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Mycobacterial disease and impaired IFN- γ immunity in humans with inherited ISG15 deficiency

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Abstract

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All of the data presented in this manuscript are tabulated in the main text and in supplementary materials, and sequence data is available from the NCBI database: accession SRA053225.

ISG15 is an interferon (IFN)- α/β -inducible, ubiquitin-like intracellular protein. Its conjugation to various proteins (ISGylation) contributes to antiviral immunity in mice. We describe human patients with inherited ISG15 deficiency and mycobacterial, but not viral diseases. The lack of intracellular ISG15 production and protein ISGylation was not associated with cellular susceptibility to any viruses tested, consistent with the lack of viral diseases in these patients. By contrast, the lack of mycobacterium-induced ISG15 secretion by leukocytes — granulocytes in particular — reduced the production of IFN- γ by lymphocytes, including natural killer cells, probably accounting for the enhanced susceptibility to mycobacterial disease. This experiment of Nature shows that human ISGylation is largely redundant for antiviral immunity, but that ISG15 plays an essential role as an IFN- γ -inducing secreted molecule for optimal antimycobacterial immunity.

The idea that life-threatening infectious diseases occurring in otherwise healthy children, during the course of primary infection, may result from single-gene inborn errors of immunity is gaining ground (1–3). From this genetic perspective, one of the most thoroughly investigated pediatric syndromes is Mendelian susceptibility to mycobacterial disease (MSMD), a rare disorder predisposing individuals to severe clinical disease upon infection with weakly virulent mycobacteria, including *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) vaccines (4). These patients are also susceptible to *Salmonella* and *M. tuberculosis* (5, 6). Genetic dissection of MSMD has revealed disease-causing germline mutations in *IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, *NEMO*, *CYBB*, and *IRF8*, the products of which are involved in IFN- γ -mediated immunity (1, 7, 8). There is considerable allelic heterogeneity at these loci, defining 15 distinct genetic disorders. However, the genetic etiology of about half the cases of MSMD has not been identified.

We combined whole-exome sequencing (9) and genome-wide linkage analysis (10) to search for new genetic etiologies of MSMD. We investigated two unrelated patients with unexplained MSMD — a 15-year-old girl from Turkey (P1) and a 12-year-old boy from Iran (P2) — both born to consanguineous parents (SOM 1). Whole-exome sequencing of P1 resulted in the identification of seven homozygous coding variants not previously reported in the 1000 Genomes, dbSNP, HapMap or our own whole-exome sequencing databases (Table S1). Only three of these variants were located in chromosomal regions linked to MSMD. The best candidate variant was a nonsense mutation in exon 2 of *ISG15*, at position c.379G>T/379G>T (p.Glu127*/ Glu127*). This nucleotide substitution was confirmed by Sanger sequencing (Fig. 1, A and B and fig. S1A).

Use of the same whole-exome sequencing approach in P2 and his 15-year-old brother (P3), who also had MSMD, led to the identification of 33 previously unreported homozygous variants, including 10 in chromosomal regions linked to MSMD. The best candidate variant was a frameshift insertion in *ISG15* (c.336_337insG/336_337insG) and this mutation did not result in a premature stop codon (p. p.Leu114fs), instead potentially leading to the production of a protein 187 rather than 165 amino acids in length (Fig. 1, A and B, fig. S1A and methods). In both families, the segregation of the *ISG15* mutant alleles was consistent with autosomal recessive MSMD. We also sequenced the *ISG15* gene in 1,056 controls from 52 ethnic groups in the HGDP-CEPH human genome diversity cell line panel, 100 Turkish and 100 Iranian additional healthy controls, none of whom carried either of the mutant *ISG15* alleles. Together with their absence in both public and our own databases (Table S1), this suggests that these two variants are not irrelevant polymorphisms. Finally, none of the known polymorphic variants of *ISG15* are nonsense or frameshift, further suggesting that the two alleles found here may be disease-causing.

ISG15 is an intracellular, IFN- α/β -inducible protein that conjugates to proteins in a ubiquitin-like fashion (11, 12). We observed normal induction of mRNA for *ISG15* and a

control IFN- α stimulated gene, *IFIT1*, in IFN- α -stimulated Epstein-Barr virus (EBV)-transformed B cells (EBV-B cells) from P1 (fig. S1B and C). Consistently, EBV-B cells from P1 responded normally to IFN- α in terms of the binding of the nuclear protein ISGF-3 to the interferon-sensitive response element (ISRE) (fig. S1D). However, we were unable to detect ISG15 protein in stimulated EBV-B cells from P1 (Fig. 1C to E). Moreover, ISG15 was not detectable in stimulated SV40-transformed fibroblasts (SV40 fibroblasts) from P1 and P2 (Fig. 1C to E), which produced interferon-induced protein with tetratricopeptide repeats 3 (IFIT3) (Fig. 2A). These data suggest that the two mutant *ISG15* alleles are loss-of-expression.

We investigated ISG15 production in various leukocytes. Surprisingly, granulocytes had the highest levels of intracellular ISG15, which was detected in control cells but not P1 cells (fig. S2A and B). Unlike live BCG, IFN- α upregulated *ISG15* mRNA in all leukocyte subsets tested (fig. S2C). We then investigated the ISGylation of intracellular proteins after stimulation with IFN- β . Fibroblasts from P1 and P2 lacked detectable IFN- β -inducible ISGylation (Fig. 2A). The transfection of fibroblasts from P1 and P2 with WT FLAG-ISG15 restored both ISG15 production and ISGylation in this assay, whereas transfection with the negative control, FLAG-ISG15AA, a mutant that cannot tag proteins, did not restore ISGylation (Fig. 2B). The two human mutant *ISG15* alleles identified in our patients were therefore loss-of-expression and loss-of-function (for ISGylation).

ISG15 is induced by IFN- α/β , which is produced in response to viral infection (13, 14), and ISG15 and ISGylation have been shown to play a role in antiviral defense (12, 15–17). The infectious phenotype of ISG15-deficient mice is characterized by enhanced susceptibility to some, but not all of the viruses tested (18–20). We cannot rule out enhanced susceptibility to other as yet unencountered viruses, but the three affected teenagers are at least normally resistant to several common viruses (Table S2 and SOM 1). We thus assessed the replication and cytopathic effects of three relevant viruses in control cells and cells from the patients (Fig. 2, C and D and fig. S3, A to H). Both cell viability and viral replication levels were normal, as was the degree of protection afforded by prior treatment with IFN- α . The lack of a viral phenotype for our patients' cells *in vitro* is thus consistent with the lack of severe viral disease *in vivo*.

Human monocytes and lymphocytes have been shown to secrete the mature form of ISG15 (21). ISG15 secretion has been detected after treatment with IFN- α/β , but the induction of ISG15 secretion by BCG has not been investigated (22). We monitored ISG15 levels within cells and in the supernatants of untreated, BCG- or IFN- α -treated leukocytes (Fig. 3A–3C). BCG induced the release of ISG15 into the supernatants of control leukocytes, with a concomitant decrease in the amount of intracellular ISG15. By contrast, the amount of intracellular ISG15 increased in IFN- α -treated leukocytes. ISG15 was not detectable in P1 leukocytes and supernatants, in any of the conditions tested. Control and P1 leukocytes secreted similar levels of tumor necrosis factor (TNF)- α upon BCG challenge (Fig. 3B). Granulocytes, which displayed the highest levels of intracellular ISG15 expression (fig. S2A and B), also secreted ISG15 upon BCG or *Staphylococcus aureus* stimulation (fig. S4A). Conversely, bacterial lipopolysaccharide (LPS), like IFN- α , did not trigger ISG15 secretion (fig. S4A).

In granulocytes, ISG15 was localized in gelatinase and secretory but not azurophilic or specific granules (23) (fig. S4B). Consistent with this finding, ISG15 was not detected in granulocytes from a patient with inherited granule deficiency (fig. S5A). We also detected secreted ISG15 in both IFN- α -treated and untreated control (C) EBV-B cell and SV-40 fibroblast supernatants. By contrast, ISG15 was not detected in the supernatants of cells from P1 and P2 (fig. S5B). The transduction of P1 EBV-B cells with a lentiviral construct

encoding WT ISG15 resulted in the restoration of both intracellular and free extracellular ISG15 levels (fig. S5B). Finally, 293T cells transfected with the WT allele but not with the *ISG15* allele of P1 produced both intracellular and extracellular proteins (fig. S5C). These experiments suggest that many cell types can secrete ISG15 and that granulocytes, via their secretory pathway, are a major source of extracellular ISG15 during phagocytosis.

Free ISG15 has been shown to induce the secretion of IFN- γ by NK cells and T cells (21, 24, 25). We also observed such an induction of secretion when we stimulated control PBMCs with recombinant human ISG15 (Fig. 3D). We found ISG15 to be effective alone or in synergy with IL-12, a cytokine well known to induce IFN- γ (Fig. 3E). Bioactive ISG15 was produced in mammalian cells and heat-inactivated ISG15 was not bioactive (Fig. 3D). The most responsive cells appeared to be CD3⁺ CD56⁺ NK cells (fig. S5D). We also stimulated PBMCs depleted of NK cells, T cells alone or NK cells alone. NK cells were the key ISG15-responsive leukocytes, in terms of IFN- γ induction (fig S5E). The mechanism underlying the synergy between ISG15 and IL-12 was unclear, but ISG15 did not influence the expression of IL-12 receptor chains on T and NK cells (fig. S6A). The IFN- γ -inducing activity of ISG15 was not dependent on ISGylation, because IFN- γ was also induced by IL-12 and ISG15 Δ GG (a mutant incapable of conjugation) (fig. S6B).

Whole blood leukocytes from P1, P2 and P3, like those from IL-12p40- and IL-12R β 1-deficient patients, produced only small amounts of IFN- γ when stimulated with BCG or BCG and IL-12. By contrast, they produced normal amounts of IL-12p40 and IL-12p70 when stimulated with BCG or BCG and IFN- γ (Fig. 4, A to C). The addition of recombinant human ISG15 restored IFN- γ concentrations to almost normal levels (Fig. 4D), a situation reminiscent of the almost complete rescue of IL-12p40 deficiency by IL-12 (26). In P1, both NK and T cells produced small amounts of IFN- γ , as demonstrated by flow cytometry (fig. S7A and B), and the addition of rhISG15 also partially rescued this phenotype (fig. S7B).

We then assessed the contribution of ISG15 to the induction of IFN- γ in whole blood leukocytes from 10 healthy donors after exposure to BCG or BCG and IL-12, by adding (or not adding) a monoclonal antibody specific for ISG15 (or its isotype control) (Fig. 4E). ISG15 blockade markedly decreased IFN- γ secretion. We monitored the intracellular levels of IFN- γ in T and NK cells in the same conditions. Extracellular ISG15 was required for the induction of IFN- γ in both T and NK cells (fig. S7C). Finally, we showed that *ISG15*^{-/-} mice succumbed earlier than their WT littermates upon infection with *M. tuberculosis* (fig. S8A and B). Collectively, these experiments suggest that the lack of secreted ISG15 in the three patients with ISG15 deficiency accounts for their low levels of IFN- γ secretion *ex vivo*, and, thus, for their MSMD phenotype *in vivo*.

The apparent differences in antiviral immunity between humans and mice lacking ISG15 may reflect the small overlap of viral infections documented. Our findings nevertheless suggest that ISGylation and its contribution to antiviral immunity are largely redundant in humans in the course of natural infections (27). By contrast, as suggested by previous human studies (21, 24, 25), we found that ISG15 was a potent IFN- γ -inducing “cytokine” playing an essential role in antimycobacterial immunity. Strikingly, the clinical and immunological phenotypes of ISG15-deficient patients resemble those of patients with IL-12p40 or IL-12R β 1 deficiency, with impaired but not abolished IFN- γ immunity and relatively mild MSMD (28). Like these and other patients with inborn errors of IFN- γ immunity, ISG15-deficient individuals may be prone to *Salmonella* infections and, possibly, to other intracellular infections of macrophages, including *bona fide* tuberculosis (28). The ISG15-IFN- γ circuit, operating principally between granulocytes and NK cells, may therefore be an “innate” complement to the more “adaptive” IL-12-IFN- γ circuit, which operates principally between mononuclear phagocytes and T cells (29).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

The familial segregation and expression pattern of the *ISG15* allele indicates recessive inheritance and an absence of protein production. Familial segregation in a family from Turkey (Kindred A) and a family from Iran (Kindred B) (A). Graphical representation of the proISG15 protein. The LRLRGG ISGylation domain, the 8-amino acid sequence (black) cleaved to yield active ISG15, and the putative proteins synthesized in the patients (B) are shown. EBV-B cells from Control 1 (C1), Control 2 (C2), a *STAT1*^{-/-} patient (negative control) and patient 1 (P1), were left untreated or treated with IFN-α. The cells were then lysed and the lysates were subjected to western blotting (C). EBV-B cells from Control 1 (C1), a *STAT1*^{-/-} patient and patient 1 (P1) were stained with ISG15 and STAT1 antibodies and analyzed by flow cytometry (D). SV-40-fibroblasts from Control 1 (C1), a *STAT1*^{-/-} patient, P1, and P2 were analyzed as in C (E). All experiments presented were performed at least 3 times.

Fig. 2.

ISGylation and viral susceptibility in cell lines derived from patients with mutations in *ISG15*. Control SV-40-immortalized fibroblasts (C1), fibroblasts derived from P1, P2 or a *STAT1*^{-/-} patient were either left untreated or treated with IFN-β for 24 hours. Cell extracts were analyzed by SDS-PAGE and immunoblotting with antibodies against ISG15, IFIT3 or tubulin (A). SV-40 fibroblast cell lines from patients P1 and P2 were mock-transfected (M), transfected with a plasmid encoding 3XFLAG-ISG15 (WT) or transfected with a plasmid encoding a form of ISG15 unable to conjugate with proteins (AA). Eighteen hours after transfection, we treated the cells with IFN-β for an additional 18 hours. Cell extracts were analyzed by SDS-PAGE and immunoblotting with FLAG, IFIT3 or tubulin antibodies (B).

(A and B are representative of at least 3 independent experiments). HSV-1 replication was monitored by assessing the fluorescence of GFP fused to a viral capsid protein, in SV40-fibroblasts from a healthy control C1, P1, P2 and a *STAT1*^{-/-} patient, infected with HSV-1 at a multiplicity of infection (MOI) of 0.2 for the times indicated. Cells were treated with either medium alone (C) or with IFN- α (D) for 24 h before infection. The results shown are the means of four independent experiments. Error bars indicate the SEM.

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Fig. 3. ISG15 secretion and the induction of IFN- γ production in leukocytes. Control and P1 leukocytes were left unstimulated or were stimulated with BCG and IFN- α 2b. After 0 h and 12 h, cells and supernatants were harvested and subjected to western blotting (A) or TNF- α ELISA (B). Resting control and P1 leukocytes were labeled extracellularly with CD16 and CD3 antibodies and intracellularly with ISG15 antibody and were subjected to ImageStreamX analysis with bright field (BF) and side scatter (SSC) also shown (C). IFN- γ secretion was measured by ELISA in PBMCs stimulated with vehicle (-), various doses of recombinant human ISG15 (including boiled recombinant human ISG15 to exclude LPS contamination) (D), recombinant human ISG15 and IL-12, alone and in combination (E). The IFN- γ secretion results shown are representative means of at least 3 independent experiments. Error bars indicate the SEM.

Fig. 4.

Impaired IFN- γ production in *ISG15*^{-/-} patients and rescue by exogenous ISG15. Cytokine production in the supernatants of whole-blood cells from local controls (n=29), travel controls (n=9), *ISG15*^{-/-} (n=3) and *IL12RB1*^{-/-} patients (n= 58), left unstimulated or stimulated with BCG alone or BCG plus cytokine (indicated), as detected by ELISA (A to C); alternatively, recombinant human ISG15 was added at the same time or 24 h before whole-blood activation by BCG and IL-12 (D). The amounts of cytokine secreted are normalized for 10⁶ PBMC on a logarithmic scale, and medians are indicated by solid bars. Differences in log-transformed IFN- γ levels after stimulation with BCG + IL-12 were assessed 1) between *ISG15*^{-/-} subjects and travel controls in Student's *t*-test (A), and 2) between *ISG15*^{-/-} subjects before and after adding recombinant human ISG15 by two-way analysis of variance, to take into account both activation by recombinant ISG15 and activation time (D). IFN- γ secretion was measured in whole blood stimulated with vehicle (-), BCG or BCG plus IL-12, in the presence of vehicle (-), a blocking antibody against ISG15, or an IgG1 isotype control (E).