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Genetic basis of chronic mucocutaneous candidiasis disease in humans

Romain Lévy

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Romain Lévy. Genetic basis of chronic mucocutaneous candidiasis disease in humans. Immunology. Université Sorbonne Paris Cité, 2017. English. NNT : 2017USPCB101 . tel-02145223

HAL Id: tel-02145223

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THESE DE DOCTORAT EN SCIENCES
UNIVERSITE PARIS DESCARTES – DIDEROT

Pour l'obtention du diplôme de

DOCTEUR DE L'UNIVERSITE PARIS V
SPECIALITE: IMMUNOLOGIE

Présentée et soutenue publiquement par

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Le 14 Novembre 2017

Sujet de thèse :

Genetic basis of chronic mucocutaneous candidiasis disease in humans

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Remerciements

Je voudrais en premier lieu remercier les membres du Jury, Sarah Gaffen, Qian Zhang, Isabelle Meyts, et Eric Oksenhendler pour avoir accepté d'évaluer ce travail. J'en suis très heureux et également honoré.

Je tiens à remercier Anne Puel, ma directrice de thèse, pour m'avoir accompagné ces dernières années, pour ses conseils, son exigence, les discussions nombreuses et sa confiance m'ayant aidé pour mener à bien ce travail de recherche.

Merci également à Jean-Laurent Casanova que j'ai rencontré en 2011, alors interne dans le service d'Immunologie-Hématologie et Rhumatologie pédiatrique de l'hôpital Necker. Je tiens à le remercier pour avoir accepté que je rejoigne son équipe entre Paris et New York. Merci pour avoir rendu possible cette formidable expérience à l'université Rockefeller. Merci à lui pour son exigence, que ce soit dans le raisonnement expérimental, la communication de mes résultats et enfin la rédaction d'un article : le « fabricating and communicating science ». J'ai appris, du moins je le crois, avec Anne et Jean-Laurent un raisonnement nouveau, une rigueur, et un langage.

Je remercie également Laurent Abel, pour son soutien, sa disponibilité, les discussions que nous avons eu, et au-delà de mon sujet même de recherche.

Je tiens aussi à remercier les membres de l'équipe que dirige Anne Puel. Mélanie Migaud pour ton enthousiasme inébranlable et ton aide indispensable ces dernières années. Kunihiko Moriya, Erika Della-Mina, Laura Israël, Ling Yun, Emile Corvillain pour toutes les discussions de tout ordre.

Un remerciement particulier à Vivien Béziat, arrivé en même temps que moi dans l'équipe, et qui sans aucun doute m'aura appris beaucoup de choses. On a fait équipe dès le départ et nos efforts ont payé. Dans chacun de nos projets respectifs et dissociés, nous avons échangé continuellement. Un compagnonnage et une amitié sincère. Merci l'ami.

Merci à tous les autres chefs d'équipe du laboratoire : Jacinta Bustamante, Emmanuelle Jouanguy, Bertrand Boisson, Stéphanie Boisson-Dupuis, Michael Ciancanelli, Shen-Ying Zhang. Un remerciement particulier à Bertrand, pour ta disponibilité, les innombrables discussions qui m'ont permis d'échanger sur mon travail, et donc d'avancer lors de mon séjour à l'université Rockefeller.

Merci à toute l'équipe du laboratoire de Paris : Maya, Caroline, Joëlle, Alejandro, Carmen, Antoine, Lazaro, Céline, Cécile, Lahouari, Jérôme, et l'équipe du « dry » lab. Merci Antoine, pour ta bonne humeur inoxydable, ton humour toujours douteux qui font du bien à tout le monde dans cette équipe. Continue !

I also want to sincerely thank all the members from the New-York branch at the Rockefeller university, and most particularly Jill, Ruben, Janet, Scott, Robert, Fabien, Serkan, Noé, Nick, Rui, Barbara, and Mark. I really enjoyed being part of your group in New-York, Barcelona, Iceland and everywhere we travelled together. Thanks to all for your help when I landed in NY, and when I

left, and in between for all the moments we shared. A special thought for Yelena and Dominick, for their help in our everyday-life, as graduate students. You both made my project feasible.

Still in New-York, I want to thank Frederic Geissmann from the Sloan Kettering Institute. Thank you for your interest in my project and all the discussions we had. Thank you very very much for having accepted that I could work in your lab, as much as I wanted. I really enjoyed to spend quite a bit of time in your lab and to meet with all your talented trainees. Here, I specially want to thank Tomi for his expertise and great help in my project.

I also want to thank Kang Liu from the Columbia University, for her collaboration as an expert in the field of dendritic cells and hematopoiesis, and to Jerry a post-doc in her lab. Jerry, thank you for your collaboration and all the skype calls we had. I wish you the best in your career.

Enfin, je voudrais remercier tous les “sponsors” : l’INSERM pour ce poste-accueil depuis 3 ans, la commission Franco-américaine Fulbright et la Phillip Foundation sans l’aide desquelles, mon projet aux Etats-Unis n’aurait pas été possible.

Evidemment, une pensée pour mes proches, mon épouse Sinja et ma fille Louise née à New-York devant Central Park. Quelle aventure, cela a été ! Merci à vous deux.

Je souhaite enfin écrire que je suis heureux de pouvoir continuer mon travail de recherche au-delà du doctorat, tout en retournant à mon métier de médecin, ceci avec le soutien de la fondation Bettencourt-Schueller, de l’équipe d’Immunologie-Hématologie et Rhumatologie pédiatrique de Necker, et de J-L Casanova. Merci à tous ces acteurs pour leur confiance !

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Abbreviations:

Abs: antibodies
AD: autosomal dominant
AD-HIES: AD Hyper-IgE syndrome
AIRE: autoimmune regulator
APS-1: autoimmune polyendocrine syndrome type 1
APC: antigen presenting cells
AR: autosomal recessive
BCR: B cell receptor
B-EBV: Epstein-Barr virus-immortalized B cell line
CARD9: Caspase recruitment domain-containing protein 9
CBAD: C/EBPs activation domain
CGD: chronic granulomatous disease
CMC: chronic mucocutaneous candidiasis
CMCD: chronic mucocutaneous candidiasis disease
iCMCD: isolated CMCD
sCMCD: syndromic CMCD
CD40L: CD40 Ligand
EAE: experimental autoimmune encephalomyelitis
GC: Germinal center
GOF: gain-of-function
IKK: inhibitor of κ B kinase
IL-12p40: Interleukin 12 p40 subunit
IL-12p35: Interleukin 12 p35 subunit
IL-23p19: Interleukin 23 p19 subunit
IL-1 β : Interleukin 1 β
IL-17A: Interleukin 17A
IL-17RA: Interleukin-17 receptor A
IL-1R: Interleukin 1 receptor
IL-23R : IL-23 receptor chain
IL-12R β 1 : IL-12 receptor β chain 1
IKK: inhibitor of κ B kinase
Iono: Ionomycin
LPS: Lipopolysaccharide
LOF: loss-of-function
MSMD: Mendelian Susceptibility to Mycobacterial Disease
NGS: Next generation sequencing
mRNA: messenger RNA
NGS: Next generation sequencing
OPC: oropharyngeal candidiasis
PHA: Phytohemagglutinin
PID: primary immunodeficiency
PMA: phorbol myristate acetate
KO: Knock-out
RORC: RAR-related orphan receptor C
SEFIR: similar expression of fibroblast growth factor and IL-17R

SEFEX: SEFIR extension domain
TB: tuberculosis
TLR: Toll-like receptor
TNF- α : Tumor necrosis factor α
TIR: Toll/IL-1 receptor
Tfh: T follicular helper
Th1: T helper type 1
Th2: T helper type 2
Th17: T helper type 17
Treg: Regulatory T cells
WES: Whole exome sequencing

Publication list

Lanternier, F., Mahdaviani, S.A., Barbati, E., Chaussade, H., Koumar, Y., **Lévy, R.**, Denis, B., Brunel, A.-S., Martin, S., Loop, M., Peeters, J., de Selys, A., Vanclaire, J., Vermylen, C., Nassogne, M.-C., Chatzis, O., Liu, L., Migaud, M., Pedergrana, V., Desoubeaux, G., Jouvion, G., Chretien, F., Darazam, I.A., Schäffer, A.A., Netea, M.G., De Bruycker, J.J., Bernard, L., Reynes, J., Amazrine, N., Abel, L., Van der Linden, D., Harrison, T., Picard, C., Lortholary, O., Mansouri, D., Casanova, J.-L., Puel, A., 2015. Inherited CARD9 deficiency in otherwise healthy children and adults with *Candida* species-induced meningoencephalitis, colitis, or both. *J. Allergy Clin. Immunol.* 135, 1558–1568.e2. doi:10.1016/j.jaci.2014.12.1930

Lévy, R., Okada, S., Béziat, V., Moriya, K., Liu, C., Chai, L.Y.A., Migaud, M., Hauck, F., Al Ali, A., Cyrus, C., Vatte, C., Patiroglu, T., Unal, E., Ferneiny, M., Hyakuna, N., Nepesov, S., Oleastro, M., Ikinciogullari, A., Dogu, F., Asano, T., Ohara, O., Yun, L., Della Mina, E., Bronnimann, D., Itan, Y., Gothe, F., Bustamante, J., Boisson-Dupuis, S., Tahuil, N., Aytakin, C., Salhi, A., Al Muhsen, S., Kobayashi, M., Toubiana, J., Abel, L., Li, X., Camcioglu, Y., Celmeli, F., Klein, C., AlKhater, S.A., Casanova, J.-L., Puel, A., 2016. Genetic, immunological, and clinical features of patients with bacterial and fungal infections due to inherited IL-17RA deficiency. *Proc. Natl. Acad. Sci. U. S. A.* 113, E8277–E8285. doi:10.1073/pnas.1618300114

Ling, Y., Cypowyj, S., Aytakin, C., Galicchio, M., Camcioglu, Y., Nepesov, S., Ikinciogullari, A., Dogu, F., Belkadi, A., **Lévy, R.**, Migaud, M., Boisson, B., Bolze, A., Itan, Y., Goudin, N., Cottineau, J., Picard, C., Abel, L., Bustamante, J., Casanova, J.-L., Puel, A., 2015. Inherited IL-17RC deficiency in patients with chronic mucocutaneous candidiasis. *J. Exp. Med.* 212, 619–631. doi:10.1084/jem.20141065

Manuscript in preparation:

Lévy, R., Beziat, V., Zhou, Y.J., Markle, J., Lazarov, T., Moriya, K., Lim, A.I., Migaud, M., Belkaya, B., Belkadi, A., Breton, S., Sadek, H., Andre-Schmutz, I., Breton, G., Boisson, B., Martinez-Barricate, R., Neven, B., Picard, C., Abel, L., Di Santo, J.P., Bousfiha, A., Blanche, S., Tangye, S., Liu, K., Geissmann, F., Bustamante, J., Casanova J.-L., Puel, A. Multiple infections and combined immunodeficiency in a patient with inherited c-REL deficiency. *In preparation*

Communications:

R. Lévy, C. Wang, N. Mahlaoui et al. ACT1 deficiency: a defect in IL-17 mediated immunity underlying chronic mucocutaneous disease (CMCD) in a multiplex consanguineous kindred. Abstract ESID-0333. ESID Biennial Meeting, Prague.

R. Lévy, V. Beziat, K. Moriya, et al. A new genetic defect in a patient with chronic mucocutaneous candidiasis and disseminated BCG disease. Abstract ESID6-0330. ESID Biennial Meeting, Barcelona, Spain.

R. Lévy, S. Okada, V. Beziat, et al. Inherited IL-17RA deficiency in 17 patients with chronic mucocutaneous candidiasis. ESID6-0105. ESID Biennial Meeting, Barcelona, Spain.

Introduction

Infectious diseases in humans are commonly seen as “environmental” in essence. The field of human genetics of infectious diseases tests the hypothesis that infectious diseases are not only environmental (dependent on the presence of the pathogen), but also genetic. The working hypothesis of our laboratory is that human genetic variability largely determines the occurrence and outcome of primary infections^{1,2}. Specifically, we suggest that life-threatening infectious diseases of childhood result from inborn errors of immunity, in at least some and, perhaps, most children. In this context, I investigated rare forms of inherited Chronic Mucocutaneous Candidiasis Disease (CMCD), either in otherwise healthy individuals (referred to as isolated CMCD, or iCMCD), or associated with other kind of clinical manifestations whether infectious or autoimmune (syndromic CMCD or sCMCD).

Chronic Mucocutaneous Candidiasis (CMC) is characterized by recurrent or persistent disease of the nails, skin, oral and genital mucosae, and mainly caused by the commensal fungus *Candida albicans*. *C. albicans* is a commensal of the human oro-gastrointestinal and vulvovaginal flora. However, in immunocompromised individuals, *C. albicans* is a major cause of mucosal and localized skin disease, or disseminated disease, usually acute (systemic, invasive candidemia)³. CMC is commonly associated with other infectious clinical manifestations in patients with impaired, primary or acquired, T cell-mediated immunity (function and/or numbers)⁴⁻⁶. These observations strongly suggest that T cells play a major role in protective mucocutaneous immunity to *C. albicans*.

CMC may also be associated with a narrower spectrum of infections, in patients with autosomal dominant (AD) Hyper-IgE syndrome (HIES)⁷, a rare complex primary immunodeficiency (PID) characterized by high plasma IgE levels, atopic dermatitis, recurrent severe skin and pulmonary infections, mostly caused by *Staphylococcus aureus*, and skeletal

abnormalities, and caused by dominant negative loss-of-function (LOF) mutations in *STAT3* gene. CMC is also an important infectious phenotype, associated with susceptibility to mucocutaneous (e.g. dermatophytosis) and systemic fungal (e.g. invasive candidiasis) infections, in patients with autosomal recessive (AR) *CARD9* (Caspase recruitment domain-containing protein 9) deficiency^{8,9}. CMC is also present in around 25% of the patients with AR *IL-12p40* (for Interleukine 12p40) or *IL-12Rβ1* (for IL-12 receptor β chain 1) deficiency. These patients are also susceptible to weakly virulent mycobacteria, such as BCG vaccine and environmental mycobacteria, a rare syndrome termed MSMD (for Mendelian Susceptibility to Mycobacterial Disease)¹⁰. CMC is also present in all the patients bearing heterozygous missense gain-of-function (GOF) mutations of *STAT1*, who also display autoimmune and infectious complications¹¹. Furthermore, CMC is the only infectious complication seen in 90% of the patients with AR APS-I (autoimmune polyendocrine syndrome type I) syndrome, resulting from mutations in the *AIRE* (autoimmune regulator) gene¹². *AIRE* is involved in T-lymphocyte tolerance, clearly accounting for the observed autoimmune endocrine phenotype.

Finally, a group of patients without any identified PID, have inherited “idiopathic” CMC, referred to as CMCD. Such condition is often severe and would affect 1 individual in a 100 000. Most cases are sporadic, however several cases are reported in a same family with an AR or an AD pattern of inheritance. The working hypothesis is that a monogenic defect explains idiopathic forms of CMC, at least in some individuals.

These past 10 years, the cellular and molecular pathogenesis of CMC has begun to be deciphered^{5,13}. This has been greatly facilitated by the development of mouse models for CMC and the discovery of IL-17 cytokines, comprising in particular IL-17A, IL-17F, secreted by two subsets of memory CD4⁺ T helper cells: Th17 cells^{14,15}, and Th1* (also producing IFN-γ)¹⁶; but also γδ T cells, natural killer (NK) cells, type 3 innate lymphoid

cells (ILC3) or invariant NK T cells (iNKT)¹⁷. Mouse models helped in characterizing the immune response against *C. albicans*, and the critical role of the IL-17A/IL-17F-mediated immunity^{18,19}. Also, studies from the laboratory suggested that a defect of IL-17A/IL-17F-mediated immunity could be the cause of CMC observed in AD-HIES, AR IL-12p40, AR IL-12R β 1 deficiencies, and AR APS-1^{20,21} (see below).

Briefly, the stimulation of the C-type lectin Receptors Dectin-1 or Dectin-2, by *C. albicans* induces through the Syk kinase and the CARD9 adaptor, the activation of the NF- κ B signaling pathway and the secretion of pro-inflammatory cytokines, such as IL-1 β , IL-6, TGF- β or IL-23. These cytokines lead to the differentiation of naïve CD4⁺ T lymphocytes toward IL-17- (IL-17A and IL-17F) secreting memory CD4⁺ T cells, so called Th17, that also secrete IL-22, IL-21 and IL-26²². IL-21 further amplifies Th17 differentiation in a positive feedback loop, by inducing IL-21 et IL-23R (IL-23 receptor chain)²³. Several transcription factors are required for Th17 cell differentiation, in particular ROR- γ t, and STAT3²². In human, specific *C. albicans* memory Th17 lymphocytes express on their surface the skin and mucosal targeting receptors CCR4 and CCR6. These cells thus confer skin and mucosal protection against pathogens (especially against extracellular bacteria and some fungi) by inducing attractive and activating factors of neutrophils and the secretion of anti-microbial peptides such as β -defensins²⁴, by epithelial cells expressing the heterodimeric receptor for IL-17A and IL-17F, composed of IL-17RA and IL-17RC (for IL-17 receptor A and C).

As (i) IL-17-producing cells, and notably Th17 cells, play a crucial role in mucosal protection to *C. albicans* in mice^{18,19}, (ii) IL-6, IL-21, and IL-23, through the activation of STAT3, induce ROR- γ t expression, which is required for Th17 differentiation, and (iii) IL-12p40 and IL-23p19 compose the functional IL-23 cytokine, which signals through the heterodimeric receptor composed of the IL-12R β 1 and IL-23R chains, Anne Puel and others have evaluated these Th17 cells in AD-HIES patients, and patients with AR IL-12p40 or IL-

12R β 1 deficiency. As expected, these studies resulted in the identification of a strong reduction in Th17 cells in AD-HIES patients that might account for the CMC²⁰. Furthermore, Th17 cells are also reduced in AR IL-12p40 and IL-12R β 1 deficient patients, probably because of a defective production of, or response to IL-23, respectively²⁰. These results strongly suggested that the impaired Th17 response observed in this three PID could underlie CMC. This idea was further extended, by hypothesizing that the CMC observed in AR APS-1 patients may also result from impaired IL-17A/IL-17F-mediated immunity. As these patients have high levels of pathogenic autoantibodies (auto-Abs), Anne Puel, and other colleagues, looked for auto-Abs against IL-17 cytokines, and have shown that APS-1 patients have indeed high levels of neutralizing auto-Abs against IL-17A, IL-17F and/or IL-22 that might account for the CMC observed in these patients^{21,25}. Finally, patients with GOF mutations in *STAT1* display low proportions of Th17 cells²⁶, although the mechanism by which these mutations impair Th17 differentiation is still not well understood.

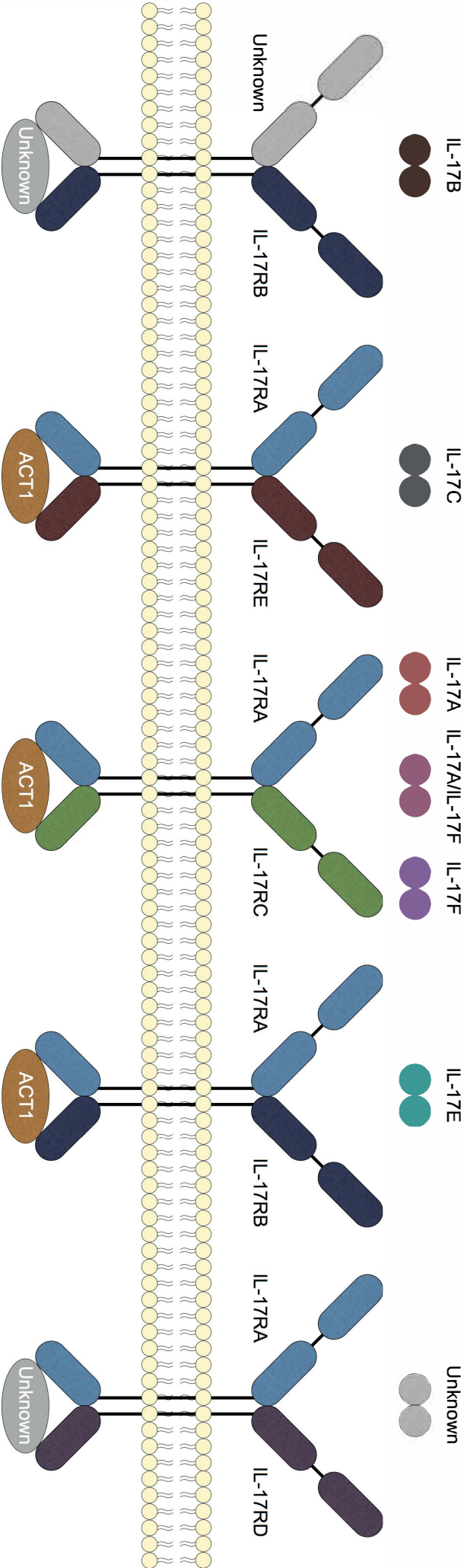
In the past decade, the laboratory has enrolled 402 patients from 289 families with isolated or syndromic forms of CMCD. During my PhD program, I identified by Whole Exome Sequencing (WES), patients with AR IL-17RA deficiency who all suffered from early onset and severe CMC. Importantly, the identification of these patients and of novel genetic forms of IL-17RA deficiency is crucial to better delineate the clinical phenotype caused by this PID, solely described in a single patient until then²⁷. I also identified in a single patient with sCMCD, a bi-allelic LOF mutation in the gene *REL*, encoding c-REL, a member of the NF- κ B family. No deleterious mutation affecting this gene have been described to date.

Autosomal recessive IL-17RA deficiency in patients with fungal and bacterial diseases: genetic, immunological, and clinical features

1. IL-17RA and the IL-17-receptors

Human IL-17RA is a 866-amino acid protein. A second and shorter isoform of 832 amino acids results from a mRNA splice variant, lacking the exon 11. IL-17RA was the first member of the IL-17-receptors family to be cloned²⁸, and remains the most studied. Since the discovery of IL-17RA, four other IL-17 Receptors have been identified: IL-17RB, IL-17RC, IL-17RD and IL-17RE²⁹. The IL-17 Receptors are single-pass transmembrane receptors and share a common structure. In particular, all receptors contain a conserved cytoplasmic functional domain, structurally similar to the TIR domain found in the TLR/IL-1R, so-called SEFIR domain (for similar expression of fibroblast growth factor and IL-17R)³⁰. The SEFIR domain is essential for mediating downstream signals as it interacts with the cytosolic adaptor molecule ACT1³¹. Also, the extracellular structure of IL-17 Receptors contains 2 fibronectin II-like domains, involved in protein-protein interaction and the binding to their ligand. IL-17RA is however distinct from the other family members as it contains additional functional motifs. IL-17RA cytoplasmic tail also contains a ~100 residue SEFEX domain (for SEFIR extension domain), that is essential for the transmission of downstream signaling, independently of ACT1 recruitment³². The distal cytoplasmic portion of IL-17RA also contains another functional domain involved in C/EBP transcriptional factors activation, and therefore termed CBAD (for C/EBPs activation domain)³³. To date, the prevailing model for IL-17 signaling is that IL-17RA pairs with another IL-17 Receptor to form a heterodimeric complex conferring ligand specificity towards a given IL-17 cytokine (**Figure 1**)³⁴. Importantly, in contrast to other IL-17 Receptors, whose expression is restricted to specific cell types, IL-17RA is broadly expressed in both immune and non-immune cells³⁵. Therefore,

Figure 1: The IL-17 cytokines and the IL-17-Receptors family



the expression profile of other IL-17 Receptors explains the target cell specificity of each IL-17 cytokine.

2. The IL-17 cytokines

The IL-17 cytokine family comprises six members, IL-17A to IL-17 F³⁴. IL-17A and IL-17F share the highest sequence homology (around 50%) and can be secreted as IL-17A and IL-17F homodimers, or IL-17A/IL-17F heterodimers, signaling through a IL-17RA/IL-17RC complex³⁶. In 2005, an important discovery was that IL-17A and IL-17F were produced by a separate lineage of memory CD4⁺ T helper cells, distinct from Th1 and Th2, so-called Th17^{14,15}. These Th17 cells are enriched in *Candida*-specific memory cells, as compared to other T helper subsets¹⁶. Besides Th17 cells, other IL-17-producing cell types present at the barrier sites, are an important and early source of IL-17A and IL-17F, such as $\gamma\delta$ T cells, ILC3 cells, iNKT cells, or NK cells¹⁷. The overlapping biological function of IL-17A and IL-17F in the setting of various pathological conditions such as host defense against pathogens, autoinflammation or autoimmunity have been studied in details (see below), and provided a rational for the development and usage of anti-IL-17A and anti-IL-17RA blocking antibodies in psoriasis or ankylosing spondylitis. As such, the fundamental studies of IL-17A and IL-17F biological functions in the past decade provide a remarkable example of translational research. IL-17E, also called IL-25, signals through a IL-17RA/IL-17RB complex³⁷. IL-17E shares 16% sequence homology with IL-17A, and has a distinct biological function from IL-17A and IL-17F. IL-17E is secreted by human eosinophils, basophils, and bronchial epithelial cells^{38,39}, and promotes the expansion of human Th2 cells, expressing IL-17RA/IL-17RB, and their production of IL-4, IL-5 and IL-13³⁸. Also, IL-17E contributes to airway eosinophilic infiltration in mice^{40,41}, and therefore would promote allergic conditions. Accordingly, IL-17E transcripts are strongly elevated in human chronic asthmatic lungs³⁸. IL-

IL-17C binds to the IL-17RA/IL-17RE heterodimer and is produced by epithelial cells in response to TNF- α , IL-1 β or agonists to TLR2 and TLR5^{42,43}. Like IL-17A and IL-17F, IL-17C drives the expression of pro-inflammatory molecules by epithelial cells and induces tissue neutrophils infiltration⁴². Finally, the respective role of IL-17B and IL-17D is still under investigation. IL-17B is suggested to have antagonistic function on IL-17E-driven inflammation in a model of colitis, possibly mediated by a competition for the IL-17RB chain⁴⁴, whereas the function and the receptor for IL-17D is unknown.

3. IL-17RA signaling and target genes: the example of IL-17A and IL-17F

IL-17A and IL-17F homo- and heterodimers signal through a dimeric IL-17RA/IL-17RC complex, and upregulate the expression of genes encoding inflammatory proteins through two mechanisms, either by inducing *de novo* mRNA transcription, or by stabilizing mRNA transcripts³⁴. The target genes of IL-17A and IL-17F include proinflammatory cytokines (such as G-CSF, IL-6, IL-1 β); chemokines (such as CXCL1, CXCL2, CXCL5) and antimicrobial peptides (S100 proteins, defensins). Importantly, IL-17A and IL-17F alone are poor mediators of inflammation. However, these cytokines synergize with other inflammatory signals, such as TNF- α , to mediate their inflammatory effector effect. Upon ligation with IL-17A and IL-17F homo- or heterodimers, IL-17RA/IL-17RC operates a conformational change enabling the recruitment of ACT1, through a homotypic SEFIR-SEFIR interaction⁴⁵. A first series of signaling events, termed the canonical pathway, lead to the activation of transcriptional factors and mRNA transcription. Briefly, ACT1 through its E3 ligase activity, mediates Lysine 63 ubiquitination of TRAF6 allowing the recruitment and activation of TAK1 and subsequently the activation of the inhibitor of κ B kinase (IKK) complex triggering NF- κ B nuclear translocation. Importantly, the recruitment of ACT1 to IL-17RA is necessary but not sufficient to allow ubiquitination of TRAF6, and downstream

signaling. Specifically, ACT1-mediated TRAF6 ubiquitination requires the presence of an additional ~100 amino acids beyond the SEFIR domain, termed SEFEX domain³². The activation of TRAF6 results in the activation of the MAPK pathway which includes p38, ERK and JNK; and of the C/EBPs pathway. A second non-canonical ACT1-dependent pathway elicited by IL-17A and IL-17F, leads to mRNA transcript stabilization: ACT1 recruits TRAF2 and TRAF5 to the receptor complex, which in turns recruit molecules involved in mRNA turnover. Precisely, these TRAF proteins can sequester mRNA-destabilizing proteins such as SF2, but also enhance mRNAs half-life through the recruitment of mRNA-stabilizing factors, such as HuR^{46,47}. In recent years, an increasing number of mechanisms negatively tuning IL-17 signaling have been deciphered. One mechanism is mediated by K63 deubiquitinase enzymes such as A20 targeting TRAF6⁴⁸, or USP25 targeting TRAF5 and TRAF6⁴⁹, impairing their respective interaction with other proteins. Another mechanism is through competition: TRAF3 interfering with ACT1/IL-17RA interaction⁵⁰, or TRAF4 competing with TRAF6 for the binding to ACT1⁵¹. Alternatively, other mechanisms have been elucidated such as the degradation of IL-17-induced mRNAs by enzymes⁵² or microRNAs; negative regulation of ACT1 mediated either by phosphorylation impairing TRAF6 recruitment⁵³, or by K48 ubiquitination leading to ACT1 degradation⁵⁴, or by microRNA⁵⁵. Importantly, the CBAD domain of IL-17RA participates to several of these negative regulatory mechanisms, as CBAD recruits TRAF3, A20, but also negatively regulates CEB β activity⁵⁶.

4. IL-17RA signaling confers host protection at the barrier sites

Since 2001, the study of mice invalidated for *Il17ra* highlighted the central role mediated by this receptor in host defense against pathogens at the barrier sites⁵⁷. Moreover, recent

studies in mice showed that IL-17RA-dependent immunity towards *C. albicans* in the oral cavity, or *Klebsiella pneumoniae* in the lungs, differs^{58,59}.

In a model of lung *K. pneumoniae* infection, *Il17ra* deficient mice show an increased susceptibility and mortality, an impaired production of several chemokines (CXCL1, CXCL2, CXCL5) and inflammatory cytokines (such as IL-6), and a failure to recruit neutrophils at the site of infection^{57,59,60}. Interestingly, neutrophils recruitment and *K. pneumoniae* clearance in the lungs are impaired in mice with a conditional knock-out (KO) of *Il17ra* or *Il17rc* in lung epithelial cells, thus suggesting that lung epithelial cells confer host protection against this pathogen⁵⁹. In contrast, *Il17re* deficient mice are not more susceptible to lung *K. Pneumoniae* infection, suggesting that IL-17RA-mediated protection is independent of IL-17C/IL-17RE signaling⁵⁹. *Il17ra* deficient mice also show an increased mortality while being challenged intratracheally with a live vaccine strain of *Francisella tularensis*, and show a higher bacterial burden in the lungs if challenged with *Staphylococcus aureus*^{61,62}.

In addition to the lung, IL-17RA signaling plays a non-redundant function in host defense against *C. albicans* in the oral cavity. Indeed, *Il17ra* deficient mice display a profound susceptibility in a model of OPC, similarly to mice invalidated for *Il23p19*¹⁸. Accordingly, analyses of the oral mucosa of these mice reveals a poor induction of IL-17 target genes such as chemokines involved in neutrophils recruitment (CXCL1, CXCL5), and consistent with the poor neutrophils infiltrate; T cell and Dendritic cell recruitment (CCL20); but also antimicrobial peptides such as β -defensin 3¹⁸. Further investigations show that both Th17 cells, and $\gamma\delta$ T cells contribute to the production of IL-17A and IL-17F in a IL-23-dependent manner, thus conferring protection in the oral cavity towards *Candida*^{18,19}. Importantly, mice invalidated for either *Il17c* or *Il17re* do not show an increased susceptibility to *Candida* in the oral cavity, thus suggesting that host protection conferred by IL-17RA in this model is

independent from IL-17C/ IL-17RE signaling⁶³. Moreover, *Il17ra* deficient mice and mice with a conditional KO of *Il17ra* in oral epithelial cells show a similar susceptibility in a model of OPC⁵⁸. In the latter case, and in contrast to the results obtained in the *K. pneumonia* lung infection model⁵⁹, β -defensin 3 production, rather than neutrophils infiltration, was identified as the core mechanism conferring immunity against *Candida*⁵⁸.

IL-17RA signaling is also important for mucocutaneous immunity to *S. aureus*. *Il17a/Il17f* double deficient mice spontaneously develop skin abscesses around nose and mouth to *S. aureus*⁶⁴, whereas *Il17ra* deficient mice show an impaired neutrophil recruitment, and develop severe skin lesions after cutaneous challenge with *S. aureus*⁶⁵. Interestingly, in the latter model, $\alpha\beta$ T cell-deficient mice are resistant, whereas $\gamma\delta$ T cell-deficient mice are susceptible to *S. aureus* skin challenge, which suggests that the response in the skin relies on resident IL-17A-producing $\gamma\delta$ T cells.

In the gut, *Il12p40*⁻, *Il23p19*⁻, *Il17a*⁻, *Il17f*⁻, and *Il17a/Il17f* double deficient mice are more susceptible *C. rodentium* infection^{64,66}. These data indirectly argue in favor of a role for IL-17RA signaling in host defenses in the intestinal barrier. Indeed, mice lacking IL-17A, or IL-17F, or IL-17A/IL-17F, show a higher bacterial burden, a production defect of β -defensins, and display more severe colonic inflammation as compared to controls. These mice spontaneously recover, however after a delayed period of time as compared to wild type controls⁶⁴. Finally, IL-17RA signaling has a protective role in different models of inflammatory colitis. *Il17a* deficient mice, and mice treated with IL-17RA blocking antibodies show a reduced accumulation of neutrophils, a colonic infiltration of proinflammatory IL-17A⁺/IFN γ ⁺ double positive CD4⁺ T cells, and a breakdown of intestinal barrier integrity⁶⁷. As well, *Il17a* deficient mice given Dextran sodium sulfate, display an increased colonic inflammation and impaired intestinal barrier integrity, secondary to

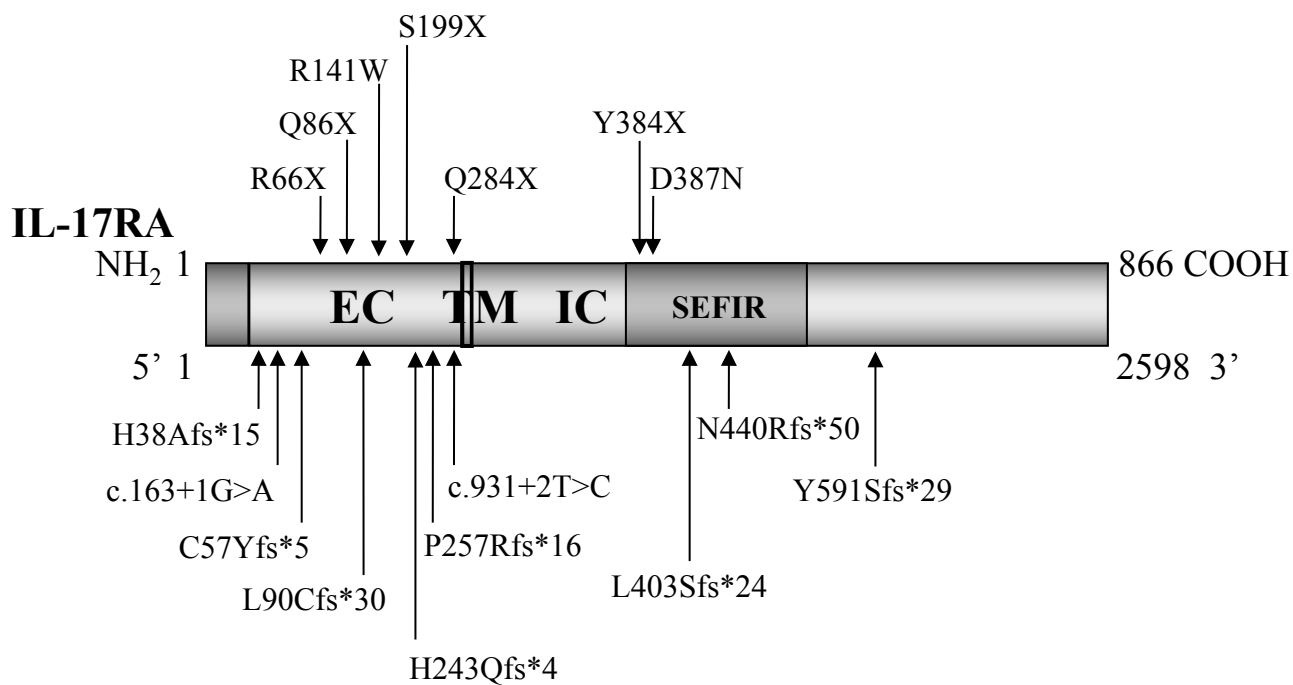
different mechanisms involving tight junction molecule localization⁶⁸, epithelial proliferation and wound repair⁶⁹.

5. AR IL-17RA deficiency in humans

a) Genetics

To date, a total of 27 patients bearing 16 different homozygous mutations in the *IL17RA* gene have been identified, with 21 patients and 12 different alleles already reported^{27,70} (**Article 1**). In terms of population genetics, not a single predicted LOF allele (stop gained; essential splice; frameshift insertion or deletion) is reported at the homozygous state in the healthy population (<http://gnomad.broadinstitute.org>). The mutations identified in the patients are either nonsense (R66X; Q86X; S199X; Q284X; Y384X); missense (R141W; D387N); frameshift deletions (H38Afs*15; L90Cfs*30; H243Qfs*4; P257Rfs*16; L403Sfs*24); frameshift insertions (C57Yfs*5; N440Rfs*50; Y591Sfs*29); or non-coding essential splice site mutations (c.163+1G>A; c.931+2T>C) (**Figure 2**). None of these alleles are reported in the public database gnomAD (comprising the WES data of 123,136 individuals), or in our in-house database of over 4,000 WES performed in patients with various infectious phenotypes. Furthermore, the combined annotation dependent depletion (CADD) scores for all these alleles predict a deleterious impact on the protein function⁷¹ (**Table 1**). The p.R141W missense, the two essential splice, four nonsense, and five frameshift mutations are located upstream from the segment encoding the transmembrane domain of IL-17RA. In contrast, the p.Y384X nonsense, the p.D387N missense, and the p.L403Sfs* and p.N440Rfs* frameshift mutations affect the intracellular SEFIR domain. In addition, the Y591Sfs* frameshift allele is located beyond the SEFIR domain, in the intracellular SEFEX domain. Interestingly, the p.D387N and the p.Y591Sfs* alleles encode for surface expressed receptors, whereas all the

Figure 2: Schematic diagram of IL-17RA protein, and the mutations responsible for AR IL-17RA deficiency



other tested alleles (highlighted in red in **Table 1**) are not detected at the surface of the patient's cells (fibroblasts or leucocytes) by flow cytometry.

b) Clinical features

IL-17RA deficiency is a fully penetrant AR disease. Accordingly, individuals bearing heterozygous LOF alleles are reported to be healthy, and all individuals homozygous for one of these LOF alleles, are sick. All patients display early onset symptoms, usually within the first year of life. However, the genetic diagnosis is usually made much later during infancy or young adulthood in our cohort. The course of the disease is chronic, and characterized by relapsing and recurrent infectious manifestations at the barrier sites. CMC is always present. Patients suffer from oral thrush, anogenital candidiasis, intertrigo, and infections of the nails (onyxis and perionyxis), due to *C. Albicans* whenever documented. In addition, 17 patients present with staphylococcal skin infections: abscesses, folliculitis, furunculosis, and disseminated crusted pustules notably affecting the scalp which can cause secondary alopecia. Importantly, if IL-17RA deficiency is usually depicted as a disease only responsible for mucocutaneous symptoms, pyogenic infections of the respiratory tract are reported, such as recurrent sinusitis (n=4), bronchitis (n=3) and otitis (n=2), but also lobar pneumonias (n=5). Therefore, IL-17RA deficiency is a disease predisposing to infections of the barrier sites, also including the upper and lower respiratory tract. Interestingly, none of the reported patients experienced symptoms suggesting a pathological process of the gastrointestinal tract. Pulmonary tuberculosis is reported in a single case, however without a microbiological proof. A second case of tuberculous meningitis in a BCG vaccinated patient is also reported, this time proven by a positive PCR and culture of the cerebrospinal fluid for *Mycobacterium tuberculosis*.

Table 1: Clinical characteristics of the 28 patients with AR IL-17RA deficiency

Patient (Kindred)	Age at diagnosis	Genotype [CADD score]	Sex	Consanguinity	Origin	Mucocutaneous features	Other clinical features
P1(9) (A)	1 mo	c.850C>T p.Q284X [40]	M	Yes	Morocco (living in France)	Skin, nails and oral mucosal candidiasis Skin pustules, folliculitis	No
P2 (B)	18 mo	c.256C>T p.Q86X [30]	F	Yes	Turkey	Genital and oral mucosal candidiasis	Suspected pulmonary tuberculosis
P3 (C)	1 mo	c.1302_1318dup p.N440Rfs*50 [22]	F	Yes	Turkey (living in France)	Scalp, genital and oral mucosal candidiasis Skin pustules, folliculitis	Eczema
P4 (C)	2 mo	c.1302_1318dup p.N440Rfs*50 [22]	M	Yes	Turkey (living in France)	Genital and oral mucosal candidiasis Skin pustules, folliculitis	Eczema, suspected tuberculous meningitis, lobar pneumonia
P5 (D)	9 yrs	c.1159G>A p.D387N [33]	M	Yes	Turkey	Scalp and oral mucosal candidiasis Skin pustules, folliculitis, furunculosis, seborrheic dermatitis	Sinusitis, lobar pneumonia
P6 (D)	4 yrs	c.1159G>A p.D387N [33]	F	Yes	Turkey	Scalp, genital and oral mucosal candidiasis Skin pustules, furunculosis, seborrheic dermatitis	Conjunctivitis
P7 (E)	1.5 yr	c.166_169dup p.C57Yfs*5 [34]	F	Yes	Turkey	Skin, scalp, nails, genital and oral mucosal candidiasis Skin pustules, folliculitis, furunculosis	Sinusitis
P8 (E)	1 yr	c.166_169dup p.C57Yfs*5 [34]	M	Yes	Turkey	Skin, scalp, nails and oral mucosal candidiasis Skin pustules, folliculitis, furunculosis	Sinusitis, conjunctivitis
P9 (F)	8 yrs	c.196C>T p.R66X [14]	F	No	Japan	Oral mucosal candidiasis Folliculitis	Eczema, bronchitis, lobar pneumonia
P10 (F)	6 yrs	c.196C>T p.R66X [14]	M	No	Japan	Skin, scalp and oral mucosal candidiasis Folliculitis	Eczema, bronchitis, lobar pneumonia
P11 (G)	25 yrs	c.112_119del p.H38Afs*15 [34]	M	Yes	Saudi Arabia	Oral mucosal candidiasis	No
P12 (G)	15 yrs	c.112_119del p.H38Afs*15 [34]	F	Yes	Saudi Arabia	Oral mucosal candidiasis	No
P13 (H)	1 mo	c.163+1G>A [25]	F	Yes	Algeria	Skin and genital mucosal candidiasis	No
P14 (I)	1 yr	c.1152C>A p.Y384X [38]	M	Yes	Argentina	Skin and oral mucosal candidiasis Skin pustules, folliculitis, furunculosis, abscess	Sinusitis, otitis, lobar pneumonia
P15 (J)	4 yrs	c.268del p.L90Cfs*30 [23]	F	Yes	Saudi Arabia	Skin, genital and oral mucosal candidiasis	No
P16 (J)	2 yrs	c.268del p.L90Cfs*30 [23]	M	Yes	Saudi Arabia	Skin, scalp, nails, genital and oral mucosal candidiasis	No
P17 (J)	15 yrs	c.268del p.L90Cfs*30 [23]	M	Yes	Saudi Arabia	Skin, scalp and oral mucosal candidiasis Folliculitis, furunculosis	No

P18 (J)	10 yrs	c.268del p.L90Cfs*30 [23]	M	Yes	Saudi Arabia	Skin, scalp and oral mucosal candidiasis Folliculitis, furunculosis	No
P19 (K)	22 yrs	c.1770_1771dup p.Y591Sfs*29 [26.7]	M	Yes	Turkey	Skin, scalp, nails and oral mucosal candidiasis Skin pustules	Otitis
P20 (L)	13 yrs	c.769_773del p.P257Rfs*16 [28]	F	Yes	Turkey	Oral mucosal candidiasis Skin abscess	No
P21 (L)	11 yrs	c.769_773del p.P257Rfs*16 [28]	F	Yes	Turkey	Oral mucosal candidiasis	No
P22 (M)	6 yrs	c.729_730del p.H243Qfs*4 [32]	M	Yes	Iran	Oral mucosal candidiasis	No
P23 (N)	23 yrs	c.596C>A p.S199X [35]	F	Yes	Maroc	Oral mucosal candidiasis Folliculitis, furunculosis	No
P24 (N)	24 yrs	c.596C>A p.S199X [35]	F	Yes	Maroc	Oral mucosal candidiasis Folliculitis, furunculosis	No
P25 (O)	35 yrs	c.421C>T p.R141W [22]	M	Yes	Iran	Skin, scalp and oral mucosal candidiasis	No
P26 (P)	23 yrs	c.1207del L403Sfs*24 [30]	M	No	Argentina	Skin, esophagal and oral mucosal candidiasis Skin pustules, folliculitis, furunculosis, abscess	Bronchitis adenitis
P27 (Q)	11 yrs	c.931+2T>C [22.8]	F	Yes	Iran	Oral mucosal candidiasis	No
P28 (Q)	3 yrs	c.931+2T>C [22.8]	F	Yes	Iran	Oral mucosal candidiasis	No

c) Immunological features

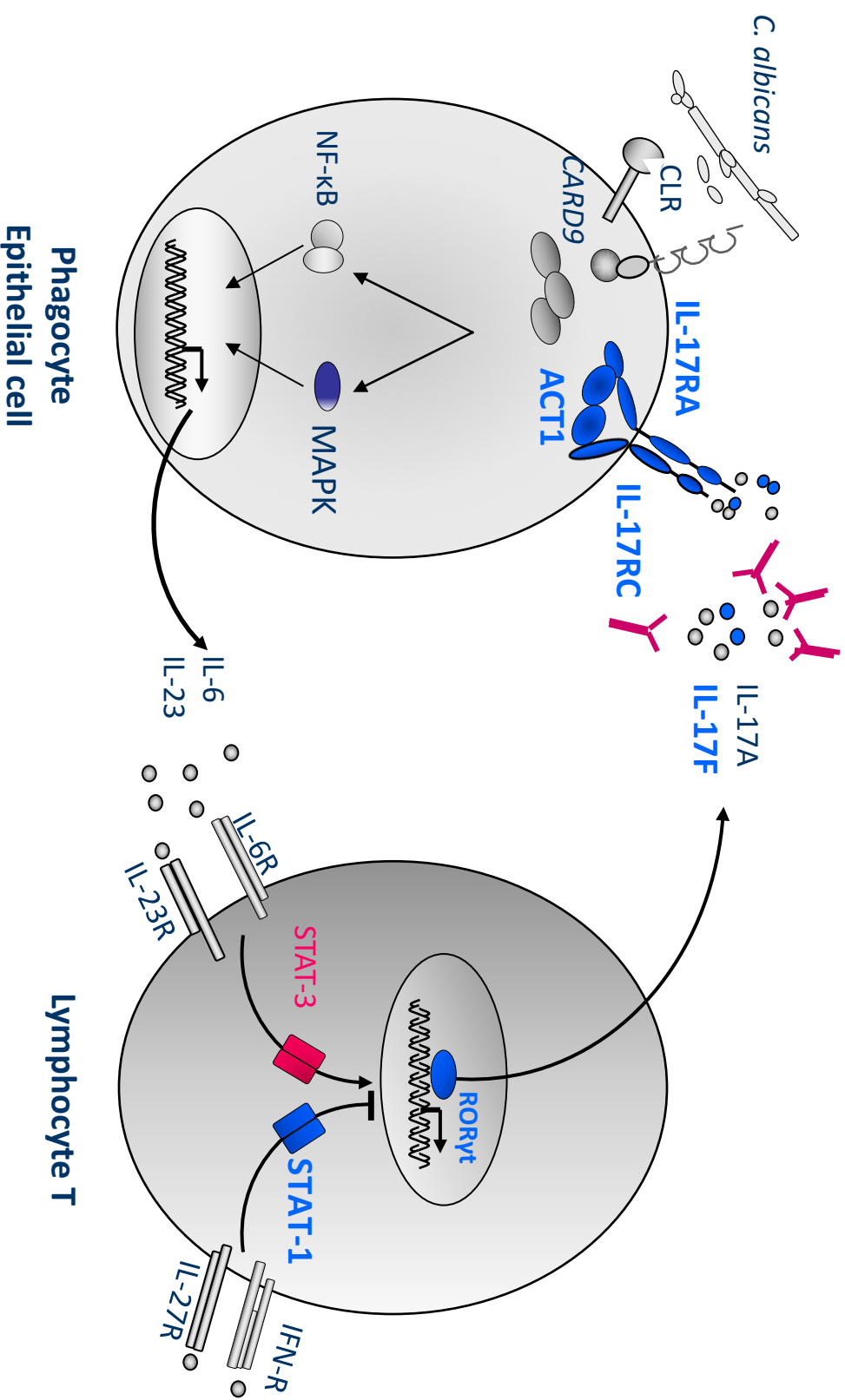
The production of IL-17A and IL-17F by ELISA in patient's whole blood after activation with phorbol myristate acetate (PMA) plus ionomycin (PMA/iono) is normal. By flow cytometry, the proportions of IL-17A⁺, and IL-17F⁺ memory CD4⁺ T cells among total CD4⁺ memory T cells, are increased in the tested patients. Finally, IL-17RA deficient patients have normal frequencies of circulating Th17 cells as assessed by the detection of CCR4 and CCR6 on the surface of CD4⁺ memory T cells, by flow cytometry.

IL-17RA deficiency abrogates the response to IL-17A and IL-17F homo- and heterodimers in fibroblasts, as well as the response to IL-17E/IL-25 in T cells. The classic method to evaluate the response to IL-17A and IL-17F in fibroblasts relies on the measurement of IL-6, CXCL1 secretion by ELISA or β -defensin 2 expression by qPCR. These target genes are induced by IL-17A, IL-17F or IL-17A/IL-17F cytokines, but their induction is strongly increased if TNF- α is added to the culture. Therefore, in patients' cells, the synergistic effect of TNF- α + IL-17A, or IL-17F, or IL-17A/IL-17F is abolished. In addition, patient's leucocytes cultured with IL-17E/IL-25, fail to secrete IL-5. As already mentioned, most mutations prevent IL-17RA expression at the cell surface and subsequently the cellular response to its ligands. Alternatively, the p.D387N expressed allele is complete LOF, as the mutation localized in the SEFIR domain abolishes the interaction of the mutated IL-17RA allele with the SEFIR domain of ACT1. Interestingly, The p.Y591Sfs* allele is expressed on patient's fibroblasts by flow cytometry. The mutation is localized in the SEFEX domain of IL-17RA. Importantly, the phenotype of the patient bearing this variant does not differ from patients with loss of expression alleles. We could hypothesize that the p.Y591Sfs* allele either affects ACT1 recruitment and the subsequent signaling events, or still can recruit ACT1 but impairs TRAF6 ubiquitination³².

6. Concluding remarks

In 2011, complete AR IL-17RA deficiency was first described in a single patient with iCMCD following a candidate gene approach²⁷. Concomitantly, AD IL-17F deficiency was found in a family with five affected individuals who suffer from iCMCD²⁷. The mutation identified in IL-17F (S65L) does not impair the ability of the mutated allele to dimerize with WT IL-17F, or IL-17A. However, S65L IL-17F homodimers do not bind to IL-17RA on control fibroblasts. Moreover, the S65L allele has a hypomorphic, dominant negative effect, when dimerized to IL-17A or WT IL-17F, in terms of IL-6 and CXCL1 production in control fibroblasts²⁷. Impaired IL-17A/IL-17F immunity was thus proven to be the key mechanism underlying CMC in humans. These discoveries paved the way to the identification of other PID associated with CMCD. Indeed, a WES approach led to the identification of AR IL-17RC and AR ACT1 deficiencies, genetic etiologies of iCMCD that impair IL-17A and IL-17F responses (AR ACT1 also impairs IL-17E/IL-25 signaling), and AR ROR- γ / γ T deficiency, a genetic etiology of sCMCD. AR ROR- γ / γ T impairs the differentiation of Th17 cells *in vitro*, and the production of IL-17A/IL-17F by memory CD4⁺ T cells *ex vivo*, thus underlying CMC. ROR- γ / γ T deficient patients also suffer from mycobacterial infections, as ROR- γ / γ T defect impairs IFN- γ production^{72–74}. AD HIES, STAT1 GOF, AR APS-1 and AR ROR- γ / γ T deficiency can be seen as syndromic forms of CMCD. In either case, impaired IL-17A/IL-17F- or IL-17RA/IL-17RC-dependent immunity is the core mechanism accounting for CMC in patients with any of these eight inherited disorders (**Figure 3**). Indeed, all patients with inborn errors of IL-17F, IL-17RA, IL-17RC, or ACT1, display CMC. These patients display dysfunctional IL-17F and IL-17A/IL-17F (AD IL-17F deficiency), or dysfunctional responses to IL-17A, IL17A/IL-17F, and IL-17F (mutations in *IL17RA*, *IL17RC*, and *ACT1*). In patients with AD HIES^{20,75–77}, AD STAT1 GOF^{78–93}, or AR ROR-

Figure 3: CMCD, a defect of IL-17 immunity



IL-17F, IL17RA, IL17RC, ACT1, RORγt, STAT3 LOF; STAT1 GOF mutations and APS-1

Eight genetic causes of inherited CMC

$\gamma/\gamma T$ deficiency⁷² the development of Th17 cells is impaired. Finally, patients with AR APS-1 have high titers of neutralizing auto-Abs against IL-17A and IL-17F^{21,25}.

IL-17RA deficiency is a rare and severe disease. IL-17RA deficiency severely impacts on patient's quality of life, as this condition requires multiple subspecialty visits and hospitalizations, laboratory monitoring, and health care costs. In addition, life-threatening complications can occur, as IL-17RA deficiency predisposes to lobar pneumonias, presumably bacterial considering the good response to antibiotics, yet not documented. Even though few patients with AD IL-17F and AR IL-17RC deficient patients have been reported so far, patients with AR IL-17RA or AR ACT1 deficiencies tend to present with a much severe disease course. In AD IL-17F deficiency, the response to IL-17A and WT IL-17F is reduced, yet not abolished, possibly explaining a milder phenotype in these patients²⁷. Human keratinocytes incubated with IL-17C, or IL-17A, induce a similar set of genes encoding for chemokines, inflammatory cytokines and antimicrobial peptides⁴². Moreover, like IL-17A, IL-17C synergizes with TNF- α in inducing β -defensin 2 from human keratinocytes⁴². IL-17C signals through a IL-17RA/IL-17RE complex, in an ACT1-dependent manner, but independently of IL-17RC³⁵. Therefore, the milder phenotype observed in IL-17RC deficiency could result from IL-17C rescuing part of the defect, as IL-17A and IL-17C have overlapping biological functions. Alternatively, IL-17E/IL-25 signaling could also play a role. However, it remains challenging to link IL-17E/IL-25 signaling to skin and mucosal immunity to *Candida* and *Staphylococcus*³⁴.

Interestingly, tuberculosis (TB) occurred in two unrelated BCG-vaccinated patients. Inborn errors of IFN- γ -mediated immunity are responsible for susceptibility to mycobacteria, including TB. Mutations in genes predisposing to tuberculosis⁹⁴ are not detected by WES in these patients: these includes monogenic defects of the respiratory burst of the phagocytic cells (X linked recessive (XR) CYBB, AR CYBA, AR NCF1, AR NCF2 and AR NCF4

deficiencies); monogenic defects responsible for MSMD, impairing the IL-12-IFN- γ pathway (AR IFN- γ R1, AR IFN- γ R2, AD STAT1, AR IL12p40, AR IL-12RB1, and AR IL-12RB2 deficiencies), or other PID associated with childhood TB (AR TYK2, XR CD40L, XR NEMO and AD GATA2 deficiencies)⁹⁴. Finally, we did not test for the presence of anti-IFN- γ auto-abs in the patient's serum, which are reported to be responsible for rare cases of childhood TB⁹⁴. However, this condition is usually associated with other infectious complications such as *Salmonella* infections, or *Cryptococcus neoformans* meningitis. *Il17ra* deficient mice or mice given anti-IL-17A blocking antibodies, are not more susceptible than controls to *Mycobacterium tuberculosis*^{95,96}. Likewise, patients who are given anti-IL-17A blocking antibodies for psoriasis, psoriatic arthritis or ankylosing spondylitis do not present a higher risk of either acute TB, or reactivation of latent TB infection⁹⁷. Alternatively, data in mice showed that IL-17-producing ESAT-specific T cells populate the lung 12 days after vaccination with ESAT-6, a peptide of *M. tuberculosis*. Upon recall with *M. tuberculosis* aerosol challenge, IFN- γ -producing CD4⁺ T cells accumulate in the lungs of WT mice, but not in the ones of *Il23p19* deficient mice, or WT mice treated with IL-17A blocking Abs. Indeed, further analysis show that IL-17A confers protection upon recall, by inducing the production of chemokines (CXCL9, CXCL10 and CXCL11) recruiting IFN- γ -producing CD4⁺ T cells⁹⁸. Therefore, we can hypothesize that IL-17RA deficient patients are more susceptible to TB, as upon infection, IL-17A signaling is important, but probably not crucial, for the optimal recruitment of IFN- γ -producing CD4⁺ T cells.

The diagnosis of AR IL-17RA deficiency is challenging. A familial history of consanguinity and/or another case among the siblings is strongly suggestive of an AR trait, although we report sporadic cases in non-consanguineous families; IL-17RA deficiency always manifests early, before the first year of life; CMC is always present; immunophenotyping of T-, B- and NK lymphocytes subsets is normal. As opposed to patients

with *STAT1*^{+/-} GOF mutations and *STAT3*^{+/-} dominant negative LOF mutations also displaying CMC, patients with IL-17RA deficiency do not display syndromic features such as autoimmunity, connective tissue abnormalities or other invasive infections. The confirmation of the diagnosis relies on the documentation of a bi-allelic mutation in *IL17RA* gene, and the demonstration of a defective response to IL-17A/IL-17F. To date, the sequencing of *IL17RA* gene is only feasible in academic laboratories or specialized centers for PID care. Alternatively, targeted NGS (Next Generation Sequencing) is a rising and interesting approach to document disease-causing mutations of over 300 genes known to be associated with particular PIDs, including *IL17RA*. A pragmatic screening approach could be to test for IL-17RA protein expression by flow cytometry on circulating leucocytes. Flow cytometry is routinely used for the screening of other PIDs (such as HLA class II; CD40; perforin; WAS; DOCK8; IL-2RG deficiencies) and offers a quick, reliable, and cost-effective answer. If IL-17RA is not expressed on the patient's cells, Sanger sequencing of *IL17RA* gene, followed by a functional *in vitro* assay should be performed to confirm the diagnosis. Importantly, IL-17RA can be expressed but not functional. In the latter case, a functional assay should be performed to confirm that the response to IL-17RA-dependent signals is abolished, if a bi-allelic mutation is documented. The response to IL-17A +/- TNF- α in the patient's fibroblasts is a reliable assay, but requires a skin biopsy. An alternative and non-invasive method is to test the response to IL-17E/IL-25 in circulating leucocytes in terms of IL-5 production by ELISA.

There are no current guidelines for the management of IL-17RA deficient patients. The management of CMC relies on sporadic antifungal treatments, with FLUCONAZOLE. Patients with recurrent staphylococcal skin infections would probably also benefit from repeated decontaminations of skin reservoirs for *S. aureus*. Importantly, IL-17RA deficient patients are prone to pyogenic infections of the upper respiratory tract, and occasionally to

lobar pneumonia. Other PID affecting antibody production classically predispose to these types of infectious complications. Immunophenotyping of B cells as well as IgG, IgA, IgM dosage are normal in all the patients that have been tested. The vulnerability of IL-17RA deficient patients to pyogenic infections of the respiratory tract, also documented in mice, probably reflects intrinsic immune functions of lung epithelial cells⁵⁹. *Il17ra* conditional KO in epithelial cells in mice, impairs CXCL5 production by lung epithelial cells (a chemoattractant for neutrophils), neutrophils infiltrate and clearance of *K. pneumoniae*⁵⁹. In light of this clinical observation, preventive measures should be considered for these patients. First, a computed tomography (CT) of the lungs could be performed at diagnosis, whether or not the patient already presented respiratory infections, to verify the presence of bronchiectasis, stigma of ancient infections. Such information is not available in our cohort. Preventive antibiotics can be discussed in a patient with bronchiectasis and/or after a first episode of lobar pneumonia and/or suffering from recurrent infections of the upper respiratory tract such as bronchitis, otitis, sinusitis. In addition, patients and household contacts should receive vaccinations notably protecting against pneumococcal and *Haemophilus influenzae* type b infections, but also seasonal influenza. β -defensin 3 in mice (the murin homologue of β -defensin 2 in humans), confers protection against *Candida* in the oral cavity⁵⁸. Whether β -defensin 3 also confers protection against *Candida* and *Staphylococcus* in the skin is unknown. We also observed that β -defensin 2 mRNA induction is abolished in the patient's fibroblast after IL-17A+ TNF- α . The development of new topical treatment with β -defensin, instead of repeated courses of antifungal drugs, could represent not only an innovative preventive option for patients with monogenic defects impairing the response to IL-17A and IL-17F, but also for patients with impaired production of IL-17A/IL-17F cytokines like in STAT3^{+/-} LOF and STAT1^{+/-} GOF mutations, who display severe CMC.

Article 1:

Inherited IL-17RA deficiency in patients

with fungal and bacterial diseases: genetic, immunological, and clinical features

Genetic, immunological, and clinical features of patients with bacterial and fungal infections due to inherited IL-17RA deficiency

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Contributed by Jean-Laurent Casanova, November 10, 2016 (sent for review August 29, 2016; reviewed by Kai Kisand and Mikko Seppanen)

Chronic mucocutaneous candidiasis (CMC) is defined as recurrent or persistent infection of the skin, nails, and/or mucosae with commensal *Candida* species. The first genetic etiology of isolated CMC—autosomal recessive (AR) IL-17 receptor A (IL-17RA) deficiency—was reported in 2011, in a single patient. We report here 21 patients with complete AR IL-17RA deficiency, including this first patient. Each patient is homozygous for 1 of 12 different IL-17RA alleles, 8 of which create a premature stop codon upstream from the transmembrane domain and have been predicted and/or shown to prevent expression of the receptor on the surface of circulating leukocytes and dermal fibroblasts. Three other mutant alleles create a premature stop codon downstream from the transmembrane domain, one of which encodes a surface-expressed receptor. Finally, the only known missense allele (p.D387N) also encodes a surface-expressed receptor. All of the alleles tested abolish cellular responses to IL-17A and -17F homodimers and heterodimers in fibroblasts and to IL-17E/IL-25 in leukocytes. The patients are currently aged from 2 to 35 y and originate from 12 unrelated kindreds. All had their first CMC episode by 6 mo of age. Fourteen patients presented various forms of staphylococcal skin disease. Eight were also prone to various bacterial infections of the respiratory tract. Human IL-17RA is, thus, essential for mucocutaneous immunity to *Candida* and *Staphylococcus*, but otherwise largely redundant. A diagnosis of AR IL-17RA deficiency should be considered in children or adults with CMC, cutaneous staphylococcal disease, or both, even if IL-17RA is detected on the cell surface.

genetics | immunodeficiency | candidiasis

Chronic mucocutaneous candidiasis (CMC) is characterized by chronic infections of the skin, nails, and oropharyngeal and genital mucosae caused by *Candida albicans*. It affects patients with various acquired T-cell immunodeficiencies, including HIV infection, who typically suffer from multiple infections. Inherited forms

Significance

Chronic mucocutaneous candidiasis (CMC) is defined as persistent or recurrent infections of the skin and/or mucosae by commensal fungi of the *Candida* genus. It is often seen in patients with T-cell deficiencies, whether inherited or acquired, who typically suffer from multiple infectious diseases. Rare patients are otherwise healthy and display isolated CMC, which often segregates as a Mendelian trait. In 2011, we described the first genetic cause of isolated CMC, with autosomal recessive (AR), complete IL-17 receptor A (IL-17RA) deficiency, in a single patient. We report here 21 patients from 12 unrelated kindreds, homozygous for 12 different mutant alleles that underlie AR IL-17RA deficiency. All patients have isolated CMC and their cells do not respond to IL-17A, -17F, and -17E/IL-25.

Author contributions: L.A., J.-L.C., and A.P. designed research; R.L., S.O., V.B., K.M., C.L., L.Y.A.C., M.M., L.Y., E.D.M., and D.B. performed research; F.H., A.A.A., C.C., C.V., T.P., E.U., M.F., N.H., S.N., M.O., A.I., F.D., T.A., O.O., F.G., J.B., S.B.-D., N.T., C.A., A.S., S.A.M., M.K., J.T., X.L., Y.C., F.C., C.K., and S.A.A. contributed new reagents/analytic tools; A.P. recruited patients; F.H., A.A.A., C.C., C.V., T.P., E.U., M.F., N.H., S.N., M.O., A.I., F.D., T.A., O.O., F.G., J.B., S.B.-D., N.T., C.A., A.S., S.A.M., M.K., J.T., X.L., Y.C., F.C., C.K., and S.A.A. recruited patients and recorded the clinical data; R.L. and Y.I. analyzed data; and R.L., J.-L.C., and A.P. wrote the paper.

Reviewers: K.K., University of Tartu; and M.S., Helsinki University Hospital.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1618300114/-DCSupplemental.

of CMC are less common and are often associated with other infectious and noninfectious complications, particularly in patients with profound T-cell deficits (1). Patients with autosomal dominant (AD) hyper-IgE syndrome (HIES), caused by heterozygous dominant negative mutations of *STAT3*, display fewer infections, and patients with autosomal recessive (AR) autoimmune polyendocrine syndrome type 1 (APS-1) are not prone to other infections (2, 3). Finally, rare patients with inherited but idiopathic forms of CMC, referred to as CMC disease (CMCD), have been described since the late 1960s (4–8). These patients may display isolated CMC, but they often also display cutaneous staphylococcal disease (nonetheless referred to as CMCD) or other infectious and/or autoimmune clinical manifestations (syndromic CMCD).

The genetic causes of CMCD described to date include AR IL-17RA deficiency in a single patient (9), AD IL-17F deficiency in a multiplex kindred (9), AR IL-17RC deficiency in three kindreds (10), and AR ACT1 deficiency in a multiplex kindred (ACT1 is a cytosolic adapter of IL-17 receptors) (11). IL-17RA and -17RC belong to the IL-17 receptor family, which also includes the IL-17RB, -17RD, and -17RE chains. These receptors form various heterodimers, through which different IL-17 cytokines signal in an ACT1-dependent manner (12). Finally, AD signal transducer and activator of transcription 1 (STAT1) gain of function (GOF) was reported in ~350 patients with syndromic CMCD (13–51) and found in approximately half of such patients in our study cohort. In patients with *STAT1* GOF mutations, CMC results, at least partly, from impairment of the development and/or survival of IL-17A/F–producing T cells, the underlying mechanisms of which remain unknown (28, 52). Patients with these mutations, who had long been known to be prone to thyroid autoimmunity, were recently found to display other infectious and autoimmune phenotypes (16, 17, 23, 37, 51). Another genetic etiology of syndromic CMCD has recently been described, with AR retinoic acid-related orphan receptors γ (ROR- γ/γ T) deficiency in three kindreds with CMC and severe mycobacterial disease (53).

AD HIES and AR APS-1 can, thus, also be seen as syndromic forms of CMCD. Alternatively, STAT1 GOF and ROR- γ/γ T deficiency can be seen as distinct entities, separate from CMCD. In either case, impaired IL-17A/F– or IL-17RA/RC–dependent immunity is the core mechanism accounting for CMC in patients with any of these eight inherited disorders. Indeed, all patients with inborn errors of IL-17F, -17RA, -17RC, or ACT1 display CMC. These patients display dysfunctional IL-17F and -17A/F (IL-17F mutations) or dysfunctional responses to IL-17A, -17A/F, and -17F (mutations in IL-17RA, -17RC, and ACT1). In patients with AD HIES (54–57), AD STAT1 GOF (13, 18, 21, 27–29, 32, 35, 36, 38, 39, 41, 42, 45, 46, 49), or AR ROR- γ/γ T deficiency (53), the development of IL-17A/F–producing T cells is impaired. Finally, patients with AR APS-1 have high titers of neutralizing auto-Abs against IL-17A and -17F (58, 59).

The pathogenesis of staphylococcal disease in CMCD patients is less clear. Staphylococcal skin disease is frequently observed in patients with ACT1 and IL-17RA deficiencies, but has not been reported in patients with IL-17F and -17RC deficiencies (9–11, 60). This observation suggests that staphylococcal disease may be partly due to an impairment of IL-17E/IL-25 responses, which normally require IL-17RA and ACT1, but neither IL-17F nor IL-17RC. However, too few patients have been described to draw firm conclusions. In particular, AR IL-17RA deficiency has been described in a single patient with CMC and cutaneous staphylococcal disease (9). We used a genome-wide approach based on whole-exome sequencing (WES) to identify 20 new patients, from 11 unrelated kindreds, bearing homozygous *IL17RA* mutations. Functional characterization of these variants showed them to be responsible for complete AR IL-17RA deficiency. We also characterized the associated clinical phenotype of the 21 patients, including the patient reported in 2011, encompassing not only CMC and

staphylococcal skin infections, but also bacterial infections of the respiratory tract.

Results

Clinical Reports. We investigated 21 patients with early onset, unexplained CMC (Fig. 1A). The patients originated from Morocco (kindred A), Turkey (kindreds B, C, D, E, K, and L), Japan (kindred F), Saudi Arabia (kindreds G and J), Algeria (kindred H), and Argentina (kindred I). The clinical features of patient 1 (P1) (kindred A), born to first cousins from Morocco, have already been reported (9). The 12 families were unrelated, and 11 were known to be consanguineous. All patients displayed CMC before the age of 6 mo, and 14 patients had also suffered from recurrent staphylococcal skin infections by the same age. CMC affected the skin (intertrigo), the scalp, mucosal sites (oral thrush; anogenital candidiasis), or nails (Table 1). These episodes were effectively managed or prevented with a combination of oral (fluconazole) and topical (nystatin) antifungal treatments. Staphylococcal skin infections were reported in 14 patients suffering from abscesses, folliculitis, furunculosis, and crusted pustules on the face and scalp, sometimes spreading to the shoulders and arms. In addition to these skin infections, eight children also had other recurrent infections, including otitis, sinusitis, bronchitis, and lobar pneumonia. Infections typically responded to antibiotics, but subsequently recurred. P2 and P4 were also treated for suspected pulmonary tuberculosis and tuberculous meningitis, respectively, without microbiological confirmation. None of the other clinical manifestations previously reported in patients with GOF *STAT1* mutations, such as autoimmune endocrinopathy, aneurysms, or mucosal carcinomas, were detected (16, 17, 23, 37, 51). Detailed phenotyping of lymphocyte subsets was performed for patients from kindreds D, E, and H and revealed no abnormality (Fig. S1).

Mutations in *IL17RA*. WES was performed for all patients and led to the detection of biallelic *IL17RA* mutations, which were confirmed by Sanger sequencing (Fig. 1A). No nonsynonymous coding sequence mutations were identified in the other five genes implicated in CMCD (*IL17F*, *IL17RC*, *ACT1*, *STAT1*, and *RORC*) or in any of the genes known to underlie related primary immunodeficiencies, including APS-1 and AD HIES. As shown in Fig. 1B, the only essential splice variant, three nonsense and four frameshift variants (and the corresponding premature stop codons), were located upstream from the segment encoding the transmembrane domain of IL-17RA. By contrast, the p.Y384X nonsense, p.D387N missense, and p.N440Rfs*50 frameshift variants affected the intracellular SEFIR (SEF/IL-17R) domain of IL-17RA, which is required for ACT1 recruitment, whereas the p.Y591Sfs*29 frameshift variant was located in the SEFEX domain (SEFIR extension domain) (61, 62). The healthy parents and siblings tested were all heterozygous for the mutant alleles or homozygous for the wild-type allele, consistent with an AR mode of inheritance with full clinical penetrance. None of the 12 mutant alleles were found in any of the various public databases (Exome Aggregation Consortium, Human Gene Mutation Database, Ensembl, National Heart, Lung, and Blood Institute Grand Opportunity Exome Sequencing Project, and 1000 Genomes Project), our in-house WES database (>3,000 exomes), or the Greater Middle Eastern Variome (63), further suggesting that the mutant alleles were causal for CMCD. The p.D387N missense mutation affected a residue conserved throughout evolution. As expected, combined annotation dependent depletion (CADD) scores predicted all mutations to be deleterious and were well above the mutation significance cutoff score for *IL17RA* (Table 1) (64, 65). These data strongly suggested that the 21 patients suffered from AR IL-17RA deficiency.

Expression and Function of the Mutant *IL17RA* Alleles. IL-17RA expression was tested on the surface of primary or SV40-transformed fibroblasts and/or lymphocyte subsets and monocytes from

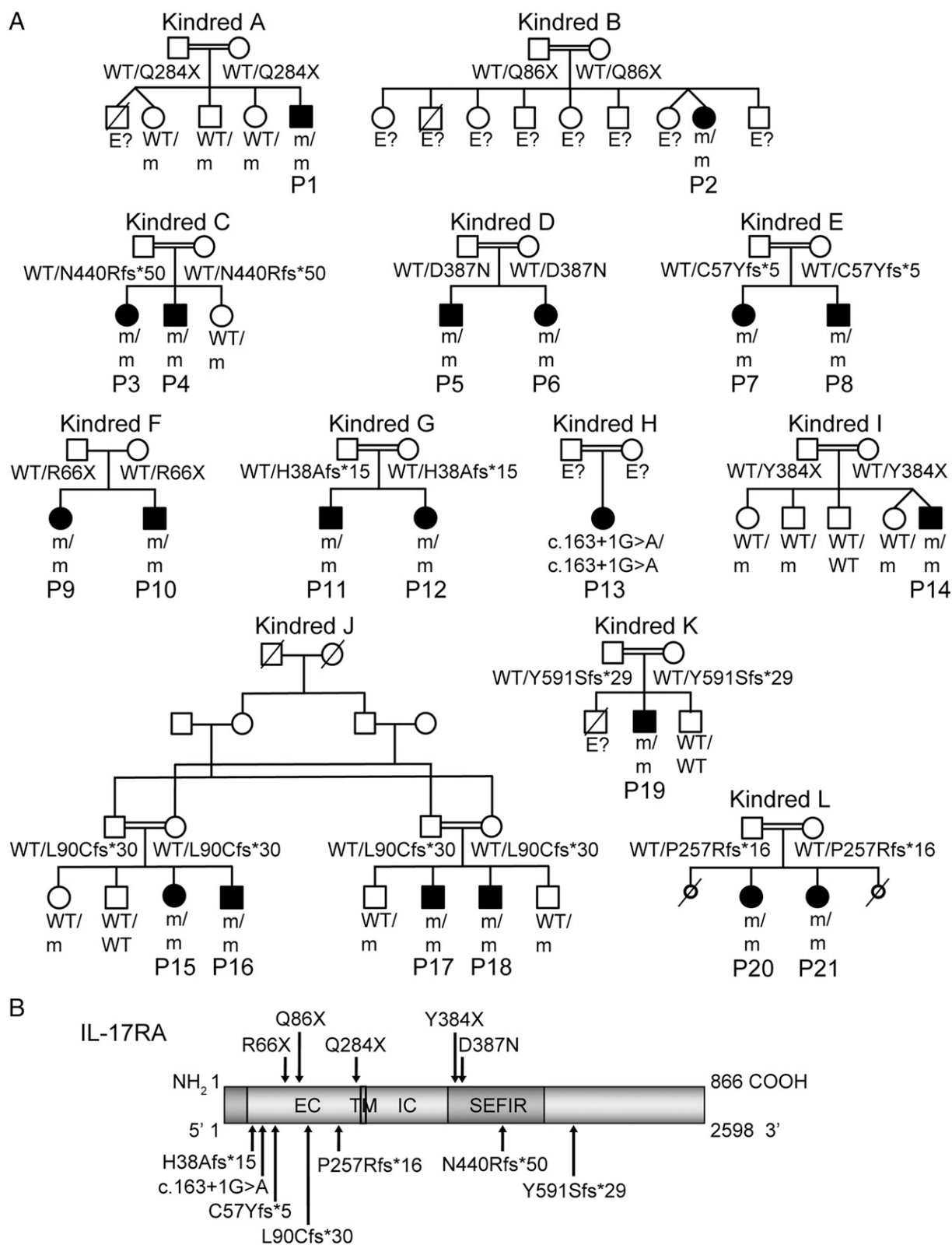


Fig. 1. The 12 kindreds with AR IL-17RA deficiency. (A) Pedigree of 12 families, with their genotypes. Kindred A has already been reported elsewhere (9). E? indicates individuals whose genetic status could not be evaluated; m, mutation. (B) Schematic diagram of the IL-17RA protein, with its extracellular (EC), transmembrane (TM), intracellular (IC), and SEFIR [SEF (similar expression to fibroblast growth factor genes) and IL-17R] domains and the positions affected by the mutations.

seven patients homozygous for six mutant *IL17RA* alleles (Fig. 2). IL-17RA expression was abolished on fibroblasts (P1, P2, and P4) (Fig. 2A) and peripheral blood mononuclear cells (PBMCs) [P1

(9), P3, and P13] (Fig. 2C), except for those from P5 (p.D387N), for whom IL-17RA was barely and normally detectable in SV40 fibroblasts and monocytes, respectively (Fig. 2A and C). In addition,

Table 1. Clinical characteristics of the 21 patients with AR IL-17RA deficiency

Patient (kindred)	Age at diagnosis	Genotype [CADD score]	Sex	Consanguinity	Origin	Mucocutaneous features	Other clinical features
P1 (9) (A)	1 mo	c.850C > T p.Q284X [40]	M	Yes	Morocco (living in France)	Skin, nails and oral mucosal candidiasis Skin pustules, folliculitis	No
P2 (B)	18 mo	c.256C > T p.Q86X [30]	F	Yes	Turkey	Genital and oral mucosal candidiasis	Suspected pulmonary tuberculosis
P3 (C)	1 mo	c.1302_1318dup p.N440Rfs*50 [22]	F	Yes	Turkey (living in France)	Scalp, genital and oral mucosal candidiasis Skin pustules, folliculitis	Eczema
P4 (C)	2 mo	c.1302_1318dup p.N440Rfs*50 [22]	M	Yes	Turkey (living in France)	Genital and oral mucosal candidiasis Skin pustules, folliculitis	Eczema, suspected tuberculous meningitis, lobar pneumonia
P5 (D)	9 y	c.1159G > A p.D387N [33]	M	Yes	Turkey	Scalp and oral mucosal candidiasis Skin pustules, folliculitis, furunculosis, seborrheic dermatitis	Sinusitis, lobar pneumonia
P6 (D)	4 y	c.1159G > A p.D387N [33]	F	Yes	Turkey	Scalp, genital and oral mucosal candidiasis Skin pustules, furunculosis, seborrheic dermatitis	Conjunctivitis
P7 (E)	1.5 y	c.166_169dup p.C57Yfs*5 [34]	F	Yes	Turkey	Skin, scalp, nails, genital and oral mucosal candidiasis Skin pustules, folliculitis, furunculosis	Sinusitis
P8 (E)	1 y	c.166_169dup p.C57Yfs*5 [34]	M	Yes	Turkey	Skin, scalp, nails and oral mucosal candidiasis Skin pustules, folliculitis, furunculosis	Sinusitis, conjunctivitis
P9 (F)	8 y	c.196C > T p.R66X [14]	F	No	Japan	Oral mucosal candidiasis Folliculitis	Eczema, bronchitis, lobar pneumonia
P10 (F)	6 y	c.196C > T p.R66X [14]	M	No	Japan	Skin, scalp and oral mucosal candidiasis Folliculitis	Eczema, bronchitis, lobar pneumonia
P11 (G)	25 y	c.112_119del p.H38Afs*15 [34]	M	Yes	Saudi Arabia	Oral mucosal candidiasis	No
P12 (G)	15 y	c.112_119del p.H38Afs*15 [34]	F	Yes	Saudi Arabia	Oral mucosal candidiasis	No
P13 (H)	1 mo	c.163+1G > A [25]	F	Yes	Algeria	Skin and genital mucosal candidiasis	No
P14 (I)	1 y	c.1152C > A p.Y384X [38]	M	Yes	Argentina	Skin and oral mucosal candidiasis Skin pustules, folliculitis, furunculosis, abscess	Sinusitis, otitis, lobar pneumonia
P15 (J)	4 y	c.268del p.L90Cfs*30 [23]	F	Yes	Saudi Arabia	Skin, genital and oral mucosal candidiasis	No
P16 (J)	2 y	c.268del p.L90Cfs*30 [23]	M	Yes	Saudi Arabia	Skin, scalp, nails, genital and oral mucosal candidiasis	No
P17 (J)	15 y	c.268del p.L90Cfs*30 [23]	M	Yes	Saudi Arabia	Skin, scalp and oral mucosal candidiasis Folliculitis, furunculosis	No
P18 (J)	10 y	c.268del p.L90Cfs*30 [23]	M	Yes	Saudi Arabia	Skin, scalp and oral mucosal candidiasis Folliculitis, furunculosis	No
P19 (K)	22 y	c.1770_1771dup p.Y591Sfs*29 [26.7]	M	Yes	Turkey	Skin, scalp, nails and oral mucosal candidiasis Skin pustules	Otitis
P20 (L)	13 y	c.769_773del p.P257Rfs*16 [28]	F	Yes	Turkey	Oral mucosal candidiasis Skin abscess	No
P21 (L)	11 y	c.769_773del p.P257Rfs*16 [28]	F	Yes	Turkey	Oral mucosal candidiasis	No

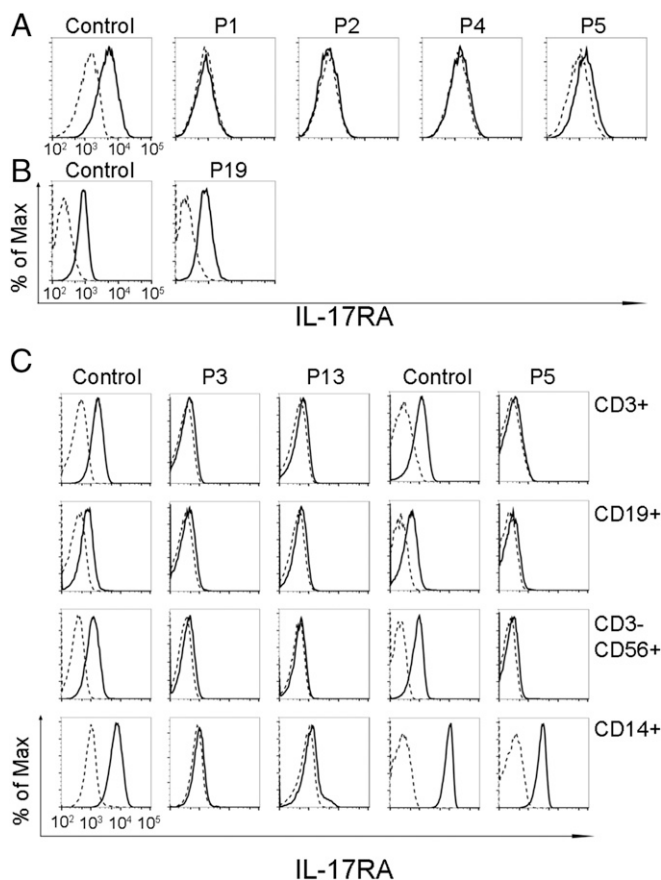


Fig. 2. IL-17RA expression. (A and B) IL-17RA expression in SV40-immortalized (A) and primary (B) fibroblasts from healthy controls and patients. (C) IL-17RA expression in T cells (CD3⁺), B cells (CD19⁺), natural killer cells (CD3⁻CD56⁺), and monocytes (CD14⁺) from healthy controls and three patients. Isotype control, dashed lines; IL-17RA-specific antibody, solid lines.

the p.Y591Sfs*29 allele (P19) was normally expressed in primary fibroblasts (Fig. 2B). The intracellular D387 residue is highly conserved and located in the SEFIR domain, which engages in homotypic dimerization with the SEFIR domain of ACT1 for IL-17RA signaling. We therefore tested HEK293T cells overproducing the p.D387N protein for interactions of this protein with ACT1, by immunoprecipitation and Western blotting; we found that the interaction of these two proteins was severely impaired in these cells (Fig. S2). We then investigated the function of p.D387N, together with several loss-of-expression alleles, by stimulating patient fibroblasts with high doses of recombinant IL-17A, -17F, and -17A/IL-17F heterodimers, with or without the addition of TNF- α . We detected no induction of IL-6 and GRO- α in any condition, whereas the induction of these two proteins was observed in control cells (Fig. 3 and Fig. S3 A and B). We measured the induction of mRNA for the antimicrobial peptide *BD2* (β -defensin 2) in patient fibroblasts stimulated with IL-17A plus TNF- α . We found no up-regulation in cells homozygous for p.D387N or p.Q284X, whereas such induction was observed in control cells (Fig. S3C). We then tested the response to IL-17E/IL-25 in the presence of IL-2 in PBMCs from P5 and P6 (p.D387N). No induction of IL-5 was observed, in contrast to the results obtained for control PBMCs (Fig. S4). Finally, the transfection of fibroblasts from P1 and P5 with a WT IL-17RA-encoding vector partially restored both surface IL-17RA expression (Fig. S5) and the response to IL-17A plus TNF- α (Fig. 4 and Fig. S6). Overall, these data indicate that p.D387N is loss of function. All patients displayed complete AR IL-17RA deficiency, with a lack of cellular responses to

IL-17A, -17F, and -17A/F in fibroblasts, as well as to IL-17E/IL-25 in PBMCs.

Abnormally High Proportions of IL-17-Producing T Cells and a Normal Response of Whole Blood to *Candida* and *Staphylococcus*. Given the critical role of IL-17A/F-producing T cells in immunity to *Candida* at barrier sites, we carried out an ex vivo assessment of the proportions of IL-17A/F-producing memory CD4⁺ T cells in patients. The patients tested (kindreds C, D, E, and H) had significantly higher proportions of IL-17A- and IL-17F-producing memory CD4⁺ T cells ex vivo than controls and healthy relatives, after stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, but similar or slightly higher proportions of IL-22-producing memory CD4⁺ T cells (Fig. 5A). By contrast, the mean values for IL-17A and -22 secretion levels in whole-blood assays were slightly higher than those for controls and healthy relatives, although this difference was not significant. This difference probably resulted from the smaller numbers of memory CD4⁺ T cells in patients than in adult controls and healthy relatives (Fig. 5B and Fig. S14). We also carried out whole-blood assays to assess the response to different stimuli, including zymosan, curdlan, lipopolysaccharide (LPS), vesicular stomatitis virus (VSV), Bacille de Calmette et Guérin (bacillus Calmette–Guérin), *Staphylococcus aureus*, and yeasts (*C. albicans*, *Saccharomyces cerevisiae*, and *Exophiala dermatitidis*), by

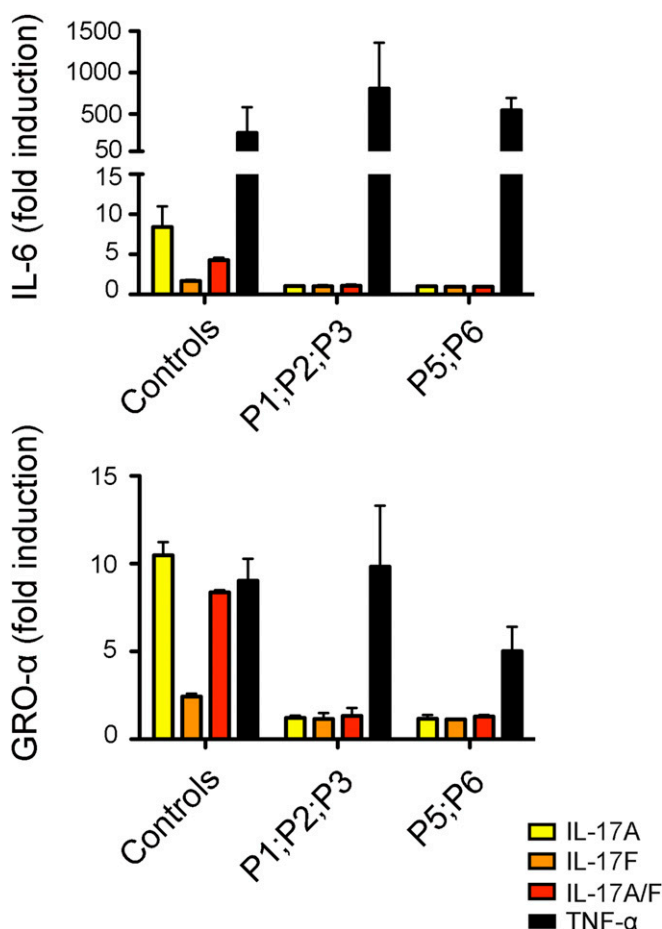


Fig. 3. Function of the mutant IL-17RA alleles. IL-6 and GRO- α fold induction measured in the supernatants of SV40-immortalized fibroblasts from two healthy controls and five patients, after 24 h of stimulation with IL-17A, -17F, -17A/F (100 ng/mL), or TNF- α (20 ng/mL), as assessed by ELISA. Means of three independent experiments are shown. Error bars represent the SD.

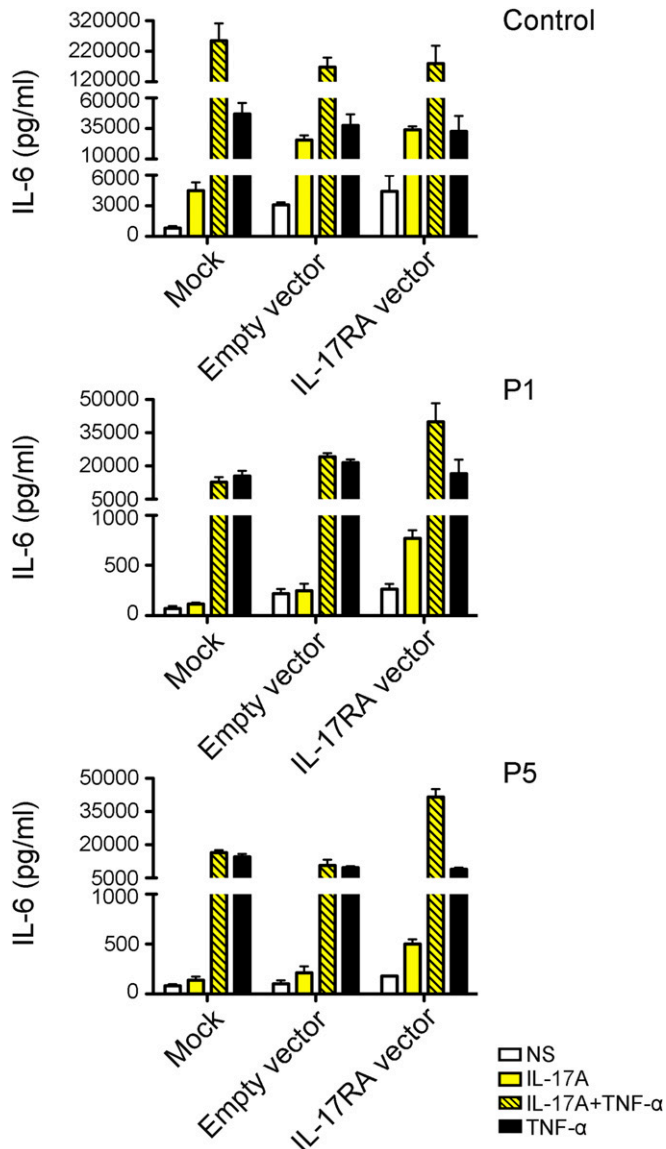


Fig. 4. Complementation of IL-17RA deficiency. IL-6 production, measured by ELISA, in the supernatants of SV40-immortalized fibroblasts from a control, P1, and P5, after transfection with the empty pORF9mcs plasmid or the pORF9-hIL17RA plasmid, after 24 h of stimulation with IL-17A (100 ng/mL) alone or in combination with TNF- α (20 ng/mL) is shown. Means of three technical replicates are shown. Error bars represent the SD. One experiment representative of the three carried out is shown. NS, not stimulated.

measuring the secretion of IL-6, -17A, and IFN- γ . Similar responses were observed for controls, the patients, and their healthy heterozygous relatives (Fig. S7). The results of these two sets of experiments suggest that the reported infectious phenotype in patients cannot be assigned to a defect in the mounting of a potent IL-17 inflammatory response or in the response to *S. aureus* and *C. albicans*, they suggest that the susceptibility to *S. aureus* and *C. albicans* reported in IL-17RA-deficient patients results from a complete lack of response to at least IL-17A, -17F, -17A/F, and -17E/IL-25.

Discussion

We report complete AR IL-17RA deficiency in 21 patients from 12 unrelated kindreds and 6 ethnic groups (9). All 12 alleles, including the 2 alleles (p.D387N and p.Y591Sfs*29) encoding

surface-expressed receptors, are loss-of-function in terms of responses to IL-17A, -17F, and -17A/F in fibroblasts. In addition, p.D387N is also loss-of-function for the response to IL-17E/IL-25 in PBMCs. Interestingly, the missense allele encodes a surface receptor in monocytes only. The clinical and cellular phenotypes of the two patients with this allele did not differ from those of patients with loss-of-expression alleles. This finding suggests that the p.D387N allele encodes a receptor that is present but not functional on monocytes, due to impairment of the SEFIR-mediated interaction with the adaptor ACT1. An alternative, but less likely, hypothesis is that IL-17RA-dependent signaling in monocytes may be redundant for mucocutaneous immunity to *Candida*. The cell-surface expression of dysfunctional receptors is the second genetic form of AR IL-17RA deficiency to be described. The detection of surface IL-17RA should not, therefore, exclude a diagnosis of IL-17RA deficiency, as previously shown for other cytokine receptors, such as IFN- γ R1 (66–69), IFN- γ R2 (70, 71), IL-12R β 1 (72, 73), and IL-10RA (74, 75). IL-17RA deficiency has recently been reported in two siblings from Sri Lanka (60). These siblings are homozygous for a large chromosomal deletion, also encompassing *CECR1* (encoding ADA2) and *XKR3* (encoding X Kell blood group-related 3). These two patients displayed

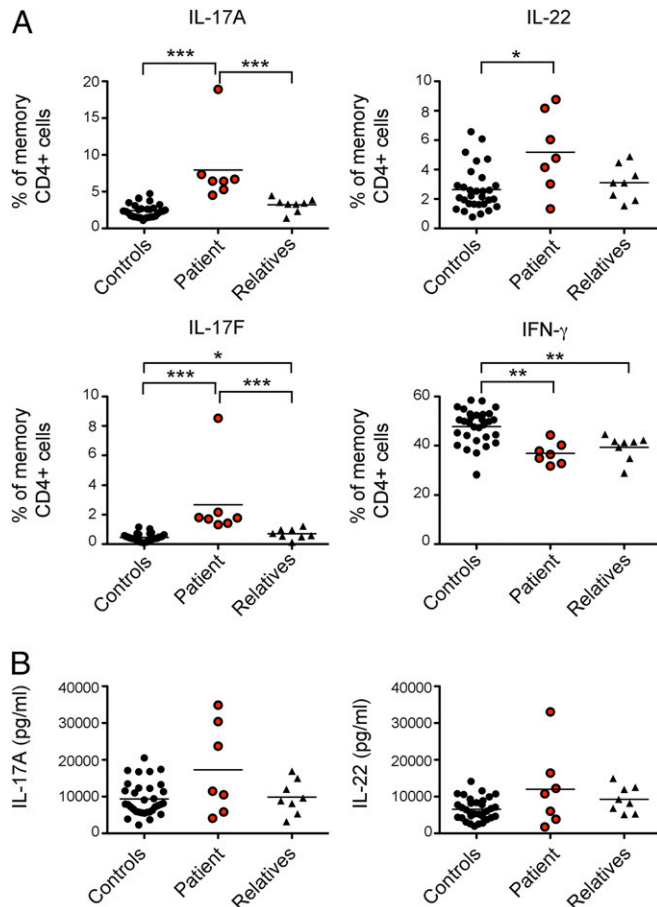


Fig. 5. IL-17-producing T cells. (A) Percentages of memory CD4⁺ T cells producing IL-17A, -22, -17F, and IFN- γ , as determined ex vivo by flow cytometry, after 12 h of stimulation with PMA and ionomycin. Horizontal lines indicate the mean value. (B) IL-17A and -22 production, measured by ELISA, in the supernatants of whole blood after 24 h of stimulation with PMA and ionomycin. Horizontal lines indicate the mean value. These two experiments were conducted in parallel in 30 healthy controls, 8 healthy relatives, and 7 patients from kindreds C, D, E, and H. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$ (two-tailed Mann-Whitney test).

CMC and staphylococcal skin infections, together with a chronic inflammatory disease possibly related to ADA2 deficiency. Collectively, these clinical observations suggest that AR IL-17RA deficiency is the second most common known genetic etiology of CMCD, after GOF STAT1, and the most common known etiology of isolated, as opposed to syndromic, CMCD (51).

All of the *IL17RA* alleles tested were null, because the responses to IL-17A/IL-17F homodimers and heterodimers in the patients' fibroblasts (cells tested displaying the best induction of IL-6 and GRO- α in controls)—and, by inference, probably in PBMCs (9)—and responses to IL-17E/IL-25 in their PBMCs were abolished. We predict that none of the cell types normally expressing IL-17RA in healthy individuals (whether hematopoietic or nonhematopoietic) respond to IL-17RA-dependent cytokines in patients. Nevertheless, the susceptibility to infection of IL-17RA-deficient patients appeared to be restricted to certain mucocutaneous barrier sites. In addition to CMC and cutaneous staphylococcal infections, several patients presented bacterial infections of the respiratory tract, which may not be coincidental (76). In a mouse model of *Klebsiella pneumoniae* infection, IL-17RA signaling has been shown to be critical for the optimal production of chemokines and granulocyte colony-stimulating factor in the lungs and for neutrophil recruitment and survival (77). The skin and mucosal phenotype of the patients may be accounted for, at least in part, by human keratinocytes and bronchial epithelial cells having a much greater dependence than other cell types on the synergistic effect of IL-17 cytokines (IL-17A and -17F in particular) and inflammatory cytokines (such as TNF- α) for the production of chemokines and antimicrobial peptides (78). It remains unclear how IL-17A, -A/F, and -F cooperate with other cytokines, but multiple ACT1-dependent mechanisms involving the promoter (as for IL-6) and/or mRNA stabilization (e.g., the GRO- α mRNA) (79, 80) are probably involved, as suggested by the similar clinical and functional impacts of human IL-17RA and ACT1 deficiencies (9, 11).

CMC is the key clinical presentation of patients with AR IL-17RA (refs. 9 and 60 and this work), AD IL-17F (9), AR ACT1 (11), or AR IL-17RC deficiency (10). Nevertheless, the heterogeneous phenotypes of these patients suggest a continuum of severity, ranging from a relatively mild phenotype in patients with IL-17F or -17RC deficiency to a more severe phenotype in patients with ACT1 or IL-17RA deficiency. Patients with ACT1 or IL-17RA deficiency are also susceptible to staphylococcal skin infections and bacterial respiratory infections, which tend to run a more chronic course. It is too early to draw firm conclusions, given the small number of patients identified. However, each of these genetic defects may have a different impact on IL-17 immunity. For example, in addition to acting in concert with IL-17RC for responses to IL-17A/F, IL-17RA acts with IL-17RB in mice (81) and with IL-17RE in mice and humans (82, 83) in responses to IL-17E/IL-25 and -17C, respectively. The function of IL-17RD is poorly defined and its ligand is unknown, but studies in mice have shown that ACT1 is essential for the signal transduction mediated by the individual IL-17RA (84, 85), IL-17RB (86), IL-17RC (87), and IL-17RE (88) subunits. Unlike those of IL-17RC-deficient patients, PBMCs from ACT1-deficient and IL-17RA-deficient patients do not respond to IL-17E/IL-25 (10, 11). The role of human IL-17E/IL-25 is unknown, in the absence of known patients bearing specific mutations, but its mouse counterpart is known to promote "Th2"-mediated responses (89, 90) and to be involved in immunity to parasitic infections (91–93). We were unable to detect cellular responses to IL-17B, -17D, and even -17C (83) in control fibroblasts, keratinocytes, or leukocytes. This finding precluded the testing of such responses in IL-17RA-deficient or other patients with CMC. Human IL-17C may play a redundant role in protective mucocutaneous immunity to *Candida*, because IL-17C- and IL-

17RE-deficient mice clear *Candida* infections normally (94). The role of each human IL-17 cytokine in vivo will be determined from the description of patients bearing mutations, as reported for IL-17F (9). Overall, our data demonstrate that human signaling via IL-17RA (in response to at least IL-17A, -17A/F, -17F, and -17E/IL-25) is essential for mucocutaneous immunity to *C. albicans* and *Staphylococcus*. They also suggest that IL-17RA-dependent signaling is important for protective immunity to various bacteria in the respiratory tract.

Materials and Methods

Massively Parallel Sequencing. Genomic DNA extracted from the peripheral blood cells of each patient was sheared with a Covaris S2 Ultrasonicator. An adaptor-ligated library was prepared with the Paired-End Sample Prep kit V1 (Illumina). Exome capture was performed with the SureSelect Human All Exon kit (Agilent Technologies). Single-end sequencing was performed on an Illumina Genome Analyzer IIx (Illumina), generating 72-base reads.

Molecular Genetics. Genomic DNA was isolated from whole blood by a phenol/chloroform extraction method. *IL17RA* gDNA was amplified with specific primers (PCR amplification conditions and primer sequences are available in Table S1). PCR products were analyzed by electrophoresis in 1% agarose gels, sequenced with the Big Dye Terminator cycle sequencing kit (Applied Biosystems), and analyzed on an ABI Prism 3700 (Applied Biosystems).

Cell Activation. For the ex vivo evaluation of IL-17A- and IL-22-producing T cells by ELISA, we used 250 μ L of whole blood diluted in RPMI (500 μ L final volume) to seed 48-well plates. We added 40 ng/mL PMA and 10^{-5} M ionomycin and incubated the plates for 24 h. The supernatants were then collected for ELISA (R&D Systems). For the evaluation of the response to IL-17E/IL-25, fresh PBMCs were cultured in the presence of 100 ng/mL thymic stromal lymphopoietin (R&D Systems; 1398-TS-010/CF0) in X-VIVO 15 (Lonza) plus 5% human AB serum (Lonza) for 24 h. PBMCs were collected, washed, and resuspended at a density of 4×10^6 cells per well in 48-well plates, in a final volume of 0.5 mL per well, in the presence of 10 ng/mL recombinant human IL-2 (R&D Systems) and 10 ng/mL recombinant human IL-17E (R&D Systems). After 3 d, IL-5 secretion was assessed by ELISA (DY205; R&D Systems). SV40-transformed fibroblasts were plated in 48-well plates at a density of 100,000 cells per well in 0.5 mL of DMEM/10% (vol/vol) FBS. They were incubated for 24 h and then left unstimulated or stimulated for 24 h with recombinant human IL-17A, -17F, and -17A/F (100 ng/mL), with or without TNF- α (20 ng/mL) purchased from R&D Systems. The supernatants were collected for ELISA for IL-6 (Sanquin) and GRO- α (R&D Systems), carried out in accordance with the kit manufacturer's instructions.

Flow Cytometry. For the ex vivo evaluation of IL-17A-, IL-17F-, IL-22- and IFN- γ -producing T cells by flow cytometry, PBMCs were dispensed into 48-well plates at a density of 3×10^5 cells per mL in RPMI/10% (vol/vol) FBS for 12 h with 40 ng/mL PMA plus 10^{-5} M ionomycin, in the presence of a secretion inhibitor (1 μ L/mL GolgiPlug; BD Biosciences). The cells were washed and surface-labeled with PE-Cy7 mouse anti-human CD3 (SK7; BD Biosciences), CD4-APC-Vio770, human (M-T321; Miltenyi Biotec), Brilliant Violet 421 anti-mouse CD197 (CCR7) (G043H7; BioLegend), PE-CF594 mouse anti-human CD45RA (HI100; BD Biosciences), and LIVE/DEAD Fixable Aqua Dead Cell (L34957; Thermo Fisher) in PBS/2% (vol/vol) FBS/2 mM EDTA for 20 min on ice. Cells were then washed twice with PBS/2% (vol/vol) FBS/2 mM EDTA, fixed by incubation with 100 μ L of BD Cytofix for 30 min on ice, and washed twice with BD Cytoperm (Cytofix/Cytoperm Plus, fixation/permeabilization kit; BD Biosciences). Cells were then incubated for 1 h on ice with antibodies purchased from Ebiosciences—anti-human IL-17A Alexa Fluor 488 (eBio64-DEC17), anti-human IL-17F PE (SHLR17), anti-human/mouse IL-22 APC (IL22JOP), and anti-human IFN gamma Alexa Fluor 700 (45.B3)—washed twice with Cytoperm, and analyzed with a FACS Gallios flow cytometer. For the evaluation of IL-17RA expression, SV40-transformed fibroblasts or PBMCs were labeled simultaneously with LIVE/DEAD Fixable Aqua Dead Cell, Alexa Fluor 647-mouse IgG1 isotype control antibodies (MOPC-21; BioLegend) or Alexa Fluor 647-anti-human IL-17AR (BG/hIL17AR; BioLegend). PBMCs were also labeled with CD14-PE-Vio770, human (TÜK4), or CD3-VioBlue, human (BW264/56), purchased from Miltenyi Biotec; APC-Cy7 mouse anti-human CD19 (SJ25C1) and PE-CF594 mouse anti-human CD56 (B159) purchased from BD Biosciences, and analyzed by flow cytometry in a FACS Gallios flow cytometer.

Cell Complementation. IL-17RA-deficient SV40-transformed fibroblasts were transfected with either empty pORF9-msc vectors or with the pORF9-*hIL17RA* vector encoding the wild-type human IL-17RA (Cayla-InvivoGen), with the Lipofectamine LTX transfection kit (Invitrogen), according to the manufacturer's instructions. At 24 h later, cells were stimulated with IL-17A (100 ng/mL), with or without TNF- α (20 ng/mL), for a further 24 h. The supernatants were collected for the assessment of IL-6 and GRO- α levels by ELISA, and the cells were collected for the evaluation of IL-17RA expression by FACS analysis.

Full-Length RT-PCR for DEFB4A and Taqman Probe Detection. Total RNA was extracted with the RNeasy minikit (Qiagen) and reverse-transcribed to generate cDNA, with the High Capacity cDNA Reverse Transcription Kit (4368813; Invitrogen). Taqman probes for *DEFB4A* (Hs00823638_m1; Invitrogen) were used to detect mRNA synthesis, with normalization on the basis of GUS expression (Human GUSB Endogenous Control VIC/MGB Probe; 4326320E; Primer Limited; Invitrogen).

Healthy Controls. The healthy controls were volunteer blood donors of European and Turkish origin.

Study Approval. The experiments described here were conducted in accordance with local, national, and international regulations and were approved by the French Ethics committee (CPP Ile-de-France 2, ID-RCB: 2010-A00636-33), French National Agency for Medicines and Health Products Safety (B100712-40), and the French

Ministry of Research (IE-2010-547). Informed consent was obtained from all patients or their families, in the case of minors, in accordance with World Medical Association rules, the Helsinki Declaration, and European Union directives.

ACKNOWLEDGMENTS. We thank the members of the laboratory for helpful discussions; Yelena Nemiroskaya, Eric Anderson, Martine Courat, and Lahouari Amar for expert secretarial assistance; and Maya Chrabieh, Lazaro Lorenzo, and Tiffany Nivare for technical assistance. This work was supported by French National Research Agency (ANR) Grants ANR GENCMCD-11-BSV3-005-01 (to A.P.), ANR EURO-CMC-14-RARE-0005-02), and "Investissement d'avenir" Program Grant ANR-10-IAHU-01; Laboratoire d'Excellence Integrative Biology of Emerging Infectious Diseases Grant ANR-10-LABX-62-IBED; the Jeffrey Modell Centers Network; the St. Giles Foundation; the Rockefeller University; INSERM; and Paris Descartes University. This study was supported in part by Japan Society for the Promotion of Science Grants in Aid for Scientific Research 16H05355 and 16K1552 and the Practical Research Project for Rare/Intractable Diseases of the Japan Agency for Medical Research and Development, AMED. R.L. was supported by the SNFMI (Bourse Marcel Simon), the INSERM PhD program for medical doctors (poste d'accueil INSERM), and a Fulbright grant (Franco-American commission). V.B. was supported by ANR Grant NKIR-ANR-13-PDOC-0025-01. K.M. was supported by a Japan Foundation for Pediatric Research fellowship grant. L.Y.A.C. was supported by the Singapore National Medical Research Council and National University Health System. F.G. is a scholar of the Else Kröner Fresenius Clinical Research School München "Rare diseases of the immune system—from pathophysiology to new therapies" (Grant 2013_Kolleg.18).

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Supporting Information

Lévy et al. 10.1073/pnas.1618300114

SI Materials and Methods

Cell Purification and Culture. Human PBMCs were isolated by Ficoll–Hypaque density gradient centrifugation (Amersham Pharmacia Biotech) from whole blood. Primary human fibroblasts, obtained from skin biopsy specimens, were immortalized with SV-40T antigen (SV-40 fibroblasts) and cultured in DMEM (Gibco BRL, Invitrogen) supplemented with 10% (vol/vol) FBS (Gibco BRL, Invitrogen).

Immunophenotyping. For the detailed phenotyping of lymphocyte subsets, CD4⁺ and CD8⁺ T cells, CD4⁺ effector T cells, B cells, natural killer (NK) cells, regulatory T cells, and fresh PBMCs were labeled with BV786 mouse anti-human CD3 (SK7), PE mouse anti-human CD4 (RPA-T4), PE-CF594 mouse anti-human CD45RA (HI100), FITC mouse anti-human CD27 (L128), BV650 mouse anti-human CD8 (RPA-T8), BV711 mouse anti-human CD161 (DX12), PE-CF594 mouse anti-human CD56 (B159), BV421 mouse anti-human CD25 (M-A251), BV711 mouse anti-human CD4 (SK3), BV711 mouse anti-human CD21 (B-ly4), APC-Cy7 mouse anti-human CD19 (SJ25C1), and Alexa Fluor 488 mouse anti-human FoxP3 (259D/C7) antibodies purchased from BD Biosciences; anti-human CD20-FITC (LT20), anti-human IgM-PE (PJ2-22H3), anti-human IgD-PE (IgD26), anti-human CD38-PE-Vio770 (REA572), anti-human CD183 (CXCR3)-VioBright FITC (REA232), anti-human CD196 (CCR6)-PE (REA190), anti-human CD194 (CCR4)-PE (REA279), anti-human CD185

(CXCR5)-PE-Vio770 (REA103), anti-human CD4-APC-Vio770 (M-T321), anti-human CD3-PE-Vio770 (BW264/56), anti-human CD159a (NKG2A)-APC (REA110), anti-human CD159c (NKG2C)-PE (REA205), anti-human TCR-V α 7.2-APC-Vio770 (REA179), and anti-human TCR γ / δ -VioBlue (11F2) antibodies purchased from Miltenyi Biotec; CD158a,h-PE IOTest (EB6B) and CD158b1/b2,j (GL183) antibodies purchased from Beckman Coulter; and Brilliant Violet 421 anti-human CD197 (CCR7) antibody (G043H7), and Brilliant Violet 421 anti-human CD27 antibody (M-T271) purchased from BioLegend.

Transfection and Coimmunoprecipitation. HEK293 cells were transiently transfected with the indicated plasmids in the presence of Lipofectamine 2000 (Thermo Fisher Scientific) before harvest for coimmunoprecipitation. After 48 h of transfection, cells were lysed in lysis buffer [1% Triton X-100, 20 mM Hepes (pH 7.4), 150 mM NaCl, 12.5 mM β -glycerophosphate, 1.5 mM MgCl₂, 10 mM NaF, 2 mM DTT, 1 mM sodium orthovanadate, and 2 mM EGTA] supplemented with protease inhibitor mixture (Complete; Roche). Cell extracts were incubated with antibody (1 μ g) and protein A beads (20 μ L). After overnight incubation, the beads were washed four times with lysis buffer and then mixed with 40 μ L of Laemmli buffer. The immunoprecipitates were resolved by SDS/PAGE and analyzed by Western blotting.

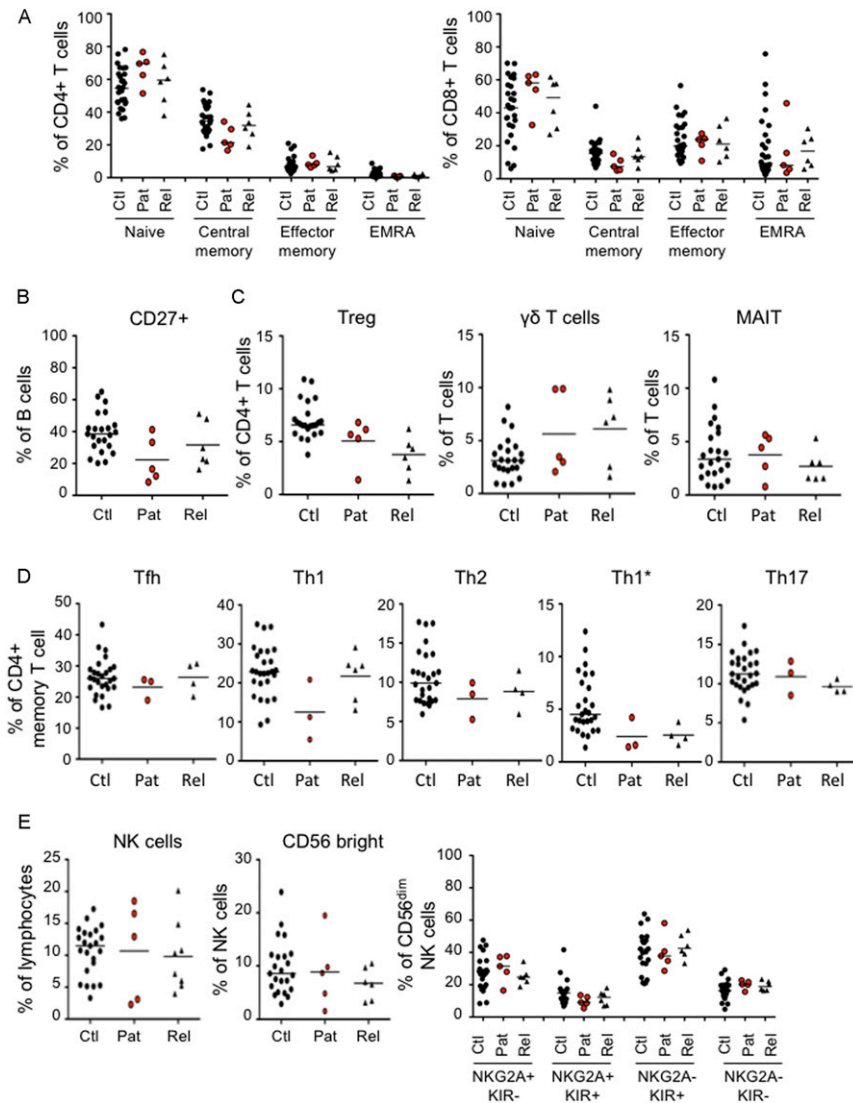


Fig. S1. Immunophenotyping. (A) Frequency of naïve (CD45RA⁺CCR7⁺), central memory (CD45RA⁻CCR7⁺), effector memory (CD45RA⁻CCR7⁻), and EMRA (CD45RA⁺CCR7⁻) cells among the CD4⁺ and CD8⁺ T cells of patients (*n* = 5), healthy relatives (*n* = 6), and controls (*n* = 28). (B) Frequency of CD27⁺ memory cells in the B-cell compartment of patients (*n* = 5), healthy relatives (*n* = 6) and controls (*n* = 23). (C) Frequency of Treg (FoxP3⁺CD25⁺) among CD4⁺ T cells and frequency of $\gamma\delta$ T cells (CD3⁺TCR $\gamma\delta$ ⁺) and mucosal-associated invariant T (CD161⁺Va7.2⁺) cells among the total CD3⁺ T cells of patients (*n* = 5), healthy relatives (*n* = 6), and controls (*n* = 23). (D) Frequency of T helper (Th) subsets within the CD4⁺ memory compartment of patients (*n* = 3), healthy relatives (*n* = 6), and controls (*n* = 28). Subsets were defined as follows: Tfh (CXCR5⁺), Th1 (CXCR5⁻CXCR3⁺CCR4⁺CCR6⁻), Th2 (CXCR5⁻CXCR3⁻CCR4⁺CCR6⁺), Th1* (CXCR5⁻CXCR3⁺CCR4⁺CCR6⁺), and Th17 (CXCR5⁻CXCR3⁻CCR4⁺CCR6⁺). (E) Immunophenotyping of NK cells showing total NK cell frequency in lymphocytes (*Left*), the frequency of CD56^{bright} cells within the NK cell compartment (*Center*), and the terminal differentiation profile of the CD56^{dim} compartment (*Right*), as assessed by the distribution of NKG2A and KIR on cells from patients (*n* = 5), healthy relatives (*n* = 6–8), and controls (*n* = 23). Horizontal bars represent the median value. Ctl, controls; Pat, patients; Rel, relatives.

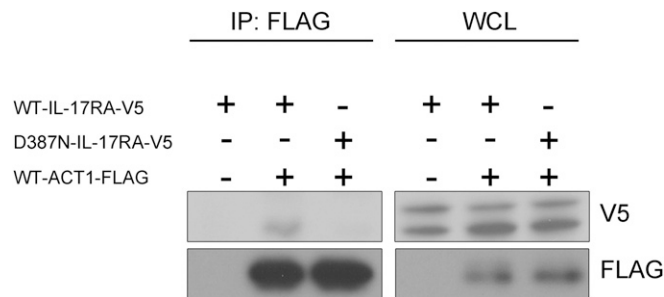


Fig. S2. The p.D387N IL-17RA allele impairs IL-17RA/ACT1 interaction. HEK293 cells were transiently transfected with a plasmid encoding FLAG-tagged ACT1 together with plasmids encoding V5-tagged WT-IL-17RA or D387N-IL-17RA. Whole-cell lysates (WCL) were subjected to immunoprecipitation with anti-FLAG antibody. Western blot analysis was performed with anti-FLAG and -V5 antibodies.

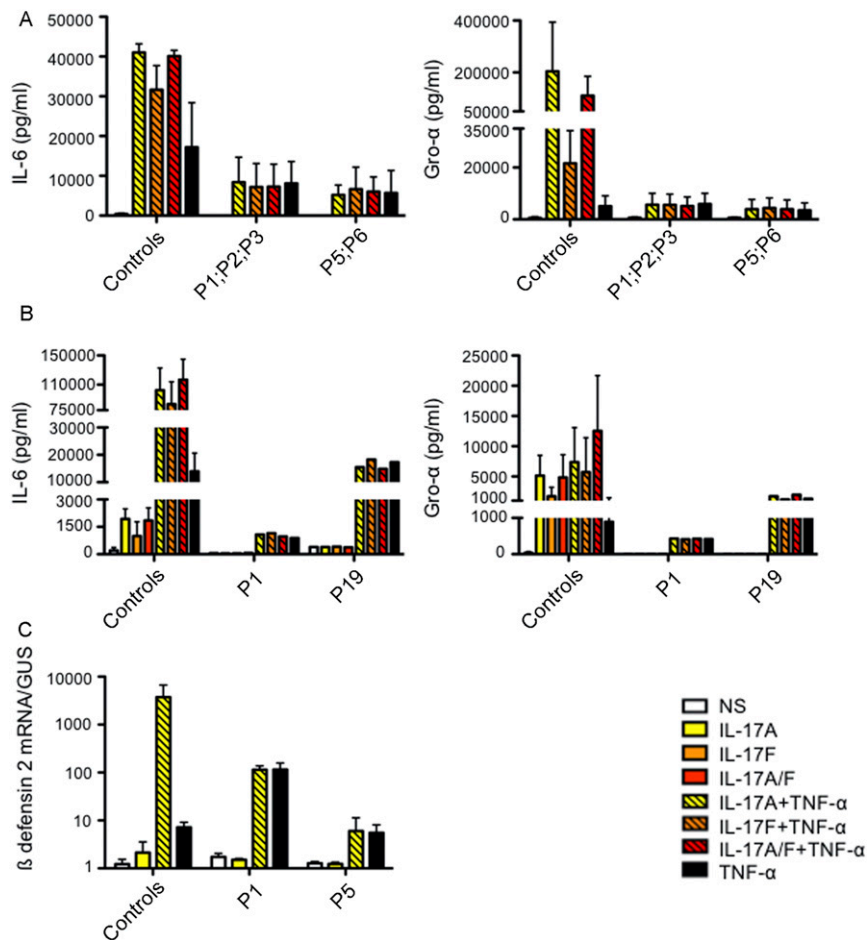


Fig. S3. Function of the mutant IL-17RA alleles. IL-6 and GRO- α production, measured by ELISA in the supernatants of SV40-immortalized (A) and primary fibroblasts (B) from two healthy controls and patients, after 24 h of stimulation with IL-17A, -17F, -17A/F (100 ng/mL), and TNF- α (20 ng/mL) alone or in combination. Means of three technical replicates are shown. Error bars represent the SD. (C) BD2 mRNA induction in SV40-immortalized fibroblasts from two healthy controls and from P1 and P5 after 24 h of stimulation with IL-17A (100 ng/mL) alone or with TNF- α (20 ng/mL). Means of three technical replicates are shown. Error bars represent the SD. Data are representative of two independent experiments. NS, not stimulated.

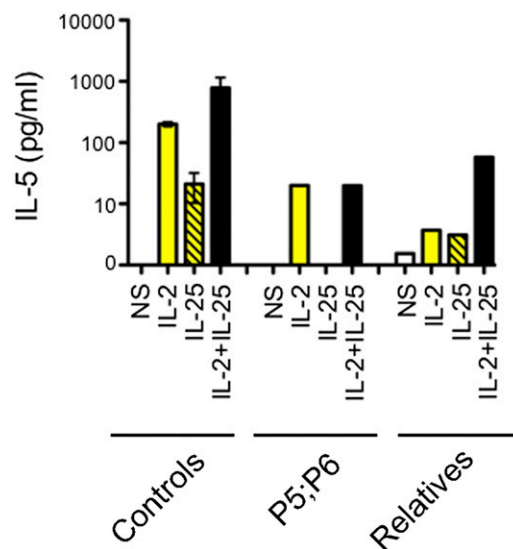


Fig. S4. The p.D387N allele is loss of function for the response to IL-17E/IL-25. PBMCs from three controls, P5, P6, and two healthy relatives were cultured with thymic stromal lymphopoietin for 24 h, harvested, and stimulated with IL-2 and -17E/IL-25 for an additional 72 h. IL-5 was determined in the culture supernatants by ELISA. Error bars represent the SD. NS, not stimulated.

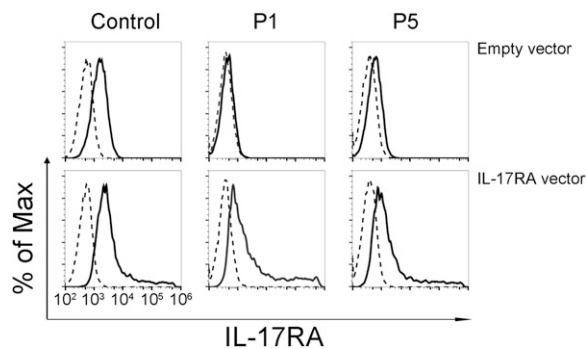


Fig. S5. Complementation of IL-17RA deficiency. IL-17RA expression in SV40-immortalized fibroblasts from a control, P1, and P5 transfected with the empty pORF9mcs plasmid or the pORF9-hIL17RA plasmid. Isotype control, dashed lines; anti-IL-17RA antibody, solid lines.

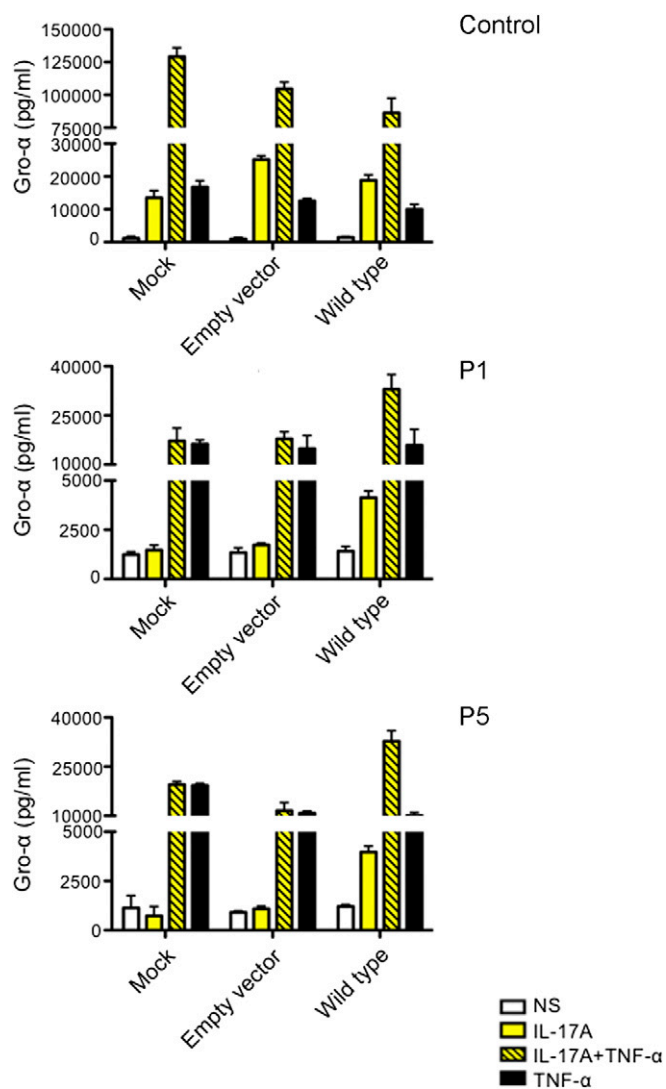


Fig. S6. GRO- α production, measured by ELISA, in the supernatants of SV40-immortalized fibroblasts from a control, P1, and P5 after transfection with the empty pORF9mcs plasmid or the pORF9-hIL17RA plasmid and stimulation for 24 h with IL-17A (100 ng/mL) alone or with TNF- α (20 ng/mL). Means of three technical replicates are shown. Error bars represent the SD. One experiment representative of the three carried out is shown. NS, not stimulated.

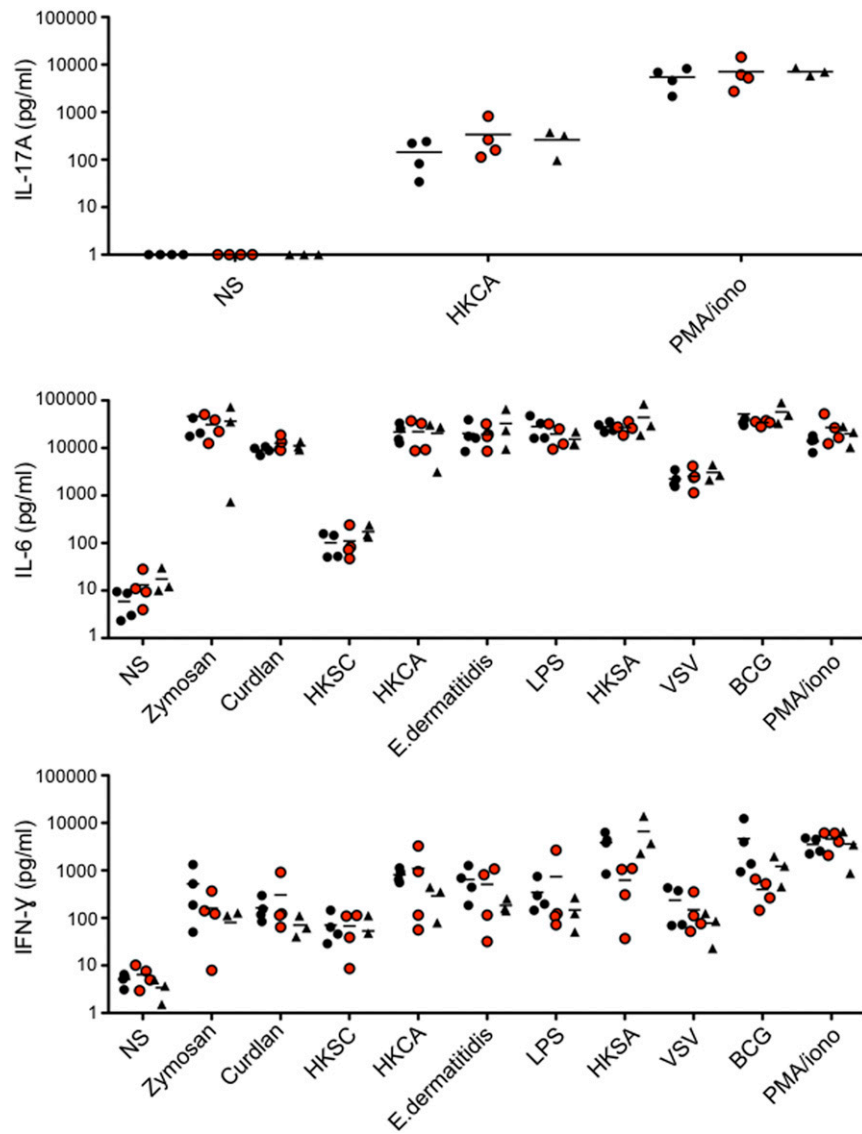


Fig. S7. Response to bacteria and yeasts in whole blood. Production of IL-17A, -6, and IFN- γ , measured by ELISA, in whole blood from four controls (black circles), four patients (red circles), and three healthy relatives (black triangles) after 3 d of stimulation is shown. Horizontal bars represent the mean value. BCG, Bacille de Calmette et Guérin; HKCA, heat-killed *C. albicans*; HKSA, heat-killed *S. aureus*; HKSC, heat-killed *S. cerevisiae*; LPS, lipopolysaccharide; NS, not stimulated; VSV, vesicular stomatitis virus.

Table S1. PCR primers and conditions for *IL17RA* genomic DNA amplification

Exon	Forward primer	Reverse primer
Exon 1	GTCCAGACTAAACTCTCC	GCAGATCCTGGCGGCTAC
Exon 2	CTGAGAATGAGCCAGTGGAG	GGCATGGCAAGACTTCTGTC
Exon 3	CTGAGCTGTTTGCTGTCTAGC	CCAATCCTGAGCCTGACTGG
Exon 4	GACACACCCAGCACTTGTC	GAAGTCTGTA CTGGTGCATC
Exon 5	GCATAGATGGGTGACAGAGGTG	GGTGCTTCCTTCTCACAGAGG
Exon 6 and 7	CAGATTTCTGAAGGCAGAGGC	GCAACGCGGTTCTGTGACAGAC
Exon 8	CTCTGGACAGCCTCTCCATG	CTCCTGTCCACTCTATAATTGC
Exon 9	GCTGCTGGCTGGAAGGCATG	CAGGTAAGGACGCAGCACAG
Exon 10 and 11	CTGGGAAGGGTTAAGAATGC	GGTGCTGGTATTAGAGCCTG
Exon 12	GTGCGACCACCTAGCACG	GGGAGTTAGAGCACAGGAG
Exon 13-a	CTCCTGGGCTGGCAGGCAC	CGTCACAGCTGACCTCGCTG
Exon 13-b	CAGCCATGAACATGATCCTCC	CTTCGAGGTGCTCCCTCAC
Exon 13-c	GCAGCAGTGGCAAAGCTGG	CAGATGAATACGTGCACACAC
Exon 13-d	GGACTCACGGAAATGGAGGAAGAG	CGTTTACATCCC GCGTGACCATC

Conditions: 2.5 mM MgCl₂; 7.5% (vol/vol) DMSO; melting temperature: 59 °C; 1 min, 1 min, 1 min × 38 cycles.

Syndromic CMCD in a patient with multiple infections and inherited c-REL deficiency

1. c-REL, a member of the NF- κ B family

NF- κ B was discovered and firstly described in B cells as a crucial transcriptional factor for their activation and development⁹⁹. We now understand NF- κ B as a family of transcriptional factors playing a role in a wide range of physiological and pathological processes¹⁰⁰. Moreover, mice studies helped to demonstrate the pivotal role of these set of proteins in the immune system, as best exemplified by KO mice models for each of the NF- κ B counterparts displaying predominantly, if not only, a variety of immunological abnormalities¹⁰¹. More recently, human genetic studies in patients bearing mutations in genes either encoding NF- κ B proteins^{102–105}, or in genes directly regulating the NF- κ B-mediated signaling^{106–118}, further highlight how the disruption of NF- κ B signaling can foster immunodeficiency, auto-inflammation, auto-immunity and lymphoproliferation. Importantly, these human studies also revealed contrasting results with the ones obtained in mice, and therefore contributed greatly to the understanding of NF- κ B function precisely in human immunology¹¹⁹.

The NF- κ B proteins family comprises p105/p50 (*NFKB1*), p100/p52 (*NFKB2*), p65 (*RELA*), Rel B (*RELB*), and c-REL (*REL*). Like Rel B and p65, c-REL is synthesized as a mature protein, whereas p50 and p52 arise from the cleavage of the precursors p105 and p100, respectively. In resting or unstimulated cells, NF- κ B dimers are localized in the cytoplasm as inactive heterodimers, bound to I κ B proteins (for Inhibitor of κ B), masking the nuclear localization sequence (NLS) and preventing NF- κ B nuclear translocation. Therefore, post-translational modification of I κ B proteins is necessary for NF- κ B activity, and can be achieved through two principal pathways, termed the canonical and alternative pathway. The

canonical pathway consists on receptors (such as T cell and B cell receptors, TLR/IL-1 Receptors) and their proximal adaptor molecules, the IKK complex (for I κ B Kinase), I κ B proteins, and NF- κ B dimers comprising p50, p65 and c-REL. Inducing stimuli trigger the activation of the IKK complex (comprising NEMO, IKK α and IKK β) leading to the phosphorylation, ubiquitination and degradation of I κ B α and I κ B β , instantaneously releasing NF- κ B dimers. These NF- κ B dimers translocate to the nucleus, and bind to the promoters/enhancers regions of genes and promote their transcription. Alternatively, the alternative pathway relies on the activation of IKK α by NIK (for NF- κ B-inducing kinase), leading to phosphorylation and proteasomal degradation of p100, and the release of p52/Rel B dimers¹¹⁹.

Human *REL* gene is localized on chromosome 2, has 11 exons, and encodes for c-REL, a 587-amino acid protein. Like other NF- κ B proteins, c-REL contains a highly conserved N-terminal Rel homology domain (RHD) of 300 amino acids enabling: dimerization with other NF- κ B proteins; DNA binding; and binding to I κ B proteins¹⁰⁰. c-REL also contains a C-terminal transactivation domain (TAD), with two subdomains (TADI: aa 424-490; and TADII: aa 518-522) that are essential for the full transcriptional activity of c-REL; and a REL inhibitory domain (RID) (aa: 323-422) between the RHD and the TAD, termed RID, as a deletion of this domain results in enhanced c-REL DNA-binding and transcriptional activity¹²⁰. A second isoform was characterized, as the product of a mRNA splice variant, lacking the exon 9 (aa 308-330) encoding for part of the RID¹²⁰.

The complexity, in which resides the remarkable function of NF- κ B signaling, relies on the capacity for these proteins to form homo- and heterodimers with distinct affinities for the κ B DNA-binding site. If redundancy at the level of promoters of genes, is a key aspect of NF- κ B biology, each of these proteins have unique functions *in vivo*, which can be appreciated in individuals lacking one of these proteins. Complete AR Rel B deficiency was

described in three patients with a combined immunodeficiency (CID)¹⁰⁴. In addition, heterozygous LOF mutations in *NFKB1* or *NFKB2* were associated with common variable immunodeficiency (CVID)^{103,105}, whereas recently published AD p65 haploinsufficiency results in chronic mucocutaneous ulcerations¹⁰². To date, patients carrying deleterious mutations in *REL* gene, have not been reported.

2. *Rel*^{-/-} mice exhibit profound immunological abnormalities

a) The first description of *Rel*^{-/-} mice

Mice invalidated for c-REL expression were reported in 1995¹²¹. In this report, *Rel*^{-/-} mice develop normally *in utero* and do not show any spontaneous infectious phenotype, or premature death, nor any morphological abnormalities. In addition, c-REL does not play an essential role for the development of mature lymphoid cells, as *Rel*^{-/-} mice display normal counts of T- and B lymphocytes. As well, the development of cells from all hematopoietic lineages is normal. However, profound functional immunological abnormalities are observed in lymphoid cells. The proliferation of *Rel*^{-/-} T cells is strongly decreased after incubation with anti-CD3, anti-CD3+anti-CD28, or the mitogen Concanavalin A (ConA). Conversely, the response to PMA/iono is marginally affected, suggesting that signals directly acting in the cytoplasm can overcome the defect. Indeed, PMA and iono both diffuse through the cell membrane: PMA directly activates PKC, and ionomycin induces Ca⁺⁺ release, respectively. The adjunction of IL-2 to the cultures rescues the impaired proliferation of *Rel*^{-/-} T cells to anti-CD3, anti-CD3+anti-CD28, or ConA in line with the IL-2 production defect observed after incubation with all these mitogens. Regarding B cells, purified *Rel*^{-/-} B cells do not proliferate in response to Lipopolysaccharide (LPS), CD40 ligand (CD40L), or B cell receptor (BCR) agonist. Serum immunoglobulin levels in unimmunized mice are reduced (IgM, IgG2b and IgG3) or undetectable (IgG1 and IgG2a). Moreover, the antibody response

in vivo to a T cell-dependent antigen (NP-KLH) is 50 to 100 folds lower in *Rel^{-/-}* mice, contrasting with a marginal reduction of antibody production in response to a T cell-independent antigen (NP-LPS). Since 1995, the function of c-REL in both lymphoid and myeloid immune cells was further investigated in mice.

b) IL-2 production defect

In mice, although IL-2 production by *Rel^{-/-}* T cells is impaired upon ConA, or antiCD3+CD28 stimulation, it is not abolished, suggesting that c-REL-independent mechanisms are also involved in the induction of *IL2* gene transcription^{121,122}. Indeed, c-REL binds to the promoter of *IL2* gene and promotes its transcription. Precisely, c-REL binds to the CD28RE region (for CD28 responsive element) within the *IL2* gene promoter, thereby facilitating its access to other transcriptional factors, by remodeling the chromatin structure^{123,124}. Further investigations show that c-REL activation in T cells is tightly controlled. TCR signaling only induces I κ B α -degrading signals leading to p50/p65 release and nuclear translocation, whereas c-REL is primarily complexed to I κ B β inhibitory protein in resting T cells¹²⁵. On the other hand, CD28 signaling triggers I κ B β degradation and c-REL nuclear translocation¹²⁶. In addition, CD4⁺ T cells primed with inflammatory cytokines such as TNF- α or IL-1 β , shift c-REL to I κ B α , therefore accessible to TCR-degradation signals¹²². Indeed, in primed T cells, I κ B α /c-REL complex are generated by a mechanism relying on nuclear export of c-REL by newly synthesized I κ B α ¹²². Accordingly, control CD4⁺ T cells primed with inflammatory cytokines significantly increase their production of IL-2 upon TCR stimulation, as compared to non-primed CD4⁺ T cells. This super-induction of IL-2 is abolished in primed *Rel^{-/-}* CD4⁺ T cells¹²². Upon antigen challenge, antigen presenting cells (APC), and notably dendritic cells (DC), provide essential help to T cells, by up-regulating CD80 and CD86 expression which bind to CD28, and by producing a wide range of inflammatory cytokines. Thus, c-REL would control the threshold of T cell activation, as it is

rapidly induced by both CD28 co-stimulation and pro-inflammatory cytokines, enabling higher and faster production of IL-2¹²². In line with this observation, mice deficient for Peli-1, a ubiquitin ligase specifically targeting c-REL and inducing its degradation, display autoimmunity secondary to c-REL nuclear accumulation and hyper-responsiveness of T cells to anti-CD3+antiCD28, and in terms of IL-2, IFN- γ production, and proliferation¹²⁷. Alternatively, in allogenic cultures, control T cells proliferate less and produce less IL-2, if being cultured with *Rel*^{-/-} dendritic cells¹²⁸. Indeed, *Rel*^{-/-} dendritic cells express less CD86 costimulatory molecule, which compromises their capacity to induce the proliferation of allogenic control T cells.

c) *Rel*^{-/-} mice exhibit multiple T helper cell defects

Studies of *Rel*^{-/-} mice show that c-REL is also critical for Th1 and Th17 cells differentiation. *Rel*^{-/-} mice are protected from experimental autoimmune encephalomyelitis (EAE), as they produce significantly less IL-17A and IFN- γ , as compared to control mice^{129,130}. In these models, further analysis show that *Rel*^{-/-} APC are unable to produce IL-12p40, IL-12p35 and IL-23p19 cytokines after LPS stimulation, which drive Th1 and Th17 differentiation¹²⁹. Similarly, the production of IL-12p40, IL-12p35 and IL-23p19 by *Rel*^{-/-} macrophages and/or DC, is significantly decreased in response to a wide range of TLR agonists (TLR2; TLR3; TLR4; TLR9) and CD40 ligand¹³¹⁻¹³⁶. These results suggest that the reduced production of polarizing cytokines by *Rel*^{-/-} myeloid cells is responsible for the Th1 and Th17 defect *in vivo*. In addition, the production of IL-17A and IFN- γ by *Rel*^{-/-} purified CD4⁺ T cells *ex vivo* is also impaired^{129,137}. Furthermore, c-REL binds to the promoter of *RORC* gene, encoding ROR- γ/γ T, a master regulator of Th17 differentiation, and induces its transcription^{130,137}. Consequently, purified naïve CD4⁺ T cells isolated from *Rel*^{-/-} mice fail to differentiate into IL-17A-producing cells *in vitro*, independently of the proliferation defect¹³⁷. *Rel*^{-/-} mice challenged into the footpad with *Leishmania major* show an impaired IL-12 and

IL-23-dependent Th1 and Th17 responses, an exacerbated leishmaniasis and parasitic burden¹³⁸. Precisely, CD4⁺ T cells from lymph nodes or spleen from *Rel*^{-/-} mice challenged with *Leishmania* produce significantly less IL-17A and IFN- γ after incubation with PMA/iono or *Leishmania* antigen, as compared to WT mice. Also, dendritic cells from challenged *Rel*^{-/-} mice produce less IL-12p40 after LPS stimulation. Finally, exogenous IL-12 restores IFN- γ production in *Rel*^{-/-} mice, which also display reduced parasitic lesions. Conversely, in another model of *Toxoplasma* infection, *Rel*^{-/-} mice are unable to produce IFN- γ , independently of an IL-12 production defect, and develop severe encephalitis¹³⁹.

Finally, c-REL is necessary for the generation and function of T follicular helper cells (Tfh), a subset of CD4⁺ T cells expressing high levels of CD40L and providing critical help to B cells in the germinal centers (GC). *Rel*^{-/-} mice exhibit decreased numbers of Tfh, as c-REL controls the transcription of IL-21, an essential cytokine for their expansion and differentiation¹⁴⁰. Accordingly, the administration of exogenous IL-21 rescues the generation of Tfh in *Rel*^{-/-} mice after antigen challenge. In addition, CD40L expression is reduced in Tfh from *Rel*^{-/-} mice, as c-REL binds to the promoter of *CD154* gene (encoding CD40L), and induces its expression¹⁴¹.

d) c-REL and regulatory T cells

Rel^{-/-} mice have diminished numbers of CD4⁺Foxp3⁺ regulatory T cells (Treg). However, *Rel*^{-/-} mice do not display autoimmunity, and *Rel*^{-/-} Treg cells show comparable suppressive activity *in vitro*, as compared to controls¹⁴². c-REL binds to an enhancer region within the promoter of *FOXP3* gene, and is essential for the assembling of an enhancer complex required for Treg differentiation *in vivo* and *in vitro*, independently of the IL-2 production defect^{143,144}. Recently, c-REL was shown to be critical in Treg-mediated suppression of anti-tumor effector responses. Consequently, melanoma growth is drastically reduced in *Rel*^{-/-}

mice, or after chemical ablation of c-REL, providing new perspectives for the development of anti-cancer treatments¹⁴⁵.

e) B cell immunity is impaired

Whereas c-REL does not appear to be essential for the development of mature B cells and the survival of quiescent B cells, it plays a critical role for optimal B cell responses to antigens *in vivo*¹²¹. B cells isolated from *Rel^{-/-}* mice proliferate poorly *in vitro* in response to mitogens such as BCR agonist, CD40L or LPS^{121,146,147}. This proliferation defect results from the inability of *Rel^{-/-}* B cells to progress through the cell cycle, and from an increased apoptosis^{146,147}. Also, total RNA, DNA and protein synthesis is strongly reduced in mitogen-activated *Rel^{-/-}* B, as compared to controls. The proliferation of *Rel^{-/-}* B cells is partially rescued if cytokines, such as IL-4 or IL-5, are added with the mitogens, by reducing apoptosis and increasing cell cycle progression. Further analysis show that c-REL controls the transcription of A1 and Bcl-xL, belonging to the Bcl-2 family, which prevent mitogen-induced cell death¹⁴⁸⁻¹⁵¹. A1 and Bcl-xL are induced in control but not in *Rel^{-/-}* B cells after BCR, CD40L, or LPS stimulation. Furthermore, the transduction of *Rel^{-/-}* B cells with A1 or Bcl-xL partially rescues the survival defect after activation with mitogens, but does not overcome the proliferation defect^{149,151}. Finally, c-REL is absolutely critical for the formation and maintenance of the GC reaction *in vivo*. Accordingly, specific ablation of c-REL in CD19⁺ B cells leads to an abortive GC reaction and abrogates T cell-dependent antibody responses, as observed in *Rel^{-/-}* mice¹⁵².

Altogether, these studies show a central role for c-REL in bridging innate and T helper cells responses *in vivo*. c-REL is critical in myeloid cells for the production of IL-12 and IL-23, in response to a wide range of stimuli *in vivo*, and *in vitro*. In T cells, c-REL is important for optimal IL-2 production, proliferation capacity, and effector functions of Th1, Th17 and Tfh. Finally, c-REL is essential for mature B cells survival, proliferation and Abs responses.

We report the case of a child who suffered from a variety of bacterial, viral, parasitic and fungal infections, suggestive of combined immunodeficiency, and for whom I identified a bi-allelic disease-causing candidate mutation in *REL*.

3. c-REL deficiency in human

a) Clinical case

The patient (P1) is a girl born in 2011 to first cousin Moroccan parents, and has no siblings. She was born at full term, with a weight within normal range. She received all recommended vaccinations, including BCG before the age of 1 year old. She was referred to the hospital for a first episode of esophagitis and oral thrush due to *C. albicans* before the age of one year old. Subsequently, P1 showed multiple episodes of mucosal candidiasis and notably ulcerative esophagitis. Concomitantly, P1 developed multiple viral infections, as she presented recurrent Herpes simplex virus 1 (HSV-1) labial herpes; one episode of thoracic Zoster; a chronic cytomegalovirus (CMV) viremia only responsible for sporadic episodes of fever; and an asymptomatic chronic digestive replication of adenovirus and enterovirus. P1 also suffered from chronic cholangitis with biliary tract dilatation due to *Cryptosporidium parvum* infection, diagnosed at 2 years old. In 2014, she was admitted for a disseminated BCG disease with osteomyelitis, lymph node and lung lesions. Antibiotherapy with ISONIAZIDE and RIFAMPICINE was initiated, enabling a quick improvement. Intravenous Immunoglobulin (Ig) replacement therapy was initiated, in association with preventive BACTRIM antibiotherapy. P1's phenotype was consistent with a combined immunodeficiency, and was referred to Necker Hospital in Paris, for further investigations. P1 did not show any extra-hematopoietic symptoms. NGS-targeted sequencing of over 300 genes associated with PIDs was performed and did not reveal any candidate mutations. P1

received a haplo-identical peripheral blood stem cell transplantation, with CD45RA⁺ T cells depletion in January 2017. Follow-up at 6 months showed a 96% donor chimerism; no signs suggestive of graft versus host disease (GVHD) under immunosuppressive treatment with NEORAL; no infectious complications (and no detectable CMV viremia) under Ig replacement therapy, BACTRIM, and ZELITREX. Immune reconstitution at 6 months, shows normal counts of CD8⁺ T cells and B cells; CD4⁺ T cell lymphopenia; no naïve CD4⁺ or CD8⁺ T cells; and NK lymphocytosis (**Table 2**).

b) Genetics

I identified by WES and confirmed by Sanger sequencing a bi-allelic mutation in *REL* gene, in P1. The mutation is a nucleotide substitution (c.395-1C>T) localized in the acceptor splice site of exon 5, an exon that encodes in part for the RHD of both described isoforms. Familial segregation is consistent with an AR inheritance, since both parents are heterozygous for the mutation. The mutation is private, as it is not reported in the gnomAD database comprising WES data from 123,136 individuals. The mutation is predicted to be damaging *in silico*, as the CADD score of 26,7 obtained for this mutation is well above the mutation significance cutoff (MSC) of 2,3. Moreover, neither this mutation, nor other bi-allelic mutations in *REL*, are present in our in-house database of approximately 4,000 WES performed in patients with various infectious diseases. Importantly, not a single predicted LOF *REL* allele (nonsense, frameshift deletions/insertions, or essential splice variants) is reported to be homozygous in gnomAD. Overall, both the family's and the population genetic study support that the mutation identified P1, is probably disease-causing. The c.395-1C>T disrupts mRNA splicing, as an aberrant cryptic splice site leads to a deletion of 14 nucleotides (c.395_408del) in exon 5, and a subsequent premature stop codon (p.Val132Alafs*3). As shown by quantitative RT-PCR, the mutation also alters *REL* mRNA stability. Consistent with these findings, c-REL expression is abolished in patient's Epstein-

Table 2: Immunophenotyping before and 6 month after HSCT in P1

	Before HSCT		M6 post HSCT		range numbers (/ul)	range %
	Number (/ul)	%	Number (/ul)	%		
Lymphocytes	4100		3367			
CD3+	2829	69	1210	33	1300-3700	56-75
CD4+	902	22	257	7	700-2200	28-47
CD8+	1271	31	917	25	490-1300	16-30
TCR $\alpha\beta$ /CD3+		66				
TCR $\gamma\delta$ /CD3+		34				
CD45RO+/CD4+ (<u>memory</u>)		14		98		
CD45RA+/CD4+		86		2		73-86
CD31+/CD4+		72		27		
CD31+CD45RA+/CD4+ (<u>naïve</u>)		67		2		57-65
CCR7+CD45RA+/CD8+ (<u>naïve</u>)		39		0		52-68
CCR7+CD45RA-/CD8+ (<u>CM</u>)		3		2		3-4
CCR7-CD45RA-/CD8+ (<u>EM</u>)		47		95		11-20
CCR7-CD45RA+/CD8+ (<u>TEMRA</u>)		11		3		16-28
CD19+	1148	28	623	17	273-860	6.1-25.2
CD27+/CD19+ (<u>memory</u>)		1.2				8.1-33.3
CD27-IgD+/CD19+ (<u>naïve</u>)		99				59.7-88.4
CD27+IgD-/CD19+ (<u>switched memory</u>)		0.2				2.9-17.4
CD27+IgD+/CD19+ (<u>MZ B cells</u>)		1				3.1-18
CD24+++CD38+++/CD19+ (<u>transitional</u>)		18				
CD24-CD38+++/CD19+ (<u>plasmablasts</u>)		0.1				
CD21 ^{low} CD38 ^{low} /CD19+ (<u>autoreactive B cells</u>)		2				
CD16+CD56+	82	2	1797	49	130-720	4-17

CM: central memory
EM: effector memory
TEMRA: terminal EM CD45RA+
MZ: Marginal zone

Barr virus-immortalized B cell lines (B-EBV) and leucocyte subsets. Overall, these results indicate that the c.395-1C>T mutation disrupt *REL* mRNA splicing, with no leakiness, and subsequently results in c-REL loss-of-expression.

c) Immunological features: preliminary results and short term plans

Before stem cell transplantation, P1 showed normal counts (/ul of blood) of circulating CD4⁺ T-, CD8⁺ T- and B lymphocytes; normal repartition of naïve versus memory CD4⁺ T cells; decreased counts of naïve and TEMRA CD8⁺ T cells; elevated counts of central memory CD8⁺ T cells, as compared to age-matched controls. In-depth *ex vivo* phenotyping of T cell subsets shows elevated proportions of $\gamma\delta$ T cells; normal proportions of MAIT (CD3⁺CD161⁺V α 7.2⁺), and iNKT (CD3⁺INKT⁺) cells; decreased proportions of Treg (CD4⁺FOXP3⁺CD25⁺CD27^{low}) cells among total CD3⁺ T lymphocytes. T helper phenotyping showed normal proportions of Tfh (CXCR5⁺), Th1 (CXCR3⁺CCR4⁻CCR6⁻), and Th17 (CXCR3⁻CCR4⁺CCR6⁺) cells; decreased proportions of Th2 (CXCR3⁻CCR4⁺CCR6⁻), and Th1* (CXCR3⁺CCR4⁻CCR6⁺) cells among total memory CD4⁺ T cells. Th1* cells are a subset of T helper cells strongly enriched in *Mycobacterium*-responsive clones, expressing both T-bet and ROR- γ T transcriptional factors, and secreting IL-17A/IL-17F and IFN- γ ¹⁶. B cells phenotyping shows that P1's B cells are essentially naïve, as CD27⁺ memory B cells are drastically reduced, with almost no CD27⁺IgD⁻ switched memory B cells. NK cell counts are slightly decreased, with a normal distribution of CD56^{dim} NK cell subsets. Overall, these data do not support a central role of c-REL deficiency in early lymphoid development.

Patient's T cells fail to proliferate *in vitro* in response to all antigens tested (CMV, candidine, tetanus toxin, tuberculin). In contrast, the response to polyclonal stimulation using beads coated with anti-CD2/anti-CD3/anti-CD28 Abs results in a strong proliferation. The response to increased plate-coated doses of OKT3 (targeting CD3) is slightly decreased. The response to Phytohemagglutinin (PHA), a lectin with mitogenic properties, was abolished

twice, and lower than in controls in a third experiment. Interestingly, the addition of exogenous IL-2 to PHA totally restores P1's T cell proliferation in two separate experiments. The production of IL-2 in P1's sorted naïve and memory CD4⁺ T cells is decreased, but not abolished, as compared to controls. Finally, P1's sorted CD3⁺ T cells proliferate similarly to controls CD3⁺ T cells in allogenic co-cultures with CD11c⁺ conventional dendritic cells (cDC) from healthy donors, suggesting that P1's T cells are intrinsically capable to produce IL-2. Overall, c-REL deficient T cells show a partial intrinsic defect to produce IL-2. Whether IL-2 production defect explains entirely the T cells defective response to antigens *in vitro* is questionable. Alternatively, a quantitative and/or functional defect of P1's myeloid cells could account for the defective proliferation to antigens. *Ex vivo* phenotyping of all dendritic cell subsets (cDC1: CD11c⁺CD141⁺; cDC2: CD11c⁺CD1c⁺; plasmacytoid DC: CD303⁺) and monocytes subsets (CD14⁺CD16⁻; CD14⁻CD16⁺) shows no quantitative defect in P1. I intend to test the function of P1's dendritic cells. To do so, we will differentiate *in vitro*, all subsets of DC from P1's and controls cryopreserved bone marrow (BM) CD34⁺ hematopoietic stem cells and test: (i) their ability to induce the proliferation of allogenic control sorted CD3⁺ T cells; (ii) the surface expression of the costimulation molecules HLA-DR, CD80 and CD86; (iii) their production of cytokines par multiplex ELISA, after incubation with TLR agonists, and CD40L. Finally, I will test whether P1's T cell proliferation to antigens is rescued in the presence of her mother's CD11c⁺ cDC, as P1 is HLA matched with her mother for HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 antigens.

P1 has normal counts of circulating CD19⁺ B cells, and almost no memory B cells. The antibody response to T cell-dependent vaccines (Diphtheria, Tetanus, Poliomyelitis) is abolished, whereas the serological response to the T cell-independent Pneumococcal polysaccharide vaccine (anti-pneumococcus IgG) is conserved. In addition, repeated dosages

of Ig showed IgG and IgM within normal ranges, but undetectable IgA. *In vitro* assays further show that P1's sorted naïve B cells cannot secrete IgA or IgG, but still can secrete IgM in response to CD40L alone or in association with CpG or IL-21; exhibit a high mortality rate after CD40L with or without IL-21, which was only partially overcome by the addition of CpG or BCR agonists; a markedly decreased proliferation to various combinations of reagents including BCR agonist and CD40L. Overall, these data corroborate the observations made in *Rel^{-/-}* mice, that c-REL is critical for the proliferation and survival of B cells, and the generation of a memory B cell response. To further address the question of the molecular impact of c-REL defect, we will perform RNA sequencing in P1's naïve B cells, to test the basal and induced mRNA expression of genes after incubation with CD40L and BCR agonist. I will confirm by qPCR experiments each target genes that is differentially expressed in P1 versus controls B cells.

P1 developed a severe disseminated BCG disease. Inborn errors of IFN- γ immunity underlie mycobacterial disease in humans¹⁰. IFN- γ can be produced by NK cells, $\gamma\delta$ T cells, CD8⁺ T cells, as well as two subsets of CD4⁺ T helper cells named Th1 and Th1*. Besides, IFN- γ production critically depends on IL-12, as best exemplified by patients with AR IL-12RB1 or IL-12p40 deficiencies suffering from MSMD¹⁰. The production of IFN- γ in P1's whole blood incubated with PMA/iono is comparable to controls, thus suggesting that collectively $\alpha\beta$ T cells (comprising both CD8⁺ and CD4⁺ T cells), $\gamma\delta$ T cells and NK cells produce IFN- γ normally. However, P1's purified memory CD4⁺ T cells (CD45RA⁻), and naïve CD4⁺ T cells cultured under Th1-polarizing conditions (antiCD2/antiCD3/antiCD28 coated beads + IL-12), produce much less IFN- γ than controls. Therefore, c-REL deficiency impairs intrinsic CD4⁺ T cells ability to produce IFN- γ in conditions of polyclonal stimulation. I then assessed *Mycobacterium*-specific IFN- γ response in P1's whole blood: P1's cells do not produce IFN- γ in response to BCG, in contrast to controls. This defect is

however rescued by the addition of exogenous IL-12. Accordingly, the production of IL-12p40 and IL-12 (comprising IL-12p40 and IL-12p35) is abolished in P1's whole blood in response to BCG alone or in association with IFN- γ . Therefore, both the IL-12 production defect and the $\alpha\beta$ CD4⁺ T cells defect may account for mycobacterial disease in c-REL deficiency. IL-12p40 and IL-12p35 are produced by myeloid cells, and predominantly by CD11c⁺ cDC. In mice, c-REL controls the production of IL-12 cytokines in response to a wide variety of ligands (see above). I will therefore test the production of IL-12 cytokines in P1's *in vitro* differentiated cDC subsets (cDC1 and cDC2), from BM CD34⁺ hematopoietic stem cells, after incubation with BCG alone or in association with IFN- γ . Also, c-REL binds to the promoter of *RORC* gene, encoding ROR- γ/γ T, and promotes its transcription in mice¹³⁷. Patients with AR ROR- γ/γ T deficiency are susceptible to mycobacteria because of a selective IFN- γ production defect by $\gamma\delta$ T cells and Th1* cells, suggesting that both these subsets are the predominant cell types responding to BCG in humans⁷². Therefore, c-REL deficiency could also result in a ROR- γ/γ T-dependent functional defect of $\gamma\delta$ T cells and Th1* cells. To address this question, we will sort P1's $\gamma\delta$ T cells and all different subsets of P1's memory CD4⁺ T helper cells, and test their response to BCG peptides in terms of proliferation (presence of *Mycobacterium*-responsive memory Th1 and Th1*, and $\gamma\delta$ T cells?) and IFN- γ production.

P1 also suffered from multiple episodes of mucosal candidiasis, suggesting that IL-17A/IL-17F-mediated immunity is impaired in c-REL deficiency. IL-17A and IL-17F are produced by Th17, Th1*, and $\gamma\delta$ T cells notably. Moreover, Th17 are enriched in *Candida*-responsive memory cells in humans, as compared to other T helper subsets¹⁶. Despite normal proportions of circulating Th17 and $\gamma\delta$ T cells, the production of IL-17A in P1's whole blood after PMA/iono, is much lower than in controls, and comparable to the levels obtained in patients with *STAT3*^{+/-} LOF, or *STAT1*^{+/-} GOF mutations, who suffer from sCMCD. P1's

purified memory CD4⁺ T cells (CD45RA⁻), and naive CD4⁺ T cells cultured under Th17-polarizing conditions (antiCD2/antiCD3/antiCD28 coated beads + TGF-β, IL-1β, IL-6, IL-21, IL-23, anti-IL-4, and anti-IFN-γ antibodies), produce much less IL-17A and IL-17F, than controls. Therefore, CD4⁺ T cells are intrinsically deficient for IL-17A and IL-17F production, which may account for the CMC observed in P1. Same results are obtained in AR ROR-γ/γT deficient patients also displaying CMC. In addition, the production of IL-17A and IL-17F by sorted CCR6⁺ memory CD4⁺ T cells (comprising Th17 and Th1*), in response to *Candida* is strongly decreased in AR ROR-γ/γT deficiency⁷². As c-REL drives ROR-γ/γT expression and Th17 differentiation in mice¹³⁷, I will test *RORC* gene mRNA expression in P1's naïve CD4⁺ T cells cultured under Th17 polarizing conditions. I will also test the response of P1's purified CCR6⁺ memory CD4⁺ T cells to *Candida* in terms of proliferation and IL-17A/IL-17F production. IL-23 production is certainly impaired in P1, as IL-23 shares with IL-12, the IL-12p40 subunit. In mice, c-REL also controls IL-23p19 expression, the second subunit of IL-23¹³⁶. Therefore, c-REL deficiency could underlie susceptibility to *Candida*, through a reduced production of IL-23, impairing IL-17 production by *Candida*-specific memory Th17 cells and γδ T cells. We will therefore test the production of IL-23p19 by *in vitro* differentiated cDCs from P1, incubated with *Candida*.

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