Penicillium marneffei infection and Impaired Interferon-gamma Immunity in humans with Autosomal Dominant Gain-of-phosphorylation STAT1 mutations

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Capsule summary:

Penicillium marneffei is an AIDS-defining illness. We provide the first identification of autosomal dominant gain-of-phosphorylation *STAT1* mutations causing defective interferon-gamma and Th17 immunity in patients with penicilliosis, an invasive mycosis endemic in Southeast Asia.

To the Editor:

Penicillium marneffei (PM) is a pathogenic fungus endemic in Southeast Asia. PM was an extremely rare pathogen in human before the HIV epidemic, but following the exponential rise in HIV prevalence in Southeast Asia, penicilliosis emerged as a clinically significant opportunistic infection and is classified as an AIDS-defining illness.[1] Less commonly, penicilliosis occurs in patients with other immunodeficiencies, such as severe combined immunodeficiency, common variable immunodeficiency, hyper-IgM syndrome, hyper-IgE syndrome, the presence of anti-IFNγ autoantibody, diabetes mellitus, immunosuppressive therapy, and solid organ or hematopoietic stem cell transplant.[1,2] Affected individuals often have disseminated disease with rapid progression to multi-organ failure and death.

We previously reported 5 Chinese HIV-negative children and teenagers with disseminated penicilliosis. Four had co-existing chronic mucocutaneous candidiasis (CMC) since infancy, and one of them was genetically confirmed to have autosomal dominant hyper-IgE syndrome (AD-HIES). For the remaining 3 patients, a search for genetic defects in *CARD9*, *AIRE*, *STAT3*, *IL12B*, *IL12RB1*, *IFNGR1* was unrevealing.[3] The co-existence of CMC and systemic penicilliosis suggested a possible functional defect of Th17 immune response in these patients. Recently, AD gain-of-function

missense mutations of *STAT1* have been identified in several multiplex kindreds displaying CMC, autoimmunity and squamous cell carcinoma.[4-9] We hypothesized STAT1 as a candidate gene, and we sought to determine the cellular response to STAT1 activation in these patients. Consent for genetic diagnosis and functional studies was obtained from parents, and the study was approved by The Institutional Review Board of The University of Hong Kong / Hospital Authority Hong Kong West Cluster.

P1, P2 and P3 were 3 unrelated Chinese children, and their clinical presentations and immunological profile were previously reported in detail.[3] The core features and genetic findings of the patients and their parents are listed in Table 1 and Fig E1 (Online Repository). Heterozygous missense mutation in *STAT1* was identified by Sanger sequencing in P1 (c.800C>T, p.A267V) and P3 (c.863C>T, p.T288I), and total exome sequencing in P2 (c.1074G>T, p.L358F; Online Repository). p.A267V is a known mutation while p.T288I and p.L358F are novel, but missense mutations involving the same amino acid residues (p.T288A and p.L358W) were reported in patients with CMC.[4-6] Multiple sequence alignment of STAT1 orthologs (HomoloGene, NCBI) showed that all residues are highly conserved in animals except zebrafish for A267 and T288, and chicken for L358.

Missense mutations affecting the STAT1 coiled-coil domain identified in patients with AD-CMC have been demonstrated to be gain-of-function mutants with increased tyrosine-701 residue phosphorylation and enhanced γ-activated sequence (GAS) promoter binding activity.[5] We compared the level of STAT1 phosphorylation in patients with healthy controls by flow cytometric analysis of intracellular phosphorylated STAT1 (pSTAT1). PBMC from patients and controls were stimulated with recombinant human IFNα (40,000IU/ml) or IFNγ (5,000 IU/ml) for 20min. Compared with normal controls, lymphocytes from all patients demonstrated significantly higher percentage of pSTAT1+ cells and increased phosphorylation intensity in response to IFNα and IFNγ stimulation (Fig 1 A and B, Fig E2 in the Online Repository). The kinetics of STAT1 dephosphorylation was studied in P1. When treated with tyrosine kinase inhibitor, almost all STAT1 in control cells was dephosphorylated by 30min; whereas about 50% and 25% of STAT1 in patient cells remained phosphorylated at 30 and 60min respectively, indicating prolonged STAT1 phosphorylation in patient cells (Fig 1C). A missense mutant affecting residue L358 was previously shown to cause delayed dephosphorylation as well.[6]

Next, we determined the proportion of IFNγ and IL17A-expressing T-cells in PMBCs activated by overnight incubation with PMA (100ng/ml) and ionomycin (1μg/ml) in the

presence of Brefeldin A. Patients had significantly lower CD3+/IFNγ+ T-cells (14.8±1.5% vs 43.3±12.8%, p<0.01) and CD3+/IL17A+ T-cells (0.30±0.11% vs 2.15±1.41%, p=0.01; Fig. 1D) compared to normal controls. Finally, we evaluated the capacity of IFNγ production towards fungal stimulation in P1 and P2. PBMCs were co-cultured with *Candida albicans* or PM for 2 days, and supernatants were collected for IFNγ assay (FlowCytomix, Bender MedSystems). Compared with normal controls, P1 and P2 produced much lower IFNγ towards both fungi (Fig. 1E). Production of other cytokines (IL1β, IL6, TNFα and MIP1α) was studied in P1, and was comparable with normal controls. (Fig E3, Online Repository).

Previous studies demonstrated that patients with CMC caused by gain-of-phosphorylation *STAT1* mutations had impaired Th1 and Th17 response as a result of defective signaling through the IL12 and IL23 pathways.[4-7, 10] Majority of these gain-of-phosphorylation mutants are located in the coiled-coil domain and two in the DNA-binding domain.[6, 7] Impaired dephosphorylation of STAT1 enhances gamma-interferon activation factor (GAF)-dependent cellular response to IFNα/β, IFNγ, and IL27, which are repressors of Th17 development from naïve T-cells. The enhanced response mediated by STAT1 probably impairs Th17 immunity.[5]

The identification of *STAT1* and *STAT3* mutations in patients with systemic penicilliosis

115 suggests the importance of Th1 and Th17 immune response against PM. It is generally
116 believed that PM establishes diseases in the lungs following inhalation of conidia, and
117 disseminates in the form of intracellular yeast via the reticuloendothelial system. The
118 activation of macrophages by IFNγ is essential for their fungicidal activity against PM
119 through the production of nitric oxide. While PM infection was self-limiting in
120 wild-type mice, all IFNγ-knockout mice died of systemic mycosis.[11] In humans,
121 individuals with anti-IFNγ autoantibody suffered from disseminated penicilliosis.[2] Our
122 experiments showed that lymphocytes of P1 and P2 exhibited defective IFNγ production
123 to PM in vitro. To our knowledge, this study shows for the first time that a primary
124 defect in IFNγ and IL17 immune response may be accountable for human PM infection.
125 Penicilliosis should be regarded as an indicator of underlying primary
126 immunodeficiency in HIV-negative individuals after excluding secondary causes.
127
128 It is worth noting that impaired IFNγ and Th17 response in patients with
129 gain-of-phosphorylation *STAT1* mutations can predispose them to invasive mycosis as
130 well as a range of bacterial and viral infections. Apart from penicilliosis, disseminated
131 aspergillosis, candidemia, disseminated histoplasmosis and recalcitrant cutaneous
132 fusariosis were reported.[6, 12] P2 and P3 had recurrent sinopulmonary infections caused
133 by respiratory viruses and encapsulated bacteria, which was also similarly described by

Uzel et al[6] and Takezaki et al.[7] Of note, P3 had tuberculous lymphadenitis, recurrent herpes zoster and EBV-associated hemophagocytosis, supporting previous observation that AD gain-of-phosphorylation STAT1 mutations are associated with susceptibility to mycobacterial and herpes virus infections.[8] Autoimmunity such as hypothyroidism, autoimmune hepatitis, systemic lupus erythematosus and type I diabetes mellitus, as well as malignancy such as esophageal carcinoma can lead to significant morbidities to this group of patients. The infectious disease susceptibility and phenotypic spectrum of AD-CMC caused by *STAT1* mutations are wider than previously believed, revealing the divergent roles of STAT1 in host-pathogen interaction, immune tolerance and carcinogenesis.

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REFERENCES

1. Supparatpinyo K, Khamwan C, Baosoung V, Nelson KE, Sirisanthana T. Disseminated Penicillium marneffei infection in southeast Asia. Lancet 1994; 344:110-3.

2. Tang BS, Chan JF, Chen M, Tsang OT, Mok MY, Lai RW, et al. Disseminated penicilliosis, recurrent bacteremic nontyphoidal salmonellosis, and burkholderiosis associated with acquired immunodeficiency due to autoantibody against gamma interferon. Clin Vaccine Immunol 2010; 17:1132-8.

3. Lee PP, Chan KW, Lee TL, Ho MH, Chen XY, Li CH, et al. Penicilliosis in children without HIV infection--are they immunodeficient? Clin Infect Dis 2012; 54:e8-e19.

4. van de Veerdonk FL, Plantinga TS, Hoischen A, Smeekens SP, Joosten LA, Gilissen C, et al. STAT1 mutations in autosomal dominant chronic mucocutaneous candidiasis. N Engl J Med 2011; 365:54-61.

5. Liu L, Okada S, Kong XF, Kreins AY, Cypowyj S, Abhyankar A, et al. Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis. J Exp Med 2011; 208:1635-48.

6. Uzel G, Sampaio EP, Lawrence MG, Hsu AP, Hackett M, Dorsey MJ, et al. Dominant gain-of-function STAT1 mutations in FOXP3 wild-type immune dysregulation-polyendocrinopathy-enteropathy-X-linked-like syndrome. J Allergy

193 Clin Immunol 2013; doi: 10.1016/j.jaci.2012.11.054.

194 7. Takezaki S, Yamada M, Kato M, Park MJ, Maruyama K, Yamazaki Y, et al. Chronic
195 mucocutaneous candidiasis caused by a gain-of-function mutation in the STAT1
196 DNA-binding domain. J Immunol 2012; 189:1521-6.

197 8. Toth B, Mehes L, Tasko S, Szalai Z, Tulassay Z, Cypowyj S, et al. Herpes in STAT1
198 gain-of-function mutation. Lancet 2012; 379:2500.

199 9. Soltész B, Tóth B, Shabashova N, Bondarenko A, Okada S, Cypowyj S et al. New
200 and recurrent gain-of-function STAT1 mutations in patients with chronic
201 mucocutaneous candidiasis from Eastern and Central Europe. J Med Genet. 2013
202 Jul 17. doi: 10.1136/jmedgenet-2013-101570.

203 10. Smeekens SP, Plantinga TS, van de Veerdonk FL, Heinhuis B, Hoischen A, Joosten
204 LA, et al. STAT1 hyperphosphorylation and defective IL12R/IL23R signaling
205 underlie defective immunity in autosomal dominant chronic mucocutaneous
206 candidiasis. PLoS One 2011; 6:e29248.

207 11. Sisto F, Miluzio A, Leopardi O, Mirra M, Boelaert JR, Taramelli D. Differential
208 cytokine pattern in the spleens and livers of BALB/c mice infected with Penicillium
209 marneffei: protective role of gamma interferon. Infect Immun 2003; 71:465-73.

210 12. Wang X, Lin Z, Gao L, Wang A, Wan Z, Chen W, et al. Exome sequencing reveals a
211 signal transducer and activator of transcription 1 (STAT1) mutation in a child with

212 recalcitrant cutaneous fusariosis. J Allergy Clin Immunol 2013; 131:1242-3.

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Table 1 Core clinical features of 3 patients with systemic *P. marneffei* infection and *STAT1* mutations.

	P1	P2	P3
Gender	M	F	F
Age of presentation	Infancy	Infancy	infancy
Family history	Nil of significance	Nil of significance	Nil of significance
Infections			
Fungus	CMC, disseminated PM	CMC, *C. albicans* and *C. tropicalis* otitis externa, disseminated PM	CMC, disseminated PM, disseminated aspergillosis
Bacteria	Nil documented	Recurrent sinopulmonary infections	Recurrent sinopulmonary infections
Mycobacteria	Nil documented	Nil documented	*M. tuberculosis* lymphadenopathy
Virus	Nil documented	H1N1 influenza A respiratory infection with prolonged carriage, CMV pneumonitis	Recurrent herpes zoster reactivation, EBV-associated hemophagocytosis
Mutation			
Nucleotide change	c.800C>t	c.1074G>T	c.863C>T
Amino acid change	p.A267V	p.L358F	p.T288I
Domain	Coiled-coil domain	DNA binding domain	Coiled-coil domain
Carrier status of parents	Not carrier	Not carrier	Mother - not carrier (father not checked)

Figure legend

Figure 1. Gain-of-phosphorylation STAT1 mutations impaired IFNγ and IL17 responses. **A,** PBMCs were stimulated with IFNα or **B,** IFNγ and analyzed for intracellular pSTAT1 expression by gating on lymphocytes. The increase in %pSTAT1+ population in stimulated cells relative to unstimulated cells was calculated. Representative histograms are shown for P1 and a normal control. **C,** PBMCs from P1 were stimulated by IFNγ followed by treatment with staurosporine for 30 or 60 minutes. The percentage of intracellular pSTAT1 expression and mean fluorescence intensity (MFI) were determined in monocytes by flow cytometry. **D,** PBMCs were stimulated with PMA plus ionomycin and intracellular expression of IFNγ and IL17A in CD3+ T-cells was analyzed by flow cytometry. **E,** PBMCs were co-cultured with *C. albicans* (MOI of 5) or *P. marneffei* conidia (MOI of 1) for 48 hours, and IFNγ in the supernatant was quantified.