p70 and IL-18 were also significantly lower ($P < 10^{-4}$) in CMCD patients than in controls (Fig. S4, C–E; and not depicted). In contrast, patients with loss-of-function mutant STAT1 alleles displayed normal levels of cytokine secretion (Fig. S4, C–E; and not depicted).
IL-12p40 production by whole blood stimulated with IFN-γ were higher in CMCD patients bearing gain-of-function STAT1 alleles than in patients bearing loss-of-function STAT1 alleles and healthy controls (Fig. 4 F and not depicted). Thus, patients with familial or sporadic AD CMCD heterozygous for mutations affecting the coiled-coil domain of STAT1, including the dominant gain-of-function R274Q mutant allele, displayed lower levels of IL-17 cytokine production by peripheral T cells, providing a molecular mechanism for the disease.

**DISCUSSION**

We have shown that several germline missense mutations affecting the coiled-coil domain of STAT1 may cause sporadic and familial AD CMCD. The underlying mechanism involves a gain of STAT1 phosphorylation caused by the loss of nuclear dephosphorylation, resulting in a gain-of-function of GAF in response to various cytokines. Impaired dephosphorylation may not be the only mechanism influencing the impact of these mutations on the transcription of STAT1 target genes, as these mutations may also affect other processes, such as the dimerization of unphosphorylated STAT1. Moreover, the gain-of-function, which manifests itself in terms of DNA-binding activity, reporter gene induction, and target gene induction, may not necessarily increase the transcription of all target genes, possibly even resulting in the repression of some genes. In addition, the various STAT1 mutations, although they all affect the coiled-coil domain and are probably all loss-of-dephosphorylation and gain-of-function, may somewhat differ from each other in terms of their functional impact. The genome-wide impact of these mutations on the transcriptome remains to be assessed in various cell types stimulated with a range of cytokines. In any case, the gain-of-function mutant STAT1 alleles were dominant for GAF activation in all cell types tested. They affected cellular responses to various cytokines, including IFN-α/β, IFN-γ, and IL-27, which predominantly activate STAT1 over STAT3, and IL-6 and IL-21, which predominantly activate STAT3 over STAT1. These mutations probably also strengthen cellular responses to IFN-α. However, they do not seem to affect STAT1-containing ISGF-3 activation by IFN-α/β, at least in the conditions tested. Moreover, STAT3 activation by IL-6, IL-21, IL-22, and IL-23 is maintained, suggesting that STAT3 activation by IL-26 is also intact.

![Graphs](https://example.com/graph.png)

**Figure 4.** Impaired development and function of IL-17- and IL-22-producing T cells ex vivo in patients with AD CMCD and STAT1 mutations. Each symbol represents a value from a healthy control individual (black circles), a patient bearing a STAT1 gain-of-function (GOF) allele (red upright triangles), or a patient bearing one or two STAT1 loss-of-function (LOF) alleles (black upside-down triangles). (A) Percentage of CD3+/IL-17A+ (A) and CD3+/IL-22+ (B) cells, as determined by flow cytometry, in nonadherent PBMCs activated by incubation for 12 h with PMA and ionomycin. (C–E) Secretion of IL-17F (C), IL-17A (D) and IL-22 (E) by whole blood cells, as determined by ELISA, in the absence of stimulation (open symbols) and after stimulation with PMA and ionomycin for 48 h (closed symbols). Horizontal bars represent medians. The p-values for the nonparametric Wilcoxon test, between patients with STAT1 GOF mutations (n = 18) and controls (n = 28) and patients with STAT1 LOF mutations (n = 6) are indicated. All differences between healthy controls and patients with STAT1 LOF alleles were not significant. (F) Secretion of IL-12p70 by whole blood cells, as determined by ELISA, in the absence of stimulation (open symbols), after stimulation with BCG (lightly colored symbols), or BCG + IFN-γ for 48 h (closed symbols). Horizontal bars represent medians. The p-values for differences between patients with STAT1 GOF mutations (n = 15) and controls (n = 23) and patients with STAT1 LOF mutations (n = 6) are indicated and were calculated in nonparametric Wilcoxon tests. All experiments were performed at least two times independently.
The mutant STAT1 alleles described herein enhance cellular responses to cytokines such as IFN-α/β, IFN-γ, and IL-27, which potently inhibit the development of IL-17–producing T cells via STAT1 (Batten et al., 2006; Yoshimura et al., 2006; Stumhofer et al., 2006; Amadi-Obi et al., 2007; Feng et al., 2008; Kimura et al., 2008; Tanaka et al., 2008; Chen et al., 2009; Ramgolam et al., 2009; Crabé et al., 2009; Diveu et al., 2009; El-behi et al., 2009; Guzzo et al., 2010; Villarino et al., 2010; Liu and Rohowsky-Kochan, 2011). These mutant alleles also increase cellular responses to IL-6 and IL-21, which normally induce IL-17–producing T cells via STAT3 rather than STAT1 (Hiirahara et al., 2010). Enhanced STAT1–dependent cellular responses to these two groups of cytokines probably impair the development of IL-17–producing T cells. It remains unclear whether this mechanism predominantly involves IL-17–inhibiting cytokines (IFN-α/β, IFN-γ, and IL-27), either individually or in combination. The available data from the mouse model suggest that IL-27 is the most potent of the three inhibitors. There is also evidence that these cytokines inhibit IL-17–producing T cell development in humans (Ramgolam et al., 2009; Liu and Rohowsky-Kochan, 2011). Enhanced STAT1 and GAF activation in response to the IL-17 inducers IL-6 and IL-21, and perhaps IL-23, may also play a key role in disease, by antagonizing STAT3 responses. The effect of the aryl hydrocarbon receptor on IL-17 T cell development might also be enhanced by gain-of-function STAT1 alleles (Kimura et al., 2008). Moreover, enhanced STAT1 activity downstream from IL-22 and IL-26 in cells, not detected in our study, might also contribute to the CMCD phenotype. Finally, thyroid autoimmunity in eight patients and systemic lupus erythematosus in another patient in our series probably resulted from the enhancement of IFN-α/β responses, as such autoimmunity is a frequent adverse effect of treatment with recombinant IFN-α or IFN-β (Oppenheim et al., 2004; Selmi et al., 2006). Importantly, no autoantibodies against IL-17A, IL-17F, or IL-22 were detected in the patients’ serum (Table I and unpublished data).

Remarkably, germline mutations in human STAT1 underlie susceptibility to three different types of infectious disease: mycobacterial diseases, viral diseases, and CMC. Patients bearing STAT1 mutations and displaying mycobacterial and/or viral disease do not suffer from CMC, and the patients with CMCD caused by other STAT1 alleles described here present no mycobacterial or viral disease. The pathogenic mechanisms involved are clearly different, with loss-of-function mutations in STAT1 underlying mycobacterial and viral diseases (Dupuis et al., 2001, 2003; Chappger et al., 2006a, 2009; Kong et al., 2010; Averbuch et al., 2011; Kristensen et al., 2011). Human AR STAT1 deficiency impairs cellular responses to IFN-α/β, IFN-γ, IFN-λ, and IL-27 (Dupuis et al., 2003; Chappger et al., 2006b, 2009; Kong et al., 2010; Kristensen et al., 2011). Viral diseases probably result from impaired IFN-α/β and, perhaps, IFN-λ immunity, although impaired IFN-γ and IL-27 immunity may also contribute to the phenotype. Patients with AD MSMD, heterozygous for loss-of-function dominant-negative mutations of STAT1, suffer from mycobacterial disease caused by the impairment of IFN-γ immunity (Chappger et al., 2006a; Dupuis et al., 2001). Overall, mutations impairing STAT1 function confer AR or AR susceptibility to intracellular agents, through the impairment of IFN-α/β (viral diseases) and/or IFN-γ immunity (mycobacterial diseases). In contrast, the gain-of-function STAT1 mutations reported here confer AD CMCD because of the enhancement of STAT1–mediated cellular responses to STAT1–dependent repressors and STAT3–dependent inducers of IL-17–producing T cells. These studies neatly demonstrate that severe infectious diseases in otherwise healthy patients may be subject to genetic determinism (Casanova and Abel, 2005, 2007; Alcâí et al., 2009, 2010). They also highlight the profoundly different effects that germline mutations in the same human gene may have, resulting in different infectious diseases through different molecular and cellular mechanisms.

MATERIALS AND METHODS

Massively parallel sequencing

DNA (3 µg) extracted from EBV-B cells from the patient was sheared with a S2 Ultrasonicator (Covaris). An adapter-ligated library was prepared with the Paired-End Genomic DNA Sample Prep kit (Illumina). The SureSelect Human All Exon kit (Agilent Technologies) was then used for exome capture. Single-end sequencing was performed on a Genome Analyzer IIx (Illumina), generating 72-base reads.

Sequence alignment, variant calling, and annotation

BWA aligner (Li and Durbin, 2009) was used to align the sequences obtained with the human genome reference sequence (hg18 build). Downstream processing was performed with the Genome analysis toolkit (GATK; McKenna et al., 2010), SAMtools (Li et al., 2009), and Picard Tools (http://picard.sourceforge.net). Substitution calls were made with a GATK UnifiedGenotyper, whereas indel calls were made with a GATK IndelGenotyperV2. All calls with a read coverage ≥2x and a Phred-scaled SNP quality of ≥20 were filtered out. All the variants were annotated with annotation software that was developed in-house. The data were further analyzed with sequence analysis software that had been developed in-house (SQL database query-driven system).

Molecular genetics

EBV-B cells and the STAT1-deficient cell line U3C were cultured as previously described (Chappger et al., 2006a). Primary fibroblasts were cultured in DME supplemented with 10% fetal calf serum. Cells were stimulated with the indicated doses (in IU/ml or ng/ml) of IFN-γ (Imukin; Boehringer Ingelheim), IFN-α2b (IntronA; Schering-Plough), IL-27 (R&D Systems), IL-21 (R&D Systems), IL-22 (R&D Systems), IL-23 (R&D Systems), and IL-6 (R&D Systems). Genomic DNA and total RNA were extracted from cell lines and fresh blood cells, as previously described (Chappger et al., 2006a). Genomic DNA was amplified with specific primers encompassing exons 6–10 of STAT1 (available upon request), sequenced with the Big Dye Terminator cycle sequencing kit (Applied Biosystems), and analyzed on an ABI Prism 3730 (Applied Biosystems). We used the various alleles of STAT1 in the pcDNA3 STAT1-V5 vector (Chappger et al., 2006a; Kong et al., 2010). We generated the various STAT1 mutations by site-directed mutagenesis (QuickChange Site-Directed Mutagenesis kit; Stratagene) with the mismatches primers listed in Table S4. U3C cells were harvested by trypsin treatment 24 h before transfection and replated at a density of 2.5 × 10⁵ cells/ml in 6-well plates. Plasmid DNA (5 µg per plate) carrying the WT or all the various mutant STAT1 alleles was used for cell transfection with the Calcium Phosphate Transfection kit (Invitrogen).

Luciferase reporter assay

U3C cells were dispersed into 96-well plates (1 × 10⁵/well) and transfected with reporter plasmids (Cignal GAS and ISRE Reporter Assay kit;
buffer, and the proteins recovered were subjected to immunoblot analysis.

in DME for 15, 30, or 60 min. The cells were then lysed with 1% NP-40 lysis buffer, and the proteins were recovered and subjected to SDS-PAGE. We used antibodies directed against phosphorylated STAT1 (pY701; BD), STAT1 (C-24; Santa Cruz Biotechnology), V5 (Invitrogen), α-tubulin (Santa Cruz Biotechnology), phosphorlated STAT3 (Cell Signaling Technology), lamin B1 (Santa Cruz Biotechnology), GAPDH (Santa Cruz Biotechnology), and STAT3 (Santa Cruz Biotechnology). EMSA was performed as previously described (Chapgier et al., 2006a). In brief, cell activation was blocked with cold 1X PBS, cells were lysed in 1% NP-40 lysis buffer, and the proteins recovered and subjected to SDS-PAGE. We used antibodies directed against phosphorylated STAT1 at Y701 (Cell Signaling Technology), lamin B1 (Santa Cruz Biotechnology), GAPDH (Santa Cruz Biotechnology), and STAT3 (Santa Cruz Biotechnology). EMSA was performed as previously described (Chapgier et al., 2006a). In brief, cell activation was blocked by incubation with cold 1X PBS, and the cells were gently lysed to remove cytoplasmic proteins while keeping the nuclear intact. We then added nuclear lysis buffer and recovered the nuclear proteins, which were subjected to non-denaturing electrophoresis with radiolabeled GAS (from the FCyR1 promoter: 5′-ATGTATTTCCCAGAAA-3′) and ISRE (from the ISG15 promoter: 5′-GATCGGGGAAGGGGAAACCTGAA-3′) probes.

Staurosporine and pervanadate treatment of cells
We assessed dephosphorylation by stimulating U3C transfectants with 10^5 IU/ml IFN-γ. The cells were then washed and incubated with 1 μM staurosporine in DMEM for 15, 30, or 60 min. The cells were then lysed with 1% NP-40 lysis buffer, and the proteins recovered were subjected to immunoblot analysis. Pervanadate was prepared by mixing orthovanadate with H2O2 for 15 min at 22°C. U3C transfectants were treated with pervanadate (0.8 mM orthovanadate and 0.2 mM H2O2) 5 min before stimulation. They were then stimulated with IFN-γ for 20 min. The protein stimulation was achieved by blocking cold 1X PBS. The proteins were recovered and subjected to immunoblot analysis.

Extraction of nuclear and cytoplasmic proteins
U3C transfectants or EBV-B cells were stimulated with IFN-γ or IFN-α for 20 min and subjected to nuclear and cytoplasmic protein extraction with NE-PER Nuclear and Cytoplasmic Extraction Regent (Thermo Scientific) according to the manufacturer’s protocol.

Immunofluorescence staining
Immunofluorescence experiments were performed as previously described (Chapgier et al., 2006a). In brief, cells (transfected U3C or SV-40 fibroblasts) were stimulated for the times indicated with 10,000 IU/ml of IFN-γ. Cells were then washed with cold PBS and fixed with 4% PFA. The cells were then washed and incubated with an antibody against STAT1, which was then detected by incubation with an Alexa Fluor 488–conjugated anti–mouse antibody.

T cell blast differentiation and stimulation
PBMCs were recovered by centrifuging blood samples on Ficoll gradients, as previously described (Chapgier et al., 2006a). They were then cultured, at a density of 1 million cells per ml in RPMI supplemented with 10% fetal calf serum and stimulated with phytohemagglutinin (1 μg/ml) for 3 days. Cells were then recovered, centrifuged on a Ficoll gradient, and stimulated with 0.2 million cells/ml to Panserin 401 supplemented with 10% FCS and glutamine 1X, and stimulated with 40 IU/ml IL-2 (Roche). Cells were then incubated for 30 min with 100 ng/ml IL-23. Activation was stopped by adding 1X cold PBS, and cells were processed for immunoblot analysis.

Whole-blood assay of the IL-12–IFN-γ circuit
Whole-blood assays were performed as previously described (Feinberg et al., 2004). Heparin-treated blood samples from healthy controls and patients were stimulated in vitro with live Mycobacterium bovis BCG (Pasteur) alone or with IFN-γ (5,000 IU/ml; Boehringer Ingelheim). Supernatants were collected after 48 h of stimulation, and ELISA were performed with specific antibodies directed against IL-12p40 or IL-12p70, using kits from R&D Systems according to the manufacturer’s instructions.

Production of IL-17A, IL-17F, and IL-22 by leukocytes
Cell activation. IL-17A– and IL-22–producing T cells were evaluated by intracellular staining or by ELISA, as previously described (de Beaucoudrey et al., 2008). In brief, PBMCs were purified by centrifugation on a gradient (Ficol-Paque PLUS; GE Healthcare) and reseeded in RPMI supplemented with 10% FBS (RPMI/10%; Invitrogen). Adherent monocytes were removed from the PBMC preparation by incubation for 2 h at 37°C, under an atmosphere containing 5% CO2.

For evalulation of IL-17– and IL-22–producing T cells by flow cytometry, we reseeded 5 × 10^6 nonadherent cells in 5 ml RPMI/10% FBS in 25 cm^2 flasks and stimulated them by incubation with 40 ng/ml PMA (Sigma-Alrich) and 10^-5 M ionomycin (Sigma-Alrich) in the presence of a secretion inhibitor (1 μl/ml Golgiplug; BD) for 12 h.

For evaluation of the IL-17– and IL-22–producing T cell blasts after in vitro differentiation, the nonadherent PBMCs were dispensed into 24-well plates at a density of 2.5 × 10^5 cells/ml in RPMI/10% FBS and activated with 2 μg/ml of an antibody directed against CD3 (Orthoclone OKT3; Janssen-Cilag) alone, or together with 5 ng/ml TGF-B1 (204-B; R&D Systems), 20 ng/ml IL-23 (1290-IL; R&D Systems), 50 ng/ml IL-6 (206-IL; R&D Systems), 10 ng/ml IL-12p40 or IL-12p70 (201-LB; R&D Systems), or combinations of these four cytokines. After 3 days, the cells were restimulated in the same activation conditions, except that the anti-CD3 antibody was replaced with 40 ng/ml IL-2 (Proleukin i.v.; Chiron). We added 1 ml of the appropriate medium, reseeded the cells by gentle pipetting, and then split the cell suspension from each well into two. Flow cytometry was performed on one of the duplicated wells 2 days later, after stimulation by incubation for 12 h with 40 ng/ml PMA and 10^-5 M ionomycin in the presence of 1 μl/ml Golgiplug. FACS analysis was performed as described in the following section. The other duplicated well was split into two, with one half left unstimulated and the other stimulated by incubation with 40 ng/ml PMA and 10^-5 M ionomycin for another 2 days. Supernatants were collected after 48 h of incubation for ELISA.

Flow cytometry. Cells were washed in cold PBS, and surface labeling was achieved by incubating the cells with PE-Cy5-conjugated anti–human CD3 antibody (BD) in PBS/2% FBS for 20 min on ice. Cells were then washed twice with 2% FBS in cold PBS, fixed by incubation with 100 μl of BD Cytofix for 30 min on ice, and washed twice with BD Cytoperm (Cytofix/ Cytoperm Plus, fixation/permeabilization kit; BD). Cells were then incubated for 1 h on ice with Alexa Fluor 488–conjugated anti–human IL-17A (S3-7179-42; Biocsense), PE-conjugated anti–human IL-22 (IC7821P; R&D Systems), or PE-conjugated anti–human IFN-γ (IC8285P; R&D Systems) antibodies, washed twice with Cytoperm, and analyzed with a FACSCanto II system (BD).

ELISA. IL-17A, IL-17F, and IL-22 levels were determined by ELISA on the supernatants harvested after 48 h of whole-blood stimulation with 40 ng/ml PMA and 10^-5 M ionomycin, or after in vitro PHA blast differentiation and
48 h of stimulation with 40 ng/ml PMA and 10−5 M ionomycin. We used anti–human IL-17A and anti–human IL-22 Duoset kits (R&D Systems) and the anti–human IL-17F ELISA Ready-SET-Go! set (eBioscience).

Statistical analysis. We assessed differences between controls, MSMD patients bearing loss-of-function STAT1 alleles, and CMCD patients bearing gain-of-function STAT1 alleles in terms of the percentages of IL-17A− and IL-22−producing T cells, as assessed by flow cytometry, and in terms of the amounts of IL-17A, IL-17F, and IL-22 produced in various stimulation conditions, as assessed by ELISA. We used the nonparametric Wilcoxon test, as implemented in the PROC NPAR1WAY of the SAS software version 9.1 (SAS Institute). For all analyses, P < 0.05 was considered statistically significant.

Online supplemental material

Fig. S1 shows that STAT1-CMCD mutants are gain-of-function alleles by loss of nuclear dephosphorylation. Fig. S2 is a schematic representation of the cytokines and transcription factors directing the development of naive CD4 cells into IL-17−producing T cells. Fig. S3 shows the normal response of CMCD patient cells to IFN-γ in terms of STAT1 nuclear translocation; and to IL-23 and IL-22 in terms of pSTAT3. Fig. S4 shows impaired in vitro differentiation of IL-17− and IL-22−producing T cell blasts in patients with CMCD and gain-of-function STAT1 mutations. Table S1 shows novel coding heterozygous variants found by whole-exome sequencing in the 6 different patients. Table S2 shows novel coding heterozygous variants found by whole-exome sequencing within genes shared by more than one patient. Table S3 lists conservation and pre-mutations, as assessed by flow cytometry, and in terms of the percentages of IL-17A− and IL-22−producing T cells, as assessed by ELISA. We used the nonparametric Wilcoxon test, as implemented in the PROC NPAR1WAY of the SAS software version 9.1 (SAS Institute). For all analyses, P < 0.05 was considered statistically significant.

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