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Impairment of immunity to *Candida* and *Mycobacterium* in humans with bi-allelic *RORC* mutations*

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Abstract

Human inborn errors of immunity mediated by the cytokines interleukin (IL)-17A/F underlie mucocutaneous candidiasis, whereas inborn errors of interferon (IFN)-γ immunity underlie mycobacterial disease. We report the discovery of bi-allelic *RORC* loss-of-function mutations in seven individuals from three kindreds of different ethnic origins with both candidiasis and mycobacteriosis. The lack of functional RORγ and RORγT isoforms resulted in the absence of IL-17A/F-producing T cells in these individuals, probably accounting for their chronic candidiasis. Unexpectedly, leukocytes from RORγ- and RORγT-deficient individuals also displayed an impaired IFN-γ response to *Mycobacterium*. This principally reflected profoundly defective IFN-γ production by circulating γδ T cells and $CD4^+CCR6^+$ $CXCR3^+$ αβ T cells. In humans, both mucocutaneous immunity to *Candida* and systemic immunity to *Mycobacterium* require RORγ, or RORγT, or both.

Introduction

Inborn errors of human IL-17A/F or IFN-γ immunity are each associated with a specific set of infections. Inborn errors of IL-17A/F underlie chronic mucocutaneous candidiasis (CMC), which is characterized by infections of the skin, nails, oral and genital mucosae with *Candida albicans*, typically in the absence of other infections. Five genetic etiologies of CMC have been reported, with mutations in five genes (1, 2). Inborn errors of IFN-γ underlie Mendelian susceptibility to mycobacterial disease (MSMD), which is characterized by selective susceptibility to weakly pathogenic mycobacteria, such as *Mycobacterium bovis* Bacille Calmette-Guérin vaccines (BCG) and environmental mycobacteria. Eighteen genetic etiologies of MSMD have been reported, involving mutations of nine genes (3, 4). Only a few patients display both candidiasis and mycobacteriosis, including some patients with IL-12p40 and IL-12Rβ1 deficiencies, which impair IFN-γ immunity in all patients and IL-17A/F immunity in some patients (4). We studied seven patients from three unrelated consanguineous families with this unusual combination of infectious diseases but with no

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known genetic disorder. A Palestinian child (Fig. 1A, Kindred A, patient P1, SOM Case Reports) died at the age of six years from disseminated BCG disease. Two other children (P2 and P3) in Kindred A had similar clinical presentations but survived and are now 7 and 4 years old, respectively. A 6-year-old Chilean child (Kindred B, P4, SOM Case Reports) had disseminated BCG infection at age 16 months. Finally, three siblings from Saudi Arabia (Kindred C, P5, P6 and P7, SOM Case Reports), aged 9, 6 and 3 years, had mycobacterial diseases, caused by BCG in two children and by *M. tuberculosis* in the third. Six of the seven patients also had mucocutaneous candidiasis, of various severities (Table S1).

Bi-allelic *RORC* mutations

We combined whole-exome sequencing (WES) and genome-wide linkage (GWL) analysis to search for homozygous genetic lesions in the three probands (P1, P4, and P6) (Fig. S1). We identified a homozygous C/T mutation in the *RORC* gene in P1, P2, and P3, resulting in a missense S38L substitution in the retinoic acid-related orphan receptors γ (RORγ) isoform, or a S17L substitution in the RORγT isoform (Fig. 1A,B, Fig. S2). In P4, we identified a homozygous *RORC* C/T mutation converting the Q329 residue of RORγ (or Q308 in RORγT) into a stop codon (Fig. 1A,B, Fig. S2). In P5, P6 and P7, we identified a homozygous C/T mutation converting the Q441 residue of RORγ (or Q420 in RORγT) into a stop codon (Fig. 1A,B, Fig. S2). In each kindred, all unaffected family members were either heterozygous or homozygous for the WT allele (Fig. 1A, Fig. S2). The familial segregation of these mutant *RORC* alleles was therefore consistent with an autosomal recessive (AR) pattern of inheritance. There were no other genes mutated in the three kindreds among the 173 genes on the 6.87 Mb interval linked with disease (maximum LOD score 6.35). The S17L mutation affects a strictly conserved residue of the DNA-binding domain (DBD) of RORγT (Fig. 1B) and is predicted to be damaging by multiple software algorithms (5). The Q308X and Q420X nonsense mutations are predicted to result in truncated proteins lacking part of the ligand-binding domain (LBD, Fig. 1B). The Q308X and Q420X alleles were not found in the NCBI, Ensembl, ExAC, and dbSNP databases, our own in-house database of over 3,000 exomes, or in 1,052 controls from 52 ethnic groups in the CEPH-HGD panel, indicating that they were very rare variants, possibly private to these two kindreds. There were no nonsense or frameshift mutants affecting isoform 2 (RORγT) in these databases. The S17L allele was found in one heterozygous individual of the ExAC database, indicating that its frequency is less than 10^{-5}. We therefore hypothesized that the biallelic *RORC* mutations found in these three kindreds were disease-causing.

Complete RORγ and RORγT deficiency

In mice and humans, the RORγ and RORγT isoforms are generated by transcription from different start sites (6-10) (Fig. 1B). Both molecules are transcription factors, but they have different expression patterns in inbred mice: RORγ is ubiquitous, whereas RORγT is restricted to leukocytes (10). RORγT plays an important role in T-cell development and function in mice (11, 12). Animals lacking only RORγT apparently have the same immunological phenotype as those lacking both isoforms (10). We first assessed the impact of *RORC* mutations, by transiently expressing wild-type (WT) and mutant RORγT and RORγ in HEK293T cells in the presence and absence of stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin. We detected both the WT and S17L RORγT

proteins, at the expected molecular weight (MW) of 56 kDa (Fig. 1C). The Q308X and Q420X RORγT mutant proteins had MW consistent with truncation at residues 308 and 420, respectively (Fig. 1C). Similar results were obtained upon expression of RORγ (Fig. S3). We then performed EMSA, to assess the ability of the mutant RORγT and RORγ isoforms to respectively bind to RORE-2 and RORE-1, the consensus binding sites in the promoter of *IL17A* (Fig. S3). The three mutations abolished DNA binding by RORγT to RORE-2 (Fig. 1C) and by RORγ to RORE-1 (Fig. S3), but not by disrupting the nuclear localization of the protein (Fig. S3). Each mutation resulted in the loss of *IL17A* promoter activation by RORγT (Fig. 1D) or RORγ (Fig. S4). Thus, each mutant allele was associated with a complete loss of function of the two encoded protein isoforms, identifying these patients as cases of human AR complete RORγ/RORγT deficiency (hereafter referred to as RORγT deficiency).

Broad immunological phenotype

Mouse RORγT is expressed in lymphoid tissue inducer (LTi) cells, innate lymphoid cells type 3 (ILC3), type 1 natural killer T cells (NKT, also designated iNKT), some γδ T cells, immature $CD4^+CD8^+$ αβ thymocytes, and IL-17A/F-producing $CD4^+$ αβ T cells (T helper (Th)17 cells) (7, 11, 13-16). LTi, ILC3, type 1 NKT and Th17 cells fail to develop in *Rorc*$^{-/-}$ mice, and $CD4^+CD8^+$ αβ thymocytes have a reduced life span (11, 14, 17). *RORC*$^{-/-}$ patients displayed clinical signs consistent with LTi deficiency, including absence of palpable axillary and cervical lymph nodes (despite visible tonsils), and had reduced thymus size (Fig. 2A). Like in *Rorc*−/− mice, ILC3 were barely detectable in the patients' blood (Fig. S5). In *Rorc*$^{-/-}$ mice, the short lifespan of $CD4^+CD8^+$ αβ thymocytes results in an inability to use the most 5' segments of the T cell receptor Vα array (12), including those encoding the Vα chains of mucosal associated invariant T (MAIT) (12) and type I NKT cells (18). High throughput sequencing of the *TRA/TRD* and *TRG* loci revealed that 5' Vα gene segment use was decreased, while Vδ and Vγ usage was normal in *RORC*$^{-/-}$ T cell clonotypes (Fig. S6). Further, these patients lacked *TRA* clonotypes utilizing 5' Vα and distal 3' Jα pairings (Fig. S6). In total *RORC*$^{-/-}$ T cell clonotypes, usage of Vγ9 was elevated (Fig. S6), consistent with antigen-driven peripheral expansion of this subset, perhaps driven by mycobacteria (19). Abolished use of the Vα segments *TRAV10* (encoding Vα24) and *TRAV1.2* (encoding Vα7.2) was confirmed by qPCR (Fig. S7) and resulted in a lack of both $CD161^+V\alpha7.2^+$ MAIT cells and $V\alpha24^+V\beta11^+$ type I NKT cells (Fig. 2B,C and Fig. S7). Some $V\alpha7.2^+$ cells other than MAIT cells have been recently shown to recognize *Mycobacterium*-derived mycolyl lipids (20); they were also missing in *RORC*$^{-/-}$ patients. Nevertheless, *RORC*$^{-/-}$ patients displayed only mild $CD4^+$ and $CD8^+$ αβ T-cell lymphopenia, with normal B- and NK-cell counts (Fig. 2D, Table S2). These patients did not, therefore, have T-cell, or "combined" immunodeficiency (CID), consistent with their lack of broad infectious and autoimmune phenotypes (21). Finally, the frequencies of circulating γδ T cells were normal (Table S2). Overall, these *RORC*$^{-/-}$ patients displayed the general immunological features characteristic of *Rorc*$^{-/-}$ mice (11, 12, 14, 22, 23). These studies also revealed that the development of MAIT and other $V\alpha7.2^+$ T cells is critically dependent on RORγT, which had been predicted but not shown in mice. No infectious phenotype can be unambiguously assigned to any of these individual immunological anomalies.

Abolished production of IL-17A/F

Given the critical role of murine RORγT in generating IL-17A/F- and IL-22-producing lymphocytes, including ILC3, γδ T cells, and Th17 cells (11, 13, 24), and the finding that patients with compromised IL-17A/F immunity are susceptible to mucocutaneous candidiasis (1) we assessed the development and function of IL-17A/F-producing lymphocytes in the patients. Circulating ILC3 cells were too few to assess their production of IL-17. $CD3^+$ T cells from *RORC*$^{-/-}$ patients displayed a severe impairment in the production of IL-17A, IL-17F, and IL-22 both at the mRNA (Fig. S8) and at the protein level (Fig. 3A) after polyclonal stimulation. $CD4^+$ αβ T cells are a major source of IL-17A/F (9). Memory ($CD45RA^-$) $CD4^+$ T cells from *RORC*$^{-/-}$patients produced much less IL-17A, IL-17F, and IL-22 than WT and heterozygous controls (Fig. 3B). In contrast, the memory $CD4^+$ T cells from these patients produced large amounts of IL-4, IL-5, and IL-13 (Fig. S8). In separate experiments, naïve ($CD45A^+$ $CCR7^+$) $CD4^+$ T cells from *RORC*$^{-/-}$ patients cultured under IL-17-polarizing conditions secreted less IL-17A and IL-17F than cells from healthy donors or heterozygous relatives (Fig. 3C). We next assessed the proliferation and cytokine secretion of highly purified WT, heterozygous, and *RORC*$^{-/-}$ $CCR6^+CD4^+$ memory αβ T cells (Fig. S9), a population enriched in IL-17A/F-secreting cells (Th17, which express CCR4), as well as cells secreting IL-17A/F and IFN-γ (herein designated as Th1*, which express CXCR3) (25), following their stimulation with *C. albicans* lysate. By monitoring the incorporation of a radioactive label, we found that $CD4^+CCR6^+$ T cells from *RORC*$^{-/-}$ patients had normal frequencies of antigen-specific cells recognizing *C. albicans* (Fig. 3D). However, these cells (including both Th17 and Th1*, whose proportions were normal, Fig. S9) secreted much less IL-17A and IL-22 than control cells (Fig. 3E). IFN-γ was also reduced, but large amounts of IL-4 were secreted, serving as control (Fig. 3E). Finally, *Herpesvirus saimiri*-transformed $CD4^+$ αβ T cells from *RORC*$^{-/-}$ patients showed abolished induction of *RORC* (Fig. 4A) and *IL17A* (Fig. 4B), but not *IFNG* serving as a control (Fig. S10). The defect in *IL17A* induction could be rescued by retroviral transduction with WT *RORC* (Fig. 4B). Collectively, these data demonstrate a profound diminution of IL-17A/F and IL-22 production by all leukocytes tested in *RORC*−/− patients. As CMC-causing germline mutations have previously been identified in *IL17F*, *IL17RA*, *IL17RC*, and *ACT1 (1, 26, 27)* we conclude that impaired IL-17A/F immunity in *RORC*$^{-/-}$ patients accounts for their development of CMC. Human IL-17A/F-producing ILC3, γδ T cells, and αβ T cells, or any of their subsets, may individually or collectively confer protection against *Candida*.

Selective defect in IFN-γ production

We then investigated the cellular mechanism underlying the patients' surprising susceptibility to mycobacteria. The patients did not display chronic granulomatous disease or severe combined immunodeficiency, which can underlie BCG disease (4). The $CD3^+$ T cells (including both γδ and αβ T cells) from *RORC*$^{-/-}$ patients produced IFN-γ normally, following the stimulation of whole blood or PBMC with PMA and ionomycin (Fig. S10 and data not shown). Likewise, total $CD4^+$ αβ T cells, memory ($CD45RA^-$) $CD4^+$ T cells, naïve $CD4^+$ T cultured under Th1-polarizing conditions, and *herpesvirus saimiri*-transformed T cells from the patients produced IFN-γ normally (Fig. S10). Overall, and in contrast to the

IL-17A/F defect, RORγT deficiency does not impair IFN-γ secretion in conditions of polyclonal stimulation. We next assessed *mycobacterium*-specific IFN-γ responses from whole blood (Fig. 5A) or PBMC (Fig. 5B) of *RORC*$^{-/-}$ patients, heterozygous family members, and healthy controls. The patients' cells produced very little IFN-γ in response to BCG plus IL-12 treatment (Fig. 5A,B). This defect was as profound as that seen in patients with IL-12Rβ1 deficiency (28). The production of IL-12p40 by *RORC*$^{-/-}$ cells was normal (Fig. S11). Impaired IFN-γ production may account for mycobacterial diseases in *RORC*$^{-/-}$ patients. This IFN-γ defect was not secondary to excessive IL-4, IL-5, or IL-13 production (Fig. S11) or to the IL-17A/F defect (Fig. S12). Many single-gene immunodeficiencies do not predispose to BCG disease despite impaired or abolished development or function of various αβ T cell subsets, including CD4^{+} T cells (29), CD8^{+} T cells (30), type 1 NKT cells (31, 32), and MAIT cells (32). Even rare patients deficient in total αβ T cell function (*ZAP70*$^{-/-}$ (33), *TRAC*$^{-/-}$ (34)) have not been reported to develop BCG disease. Whole blood or PBMC from such patients responded to BCG plus IL-12 normally, except for patients lacking all functional αβ T cells (Fig. S12). As MAIT cells were shown to respond to mycobacteria (35), we purified these cells from WT donor PBMC and added them to PBMC from *RORC*$^{-/-}$ patients before BCG stimulation. The lack of MAIT cells in *RORC*$^{-/-}$ patients did not account for their impaired IFN-γ production (Fig. S13). Overall, the absence of type 1 NKT and MAIT cells, the mild T-cell lymphopenia, and the poor development of IL-17A/F T cells may contribute marginally to mycobacterial susceptibility, but do not account for the low level of IFN-γ production by *RORC*$^{-/-}$ leukocytes stimulated with BCG and IL-12, and probably not for the patients' mycobacterial disease.

Impaired IFN-γ production by γδ T cells

We thus systematically characterized the consequences of leukocyte population depletions on BCG-dependent IFN-γ production by PBMC in healthy controls. We found no overt IFN-γ defect as a consequence of depleting NK cells, CD14^{+} cells, CD4^{+} or CD8^{+} T cells. Depletion of αβ T cells, γδ T cells, or both resulted in diminished IFN-γ production (Fig. S14). To probe the kinetics of this phenotype, a similar experiment was repeated and supernatant was assessed at 6, 12, 18, 24 and 48h post-stimulation (Fig. S14). The effect of γδ T cell depletion was most apparent at 24h (Fig. S14). We observed high expression of *RORC* isoform 2 mRNA in both αβ and γδ T cells of healthy donors (Fig. S15), prompting further analyses of γδ T cell function. Flow cytometry analyses revealed that the TCR^{high} γδ T cells from *RORC*$^{-/-}$ patients could not secrete IFN-γ in response to PMA-ionomycin, unlike TCR^{low} γδ T cells (Fig. S15). TCR Vδ2^{+} cells have been reported as the predominant cells responding to human BCG vaccination (19). *RORC*$^{-/-}$ patients had normal frequencies of TCR Vδ2^{+} cells, but these cells were unable to secrete IFN-γ when stimulated with PMA and ionomycin (Fig. S15), suggesting a possible contribution of this γδ T cell subset defect to mycobacterial susceptibility in *RORC*$^{-/-}$ patients. Overall, RORγT deficiency diminishes the IFN-γ-producing capacity of γδ T cells, which normally produce this cytokine in response to *Mycobacterium* stimulation.

The patients' CCR6^{+} CD4^{+} αβ T cells produce little IFN-γ in response to BCG

Previous studies have demonstrated that the T-bet- and RORγT-expressing, IFN-γ and IL-17A/F-producing Th1* CCR6^{+}CXCR3^{+} subset is strongly enriched for *Mycobacterium*-

responsive $CD4^+$ αβ T cells, unlike $CCR6^+CCR4^+$ Th17 cells that only express RORγT and IL-17A/F and are enriched for *Candida*-responsive T cells (25). We therefore purified $CCR6^+$ cells (Fig. S9), and assessed their proliferation and cytokine production in response to a pool of BCG peptides. $CD4^+CCR6^+$ αβ T cells from *RORC*$^{-/-}$ patients had a normal or high frequency of antigen-specific cells recognizing BCG peptides, as demonstrated by the induction of proliferation (Figs. 5C and S16). However, although $CD4^+CCR6^+$ T cells from *RORC*$^{-/-}$ patients responded to mycobacterial antigens, they secreted much less IFN-γ than $CD4^+CCR6^+$ αβ T cells from normal donors (Fig. 5D). The normal proliferation and cytokine production of other $CD4^+$ memory subsets in response to *Candida* and *Mycobacterium* (Fig. S17), and to irrelevant viral stimuli (Fig. S18), indicate a selective RORγT-dependent functional defect in *Mycobacterium*-specific $CD4^+CCR6^+$ αβ T cells. Although we did not purify and test Th1* cells, they were present in normal proportions in the patients (Fig. S9), implying that they are functionally defective for IFN-γ production. Collectively, these data suggest that mycobacterial diseases in *RORC*$^{-/-}$ patients may result from the poor production of IFN-γ by γδ T cells, $CD4^+CCR6^+CXCR3^+$ αβ Th1* cells, or both, in response to mycobacteria. IFN-γ treatment may therefore be beneficial for *RORC*$^{-/-}$ patients. This combined defect probably also accounts for mycobacterial disease in SCID patients, as patients with various forms of CID are normally resistant to BCG (28, 34). Finally, the lack of MAIT and type 1 NKT cells, reduction in ILC3, and possibly of other lymphocytes not analyzed using blood samples (e.g. LTi), may aggravate the mycobacterial phenotype of *RORC*$^{-/-}$ patients.

Conclusion

Collectively, these data demonstrate that human *RORC* play a surprising dual role in host defence. These findings are clinically, immunologically, and genetically robust, as they were consistent in seven patients, from three ethnic groups, homozygous for three different *RORC* mutations that are loss-of-function for both isoforms. Interestingly, although the two infectious phenotypes are purely recessive, some immunological phenotypes showed co-dominant or dominant inheritance. The mild T-cell lymphopenia, small thymus, lack of palpable axillary and cervical lymph nodes, and absence of MAIT and type 1 NKT cells in *RORC*$^{-/-}$ patients were consistent with the phenotype of *Rorc*$^{-/-}$ mice (Table S3). Likewise, impaired IL-17A/F immunity was predicted to account for impaired protection against *Candida albicans* (36), as *Rorc* is the master gene controlling Th17 differentiation in inbred mice (11) and mutations affecting human IL-17A/F immunity underlie isolated CMC (1, 27, 37). The IL-17A/F defect therefore underlies CMC in RORγT-deficient patients, probably but not necessarily because of T cells as other cells can normally produce these cytokines. We expected these patients to be susceptible to candidiasis, but their susceptibility to mycobacterial disease, and its severity, were unanticipated. This phenotype does not seem to be human-specific, as we also found that mice deficient for *Rorc* (14) are susceptible to mycobacterial infection (Fig. S19). Our data conclusively demonstrate that human *RORC* plays an indispensable role in the induction of IFN-γ-dependent anti-mycobacterial systemic immunity. The mechanism underlying disease in these patients probably involves an impairment of the induction of IFN-γ production by γδ T cells, or $CD4^+CCR6^+CXCR3^+$ αβ Th1* cells, or both, in response to mycobacteria. Other

mechanisms may also be at work. Human *RORC* is essential not only for the development of IL-17A/F-producing lymphocytes protecting the mucocutaneous barriers against *Candida*, but also for the activation of IFN-γ-producing T cells, and for systemic protection against *Mycobacterium*.

Supplementary Material

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37. Material and methods are available as supplementary materials on Science online.

One-Sentence Summary

Human RORγ and RORγT deficiency impairs both IL-17A/F-dependent mucocutaneous immunity to *Candida* and IFN-γ-dependent systemic immunity to *Mycobacterium*.

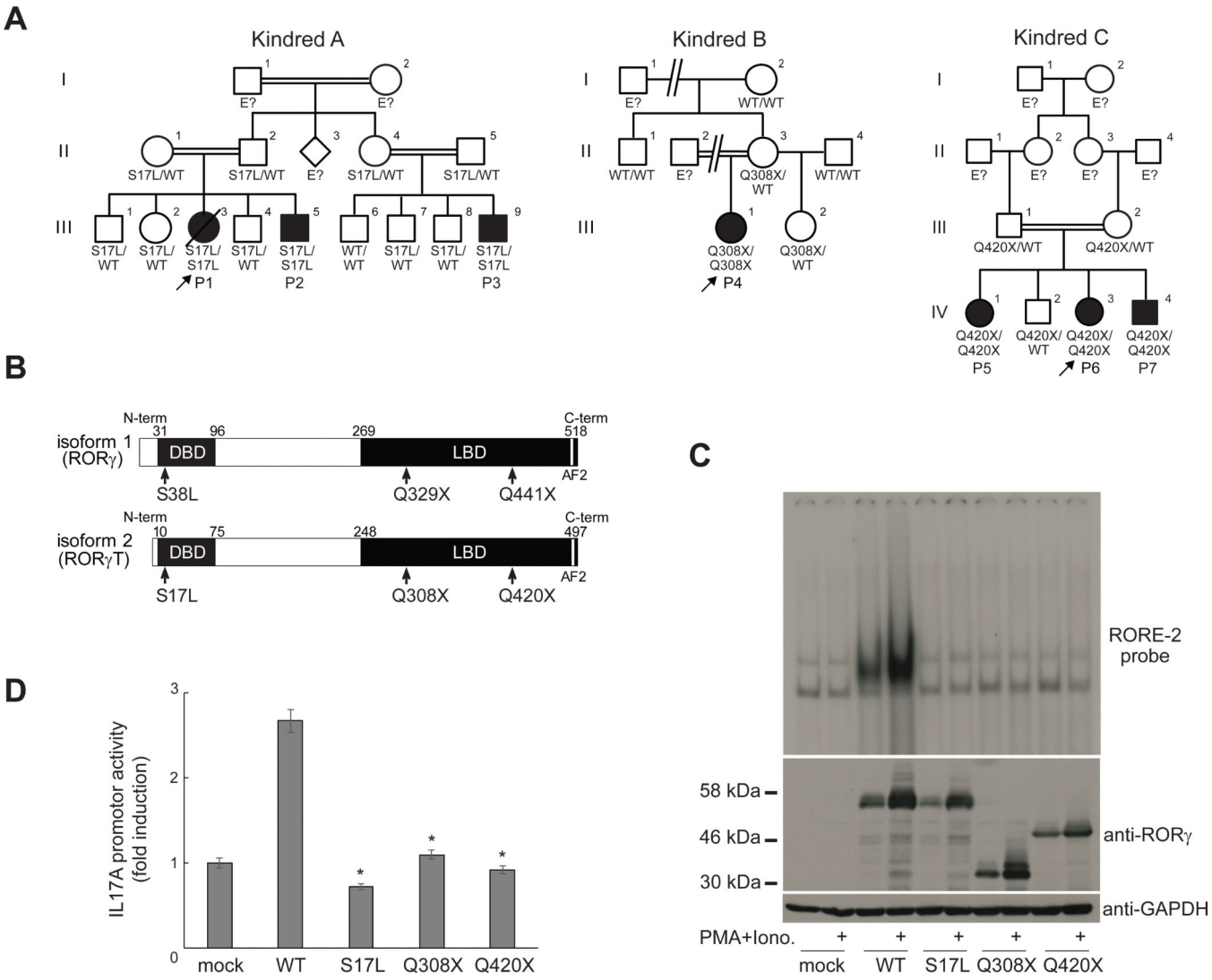

Fig. 1.

Identification of homozygous loss-of-function mutations affecting the human RORγT protein. (**A**) Sanger sequencing results and familial segregation of previously unidentified homozygous *RORC* mutations in three unrelated consanguineous families, indicating an autosomal recessive pattern of inheritance, with complete clinical penetrance. (**B**) Graphical representation of the RORγ and RORγT proteins, encoded by *RORC* isoforms 1 and 2, respectively. AF2, activation function 2 domain. Red arrows indicate the location of the sites affected by the *RORC* mutations found in the families. (**C**) HEK293T cells were either mock-transfected or transfected with the indicated plasmids. After 24 hours, cells were either left untreated or were stimulated with PMA and ionomycin. Whole-cell lysates were obtained and subjected to western blotting (**lower panel**), and nuclear lysates were subjected to EMSA with a ^{32}P-labeled RORE-2 probe derived from the *IL17A* promoter sequence (**upper panel**). (**D**) *IL17A* reporter plasmids, the pRL-SV40 vector and WT or mutant *RORC* plasmid were used to transfect HEK293T cells. After 24 hours, cells were stimulated with PMA and ionomycin as in (C), then subjected to luciferase assays. Experiments were

performed in triplicate, and *IL17A* promoter activity is expressed as fold-induction relative to mock-transfected cells.*p<0.05 versus WT controls, in two-tailed Mann-Whitney tests.

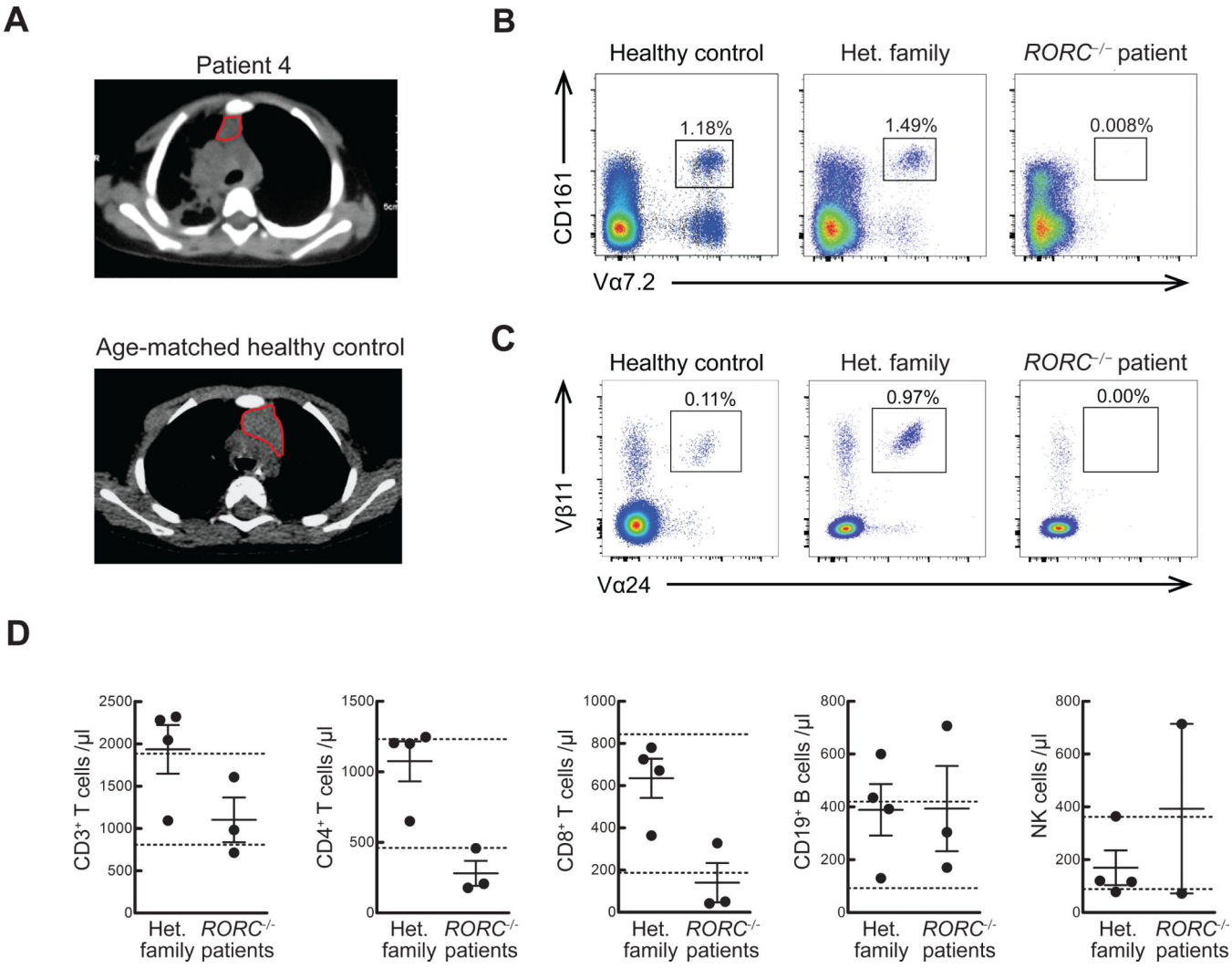

Fig. 2.

RORC$^{-/-}$ patients display abnormal thymus size and TCRα rearrangement, in line with their mild T-cell lymphopenia with a complete absence of MAIT and type 1 NKT cells. **(A)** Chest CT scan of P4 at the age of 16 months, revealing right lung infiltrate and thymic hypoplasia. (**B, C**) PBMCs from WT, heterozygous family members or from *RORC*$^{-/-}$ patients were analyzed for MAIT (B) and type 1 NKT (C) cell frequencies by flow cytometry. Each plot is representative of *n*=3 experiments. (**D**) Cell counts were performed on fresh blood samples from heterozygous family members (*n*=4) and *RORC*$^{-/-}$ patients (*n*=3). Dotted lines indicate the normal ranges for each lymphocyte population per µL of blood, based on the results for healthy individuals tested at the Necker Hospital for Sick Children, Paris, France.

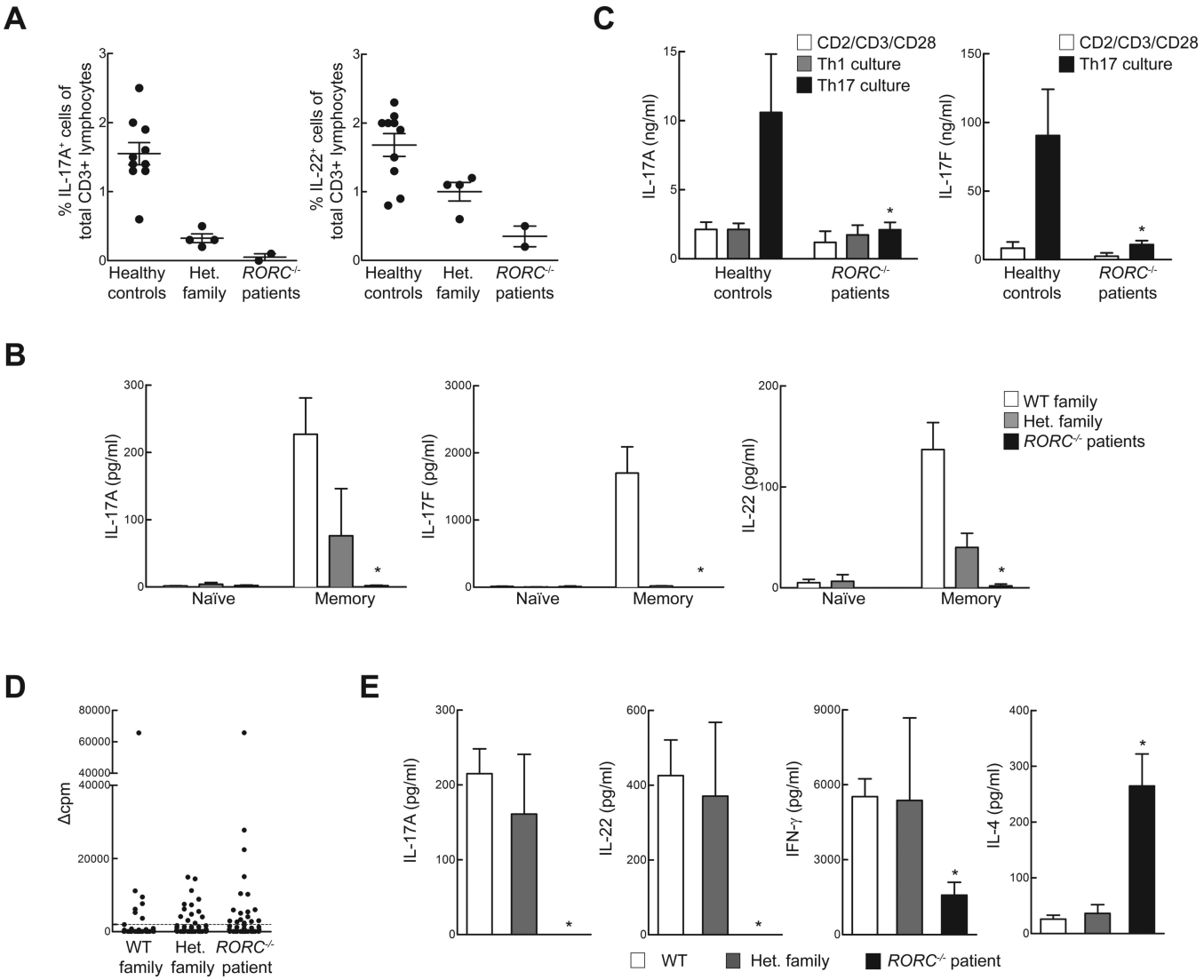

Fig. 3.

Cellular mechanisms of compromised IL-17 immunity and CMC in *RORC*$^{-/-}$ patients. (**A**) Whole blood from healthy WT donors, heterozygous family members, or *RORC*$^{-/-}$ patients was activated by PMA and ionomycin in the presence of brefeldin A, then assessed by intracellular flow cytometry for the production of IL-17A and IL-22. (**B**) Naïve and memory CD4^{+} T cells from WT controls (*n*=7), heterozygous family members (*n*=2) and *RORC*$^{-/-}$ patients (*n*=3) were cultured with T-cell activation and expansion (TAE) beads, and the culture supernatants were then assessed for secretion of the cytokine indicated (38). (**C**) Cytokine production by *in vitro*-differentiated CD4^{+} T cells from control donors and *RORC*$^{-/-}$ patients. Naïve (CD45RA^{+}CCR7^{+}) CD4^{+} T cells were purified from the PBMCs of WT controls (*n*=6) or *RORC*$^{-/-}$ patients (*n*=3), then cultured in the presence of TAE beads alone or TAE beads together with polarizing stimuli to induce the differentiation of Th1- or Th17-type cells (38). After 5 days, culture supernatants were assessed for the secretion of the cytokine indicated. (**D**) Sorted CCR6^{+} memory CD4^{+} T cells from WT controls, heterozygous family members and *RORC*$^{-/-}$ patients were initially polyclonally

stimulated to generate T-cell libraries, then cultured with autologous irradiated B cells, with or without a 3 h pulse with *C. albicans* lysate (CA, 5 μg/mL)(38). Proliferation was assessed by evaluating radiolabel incorporation on day 4, and is expressed as Δcpm values (38). Dotted lines represent the cutoff values. The frequencies of specific T cells using the Poisson distribution were $315/10^6$, $631/10^6$, $874/10^6$ in WT, Het. family and *RORC*$^{-/-}$ patient, respectively. (**E)** Concentration of the indicated cytokines were measured in the supernatants from positive cultures (Δcpm values above the cut-off value) from experiments performed as in (D) with cells from WT controls, heterozygous family members, and *RORC*$^{-/-}$ patients (n=2 each) The number of wells included was *n*=45-64 for WT controls, *n*=4-10 for heterozygous family members, and *n*=14-23 for *RORC*$^{-/-}$ patients. **p*<0.05 versus WT controls, in two-tailed Mann-Whitney tests.

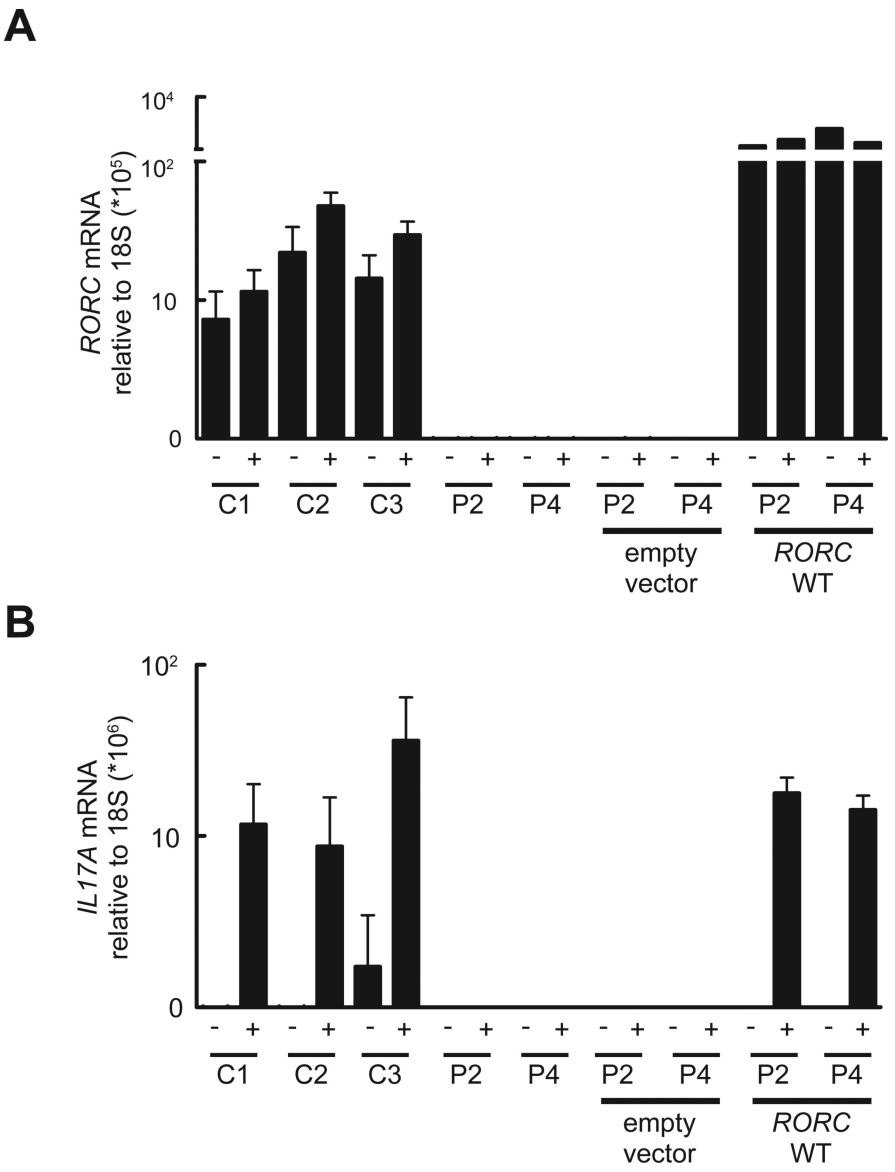

Fig 4.

T cell lines from *RORC*$^{-/-}$ patients fail to induce *IL17A* after mitogen stimulation. (**A**) *Herpesvirus saimiri*-transformed T cells from healthy donors (C1, C2, C3) or *RORC*$^{-/-}$ patients (P2, P4) were cultured in the presence (+) or absence (−) of PMA and ionomycin and then total RNA was extracted and used for qRT-PCR for total *RORC*. T cell lines from *RORC*$^{-/-}$ patients were transduced with retrovirus encoding either a tag only (empty vector), or tagged WT *RORC* isoform 2. (**B**) *IL17A* expression was assessed in the same RNA samples presented in (A). n=3, error bars represent SEM.

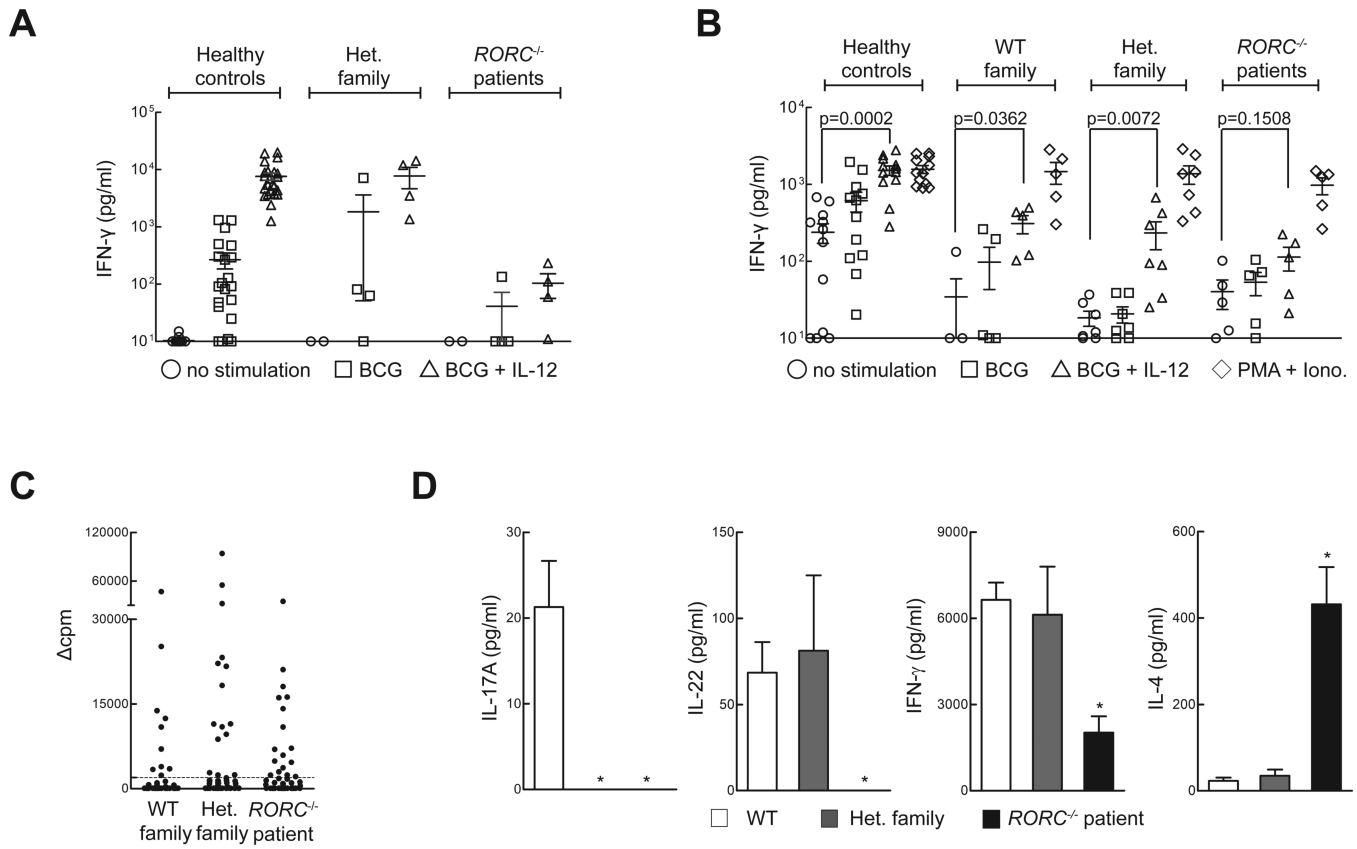

Fig. 5.

Cellular mechanisms of impaired IFN-γ immunity to Mycobacterium in *RORC*$^{-/-}$ patients. (**A**) Whole-blood samples from healthy controls (*n*=23), heterozygous family members (*n*=4), or *RORC*$^{-/-}$ patients (*n*=4) were incubated for 48 h under three different sets of activation conditions: medium alone, live *Mycobacterium bovis*-BCG (BCG) at a MOI of 20 BCG cells/leukocyte, and with BCG plus 20 ng/ml IL-12. The IFN-γ levels of culture supernatants were determined by ELISA. (**B**) Equal numbers of live PBMCs from healthy controls, WT family members, heterozygous family members, or *RORC*$^{-/-}$ patients were cultured in the presence of live BCG, BCG and IL-12, or PMA/ionomycin for 48 hours. IFN-γ concentration in the culture supernatant was assessed by ELISA. (**C**) Sorted CCR6^{+} memory CD4^{+} T cells were polyclonally stimulated with PHA in the presence of irradiated allogeneic feeder cells and IL-2, to generate T-cell libraries, as in Fig. 3D. Library screening was performed 14-21 days after initial stimulation, by culturing thoroughly washed T cells with autologous irradiated B cells, with or without a three-hour pulse with *Mycobacterium bovis*-BCG peptide pools. Proliferation was measured by radiolabel incorporation on day 4, and is expressed as Δcpm values. Each symbol illustrates one culture. Dotted lines represent the cutoff value. The frequencies of specific T cells calculated using the Poisson distribution were 467/10^{6}, 749/10^{6}, 875/10^{6} in WT, Het. family and *RORC*$^{-/-}$ patient, respectively. (**D**) The cytokines indicated were determined in the culture supernatants from (C), for wells with Δcpm values above the cutoff value. The number of wells included was *n*=45-64 for WT

controls, n=4-10 for heterozygous family members, and n=14-23 for *RORC*$^{-/-}$ patients. *p<0.05 versus WT controls, in two-tailed Mann-Whitney tests.