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

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Abbreviations used: AD, autosomal dominant; AR, autosomal recessive; CMC, chronic mucocutaneous candidiasis; CMCD, CMC disease; EMSA, electrophoretic mobility shift assay; GAS, γ-activated sequence; ISRE, IFN-stimulated response element; MCM, 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 germ-line 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-21 enhance the impairment of IL-17A, IL-17F, and IL-22, 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, presumably 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 circulating IL-17A– and IL-22–producing T cells, presumably because of impaired responses to 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 (Glocker 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 CMC. 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 CMC. In a consanguineous family from Morocco, a child with CMCD was found to display AR complete IL-17RA 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 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 not genotyped. The intrafamilial segregation of the mutations was consistent with an AD trait, as all patients with CMCD from the kindreds tested were heterozygous, whereas none of these mutations was found in the heterozygous state in any of the healthy relatives tested (Fig. 1 B). Moreover, the STAT1 haplotypes for common SNPs indicated that the five recurrent mutations were caused by mutation hotspots rather than founder effects (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 Polymorphisme 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 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; Table S3). None of the previously described nine patients with AD STAT1 deficiency and MSMD was heterozygous for mutations affecting the coiled-coil domain (Fig. 1, A and C; Chapgier et al., 2009; Hoshino et al., 2006; Mertens et al., 2006). In contrast, the other two morbid missense mutations affecting residues located on the other side of the coiled-coil domain (Fig. 1 C). Moreover, these two hypomorphic alleles were shown to be pathogenic not because they were missense, but because they promoted the splicing out of exon 8, resulting in AR partial STAT1 deficiency, with the production of small amounts of intrinsically functional STAT1 molecules (Kong et al., 2010; Kristensen et al., 2011). These genetic data strongly suggest that heterozygous missense mutations in the coiled-coil domain of STAT1 may cause AD CMCD in a large fraction of patients. Nevertheless, the occurrence of other germline mutations in STAT1 in patients without CMC and with an AD or AR predisposition to other infectious diseases raised questions about whether these mutations were really responsible for CMCD and the underlying mechanism of disease.

We functionally characterized the CMCD-causing STAT1 allele R274Q, which was found in four kindreds (Fig. 1 B and Table I). We compared it with a WT and an MSMD-causing loss-of-function STAT1 allele (L706S; Dupuis et al., 2001). We transfected STAT1-deficient U3C fibrosarcoma cells with WT, R274Q, or L706S STAT1 alleles. Upon stimulation with IFN-α, IFN-γ, 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 α 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/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

| Patient | Age at presentation | Origin | Clinical features of CMC | Cause of death (age/yr) | Autoimmunity | Genotype |
|---------|---------------------|--------|--------------------------|--------------------------|--------------|----------|
| A-I-1   | -                   | France | Nails                    | Not related to the disease (old age) | None         | -        |
| A-II-1  | -                   | France | Nails                    | Not related to the disease (old age) | None         | -        |
| A-III-1 | 1 mo                | France | Nails, oral cavity, oropharynx, genital mucosa | None | WT/R274Q |
| A-III-3 | -                   | France | Nails, oral cavity       | Not related to the disease (40) | None         | -        |
| A-III-4 | 1 mo                | France | Nails, oral cavity, oropharynx | None | WT/R274Q |
| B-II-1  | -                   | France | -                        | None                       | None         | -        |
| B-III-2 | 3 yr                | France | Skin, nails, oral cavity, oropharynx, genital mucosa | None | WT/K286I |
| B-IV-1  | 5 yr                | France & Congo | Skin, nails, oral cavity, oropharynx | None | WT/K286I |
| B-IV-2  | 5 mo                | France & Congo | Skin, nails, oral cavity, oropharynx | Cerebral aneurysm (8) | None | -        |
| C-III-1 | -                   | Turkey | Nails, oral cavity, genital mucosa | Cerebral aneurysm (34) | Thyroid autoimmunity | WT/R274Q |
| C-IV-1  | -                   | Turkey | Nails, oral cavity       | None                       | WT/R274Q |
| D-II-1  | -                   | France | Nails, oral cavity, genital mucosa | None | WT/M202V |
| D-III-2 | 7 yr                | France | Skin, oral cavity, oropharynx | None | WT/M202V |
| D-IV-2  | 1 mo                | France | Skin, nails, oropharynx  | Thyroid autoimmunity      | WT/M202V |
| E-II-1  | 1 yr                | Germany | Skin, oral cavity, oropharynx | Squamous cell carcinoma (54) | - | - |
| E-III-2 | 1 yr                | Germany | Nails, oral cavity, oropharynx, genital mucosa | Thyroid autoimmunity | WT/C174R |
| E-III-3 | 9 mo                | Germany | Skin, nails, oral cavity, oropharynx, genital mucosa | Thyroid autoimmunity | WT/C174R |
| E-IV-1  | 18 mo               | Germany | Skin, oral cavity, oropharynx, genital mucosa | Thyroid autoimmunity | WT/C174R |
| E-IV-2  | 2 yr                | Germany | Skin, oral cavity, oropharynx | Thyroid autoimmunity | WT/C174R |
| E-IV-4  | 2 yr                | Germany | Skin, oral cavity, oropharynx, genital mucosa | None | WT/C174R |
| E-IV-5  | 1 yr                | Germany | Skin, nails, oral cavity, oropharynx | None | WT/C174R |
| F-III-2 | 1 mo                | Argentina | Nails, oral cavity, oropharynx, genital mucosa | None | WT/R274W |
| F-IV-2  | 1 mo                | Argentina | Skin, nails, oral cavity, oropharynx | None | WT/R274W |
| F-IV-3  | 6 mo                | Argentina | Nails, oral cavity, genital mucosa | None | WT/R274W |
| G-II-1  | 3 mo                | Ukrainian | Nails, skin, oral cavity, oropharynx, esophagus | None | WT/D165G |
| H-I-2   | 1 yr                | Japan   | Skin, oropharynx, esophagus | None | WT/R274Q |
| H-II-2  | 5 yr                | Japan   | Oral cavity, oropharynx | None | WT/R274Q |
| I-II-3  | 9 mo                | Mexico  | Skin, nails, oral cavity, genital mucosa | None | WT/T288A |
| J-I-2   | -                   | Switzerland | Oral cavity, oropharynx | None | WT/T288A |
| J-II-2  | 3 mo                | Switzerland | Oral cavity, oropharynx | None | WT/T288A |
| K-II-2  | 11 mo               | Switzerland | Nails, oral cavity, oropharynx | Thyroid autoimmunity | WT/Y170N |
| L-I-2   | 7 yr                | France   | Skin, nails, oropharynx, esophagus | Thyroid autoimmunity | WT/R274Q |
| L-II-1  | 1 mo                | France   | Skin, nails, oropharynx, esophagus | None | WT/R274Q |
| M-II-2  | 6 mo                | Germany   | Skin, nails, oropharynx, genital mucosa | Thyroid autoimmunity | WT/D165H |
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 variant sporine, but less than that of the known dephosphorylation variant.

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; Kastelein 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 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-22. The mutation may also affect IFN-λ responses.

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

| Patient | Age at presentation | Origin | Clinical features of CMC | Cause of death (age/yr) | Autoimmunity | Genotype |
|---------|---------------------|--------|--------------------------|-------------------------|--------------|----------|
| N-II-2 | 1 yr                | Germany | Skin, nails, oropharynx  | Squamous cell carcinoma (54) | None | WT/R274W |
| O-II-1 | 18 mo               | Germany | Oral cavity, oropharynx  | Not related to the disease (46) | None | WT/M202I |
| P-I-1  | 1 yr                | Israel  | Oropharynx, genital mucosa | squamous cell carcinoma (55) | None | WT/A267V |
| P-II-1 | <2 yr               | Israel  | Skin, nails, oropharynx  | Systemic lupus erythematosus (M202V) | None | WT/M202V |
| P-II-2 | <2 yr               | Israel  | Skin, nails, oropharynx  | Systemic lupus erythematosus (54) | None | WT/M202I |
| Q-II-1 | 1 yr                | France  | Skin, oral cavity, oropharynx | Squamous cell carcinoma (41) | None | WT/Q271P |
| R-I-1  | 4 yr                | France  | Skin, nails, oropharynx  | Squamous cell carcinoma (55) | None | WT/R274W |
| R-II-1 | 18 mo               | France  | Lips, oropharynx         | Squamous cell carcinoma (54) | None | WT/M202I |
| S-I-2  | 6 mo                | France  | Skin, oral cavity, oropharynx | Squamous cell carcinoma (41) | None | WT/M202I |
| S-II-2 | 1 yr                | France  | Nails                    | Squamous cell carcinoma (55) | None | WT/R274W |
| S-II-3 | 1 mo                | France  | Skin, oropharynx         | Squamous cell carcinoma (54) | None | WT/M202I |
| T-II-3 | 1 yr                | Germany | Skin, nails, oropharynx  | Squamous cell carcinoma (54) | None | WT/Q271P |

None of the patients displays autoantibodies against IL-17A, IL-17F, and IL-22. -, unknown.
IL-27 is a potent inhibitor of the development of IL-17–producing T cells in mice (Batten et al., 2006; Stumhofer et al., 2006; Yoshimura et al., 2006; Amadi-Obi et al., 2007; Diveu et al., 2009; El-behi et al., 2009; Villarino et al., 2010) and humans (Diveu et al., 2009; Liu and Rohowsky-Kochan, 2011), through a mechanism dependent on STAT1 (Amadi-Obi et al., 2007; Batten et al., 2006; Diveu et al., 2009; Liu and Rohowsky-Kochan, 2011; Stumhofer et al., 2006; 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 × 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).

Figure 3. The mutant R274Q STAT1 allele is dominant for GAF-dependent cellular responses at the cellular level. The responses of the patient’s EBV-B cells (R274Q/WT) were evaluated independently at least twice, by EMSA, with a GAS probe (A, C, E, and G), and by Western blot (B, D, F, and H). This response was compared with that of one or two healthy controls (WT/WT and WT/WT2), heterozygous cells with a WT and a loss-of-function STAT1 allele (STAT1+/-), cells heterozygous for a dominant loss-of-function mutation of STAT1 (L706S/WT), cells with complete STAT1 deficiency (STAT1-/-), and cells from two patients heterozygous for dominant loss-of-function mutations of STAT3 (STAT3+/- and STAT3+/-). Cells were left nonstimulated (NS) or stimulated, as indicated, with IFN-γ, IFN-α, IL-6, IL-27, IL-21, and IL-23. pSTAT is an antibody specific for STAT with a phosphorylated tyrosine residue. (I) The nuclear and cytoplasmic fractions of EBV-B cells from a control (WT/WT), a CMCD patient (R274Q/WT), a heterozygous patient with a dominant loss-of-function mutation of STAT1 (L706S/WT), and a patient with complete STAT1 deficiency (-/-) stimulated with IFN-γ and IFN-α were tested for the presence of phosphorylated STAT1 and STAT1 by WB. Antibodies directed against GAPDH and Lamin B1 were used to normalize the amount of cytoplasmic and nuclear proteins, respectively. The experiment was performed twice.
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-β. 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.

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, 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.

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 and B) 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 potentially 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 (Hirahara 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 cells via STAT3 rather than STAT1 (Hirahara et al., 2010). 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 via STAT3 rather than STAT1 (Hirahara et al., 2010). 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 via STAT3 rather than STAT1 (Hirahara et al., 2010).

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buffer, and the proteins recovered were subjected to immunoblot analysis.

First, the cells were stimulated with IFN-γ (500 and 1,000 IU/ml), IL-27 (20 and 100 ng/ml), and IFN-α (500, 1,000, and 5,000 IU/ml) for 16 h and subjected to luciferase assays with the Dual-Glo luciferase assay system (Promega). Experiments were performed in triplicate and firefly luciferase activity was normalized with respect to Renilla luciferase activity. The data are expressed as fold induction with respect to nonstimulated cells.

Immunoblot analysis and electrophoretic mobility shift assays

The following optimal stimulation conditions were used. EBV-B or U3C cells were stimulated by incubation for 20 min with 100 μg/ml IL-21 or 25 ng of IL-22; 30 min with 10^3 or 10^5 IU/ml IFN-γ and IFN-α; 15 min with 50 ng/ml IL-6; or 30 min with 50 or 100 ng/ml IL-27. WB was performed as previously described (Dupuis et al., 2003). In brief, cell activation was blocked with cold 1X PBS, cells were lysed in 1% NP-40 lysis buffer, and the proteins were recovered and subjected to SDS-PAGE. We used antibodies directed against phosphorylated STAT1 (γY701; BD), STAT1 (C-24; Santa Cruz Biotechnology), V5 (Invitrogen), α-tubulin (Santa Cruz Biotechnology), phosphorylated 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 by incubation with cold 1X PBS, and the cells were gently lysed to remove cytoplasmic proteins while keeping the nucleus intact. We then added nuclear lysis buffer and recovered the nuclear proteins, which were subjected to non-denaturing electrophoresis with radiolabeled GAS (from the FCγR1 promoter: 5'-ATGATTTTCCTGACAGAA-3') and ISRE (from the ISG15 promoter: 5'-GATCGGGGAAAGGGGAACCGGAACCTGAA-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 stimulation was stopped by adding 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 Regents (Thermo Fisher 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 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 cultivated, at a density of 1 million cells per ml in RPMI supplemented with 10% fetal calf serum and supplemented with phytohemagglutinin (1 μg/ml) for 3 d. Cells were then recovered, centrifuged on a Ficoll gradient, cultivated at (a density of 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.

Modeling

Images of the three-dimensional structure of STAT1 (Chen et al., 1998) were generated with the 2002 PyMOL Molecular Graphics System (DeLano Scientific), using PDB accession no. 1BF5.

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 (Ficoll-Paque PLUS; GE Healthcare) and resuspended in RPMI supplemented with 10% FBS (RPMI/10% FBS; Invitrogen). Adherent monocytes were removed from the PBMC preparation by incubation for 2 h at 37°C, under an atmosphere containing 5% CO2.

For ex vivo evaluation of IL-17- and IL-22–producing T cell flow cytometry, we reseeded 5 × 10^6 nonadherent cells in 5 ml RPMI/10% FBS in 25 cm² flasks and stimulated them by incubation with 40 ng/ml PMA (Sigma-Aldrich) and 10^-5 M ionomycin (Sigma-Aldrich) in the presence of a secretion inhibitor (1 μl/ml GolgPlugs; 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 × 10^6 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 TGFR-β1 (201-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-1β (201-LB; R&D Systems), or combinations of these four cytokines. After 3 d, the cells were reseeded in the same activation conditions, except that the anti-CD3 antibody was replaced with 40 IU/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 d 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 GolgPlugs. 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 iono- mycin for another 2 d. 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-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 10 μl of BD Cytotox 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; Bioscience), PE-conjugated anti-human IL-22 (IC821P; R&D Systems), or PE-conjugated anti-human IFN-γ (IC825P; 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

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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, MSSMD 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-α in terms of ISGF3 activation, 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 predictions on the function of the mutant STAT1 alleles associated with CMCD. Table S4 lists the STAT1 GOF mutation created, and the pair of primers used. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.201101958/DC1.

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