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RESEARCH HIGHLIGHT

ISG15 regulates IFN-γ immunity in human mycobacterial disease

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Interferon-gamma (IFN-γ) is crucial for immunity against different pathogens due to its broad effects on the multiple arms of the immune system. The regulation of IFN-γ immunity is of extensive interest to research as well as practical activity for drug discovery. New evidence supports previous findings that ubiquitin-like protein ISG15 acts as an extracellular cytokine and promotes IFN-γ production, providing intriguing insights of the importance of ISG15 into the control of human mycobacterial disease.

ISG15 is an interferon-stimulated gene (ISG) [1]. It is strongly upregulated by interferons (IFNs), especially type I IFN (IFN-α/β), pathogen infections, and cellular stresses that activate IFN production [2]. ISG15 is the first identified ubiquitin-like modifier, which can covalently conjugate to other cellular proteins to form ISGylated proteins. Free ISG15 is detected as both extracellular and intracellular protein. The mature form of ISG15 contains two ubiquitin-like domains with a calculated molecular weight of 17 145 Daltons. It lacks an N-terminal methionine, and has a C-terminus ending with the amino acid sequence 'Leu Arg Leu Arg Gly Gly' (LRLRGG), which is the same six amino acid sequence as mature ubiquitin. As with the ubiquitin system, there are a series of distinct enzymes involved in the process of protein ISGylation, including ISG15 activating enzyme (E1), UBE1L — conjugating enzyme (E2), UBCH8 — protein ligases (E3), and ISG15-specific protease USP18. However, unlike ubiquitin and many ubiquitin-like proteins, such as SUMO1 and NEDD8, which can be found in all species of eukaryotic cells, ISG15 is only identified in vertebrates, including human, monkey and mouse, *etc*. Moreover, compared with ubiquitin, which has virtually 100% cross-species conservation, ISG15 protein has relatively low cross-species conservation, ranging from a high of 98% (chimpanzee to human) to a low of 42% (opossum to human) in mammals. Together, the relatively low cross-species conservation and the absence in many eukaryotic species indicate that *ISG15* is not an essential housekeeping gene but may be an evolutionary adaptation to particular conditions [2].

Significant efforts have been made to identify the substrates of ISG15 conjugation and their roles in pathogen infection and tumorigenesis [3, 4]. For example, the molecular basis of antiviral effect of ISG15 was described in a recent report of the influenza A viral protein NS1 (NS1A). Multiple lysines of the NS1A protein could be modified by ISG15, with K41 in the N-terminal domain of this protein acting as the main modification site. ISG15 modification on NS1A resulted in a disruption of normal function of NS1A, thus inhibiting the virus infection [3]. Meanwhile, a recent report revealed an important role of ISG15 in the enhancement of chemosensitivity during cancer therapy [4]. Treatment of cells with several chemotherapeutic drugs, including doxorubicin and camptothecin, increases ISG15 expression and results in ISGylation of ΔNp63α, an alternative splice variant of p53 family protein p63. The ISGylated nuclear ΔNp63α is subjected to the cleavage by caspase-2 and subsequently releases its inhibitory domain to the cytoplasm, resulting in the inactivation of ΔNp63α. Since ΔNp63α inhibits the activity of p53 family members in a dominant-negative manner to increase cell proliferation and tumorigenesis, ISGylation of ΔNp63α ablated the ability of ΔNp63α to promote anchorage-independent cell growth and tumor formation *in vivo*.

Besides protein ISGylation, significant amount of free ISG15 was also detected in both intracellular and extracellular compartments. Several studies suggested a potential role of free ISG15 as a cytokine or chemokine [5-7]. An early study in 1996 demonstrated that free ISG15 is secreted from multiple cells, including primary cultures of peripheral blood $CD3^+$ cells and viable cell lines of monocyte, T lymphocyte, B lymphocyte, and epithelial origins, and is detectable in serum from healthy human volunteers treated with IFN-β [6]. The potential modulation of immune cell function by ISG15 was reported later in the same year. ISG15 stimulates the production of IFN-γ from $CD3^+$ T cells to enhance the proliferation and cytotoxicity of natural killer (NK) cells [5]. In the year of 2003, ISG15 was shown as a neutrophil chemotactic factor in *Plasmodium yoelii*-infected red blood cells [7]. However, a primary challenge to the cytokine properties of ISG15 is that cytokine- or chemokine-related defects have not been identified

in *ISG15* knockout mice [8]. Most recently, Bogunovic and colleagues published one critical paper in *Science*, providing new evidence to confirm that ISG15 acts as an extracellular cytokine and promotes IFN-γ production. More importantly, this paper shows an essential role of ISG15-regulated IFN-γ immunity against mycobacterial disease in human [9].

This new facet of ISG15 was uncovered by studying the genetic etiology of Mendelian susceptibility to mycobacterial disease (MSMD), a rare disorder — individuals have a predisposition to clinical disease caused by weakly virulent mycobacteria, such as *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) vaccines [10]. Whole-genome sequencing and genome-wide linkage analysis were used to search for the genetic etiologies of MSMD in two unrelated patients, a 15-year-old girl from Turkey and a 12-year-old boy from Iran. Both of them were born to consanguineous parents and developed clinical symptoms after BCG vaccination. Subsequent results suggested a common deficiency of ISG15 in these two patients. One has a nonsense mutation in exon 2 of *ISG15*, while the other one and his brother have a frameshift insertion in the *ISG15* allelels. Both mutant *ISG15* alleles identified in these patients were homozygous mutation, loss-of-expression and loss-of-function (for ISGylation). Considering the facts that ISG15 could be a potential cytokine for regulating IFN-γ production and individuals with inherited disorders of IFN-γ-mediated immunity appear to be specifically vulnerable to mycobacterial infections [11], the ISG15 deficiency-caused impairment of IFN-γ immunity could provide the molecular basis of MSMD in these patients.

To further support the involvement of ISG15 as a cytokine in MSMD, more detailed studies, including ISG15 induction and secretion and ISG15-induced IFN-γ production, were examined using the cells derived from the valuable patient samples. The results demonstrated that granulocytes have the highest levels of intracellular ISG15 upon IFN-α/β stimulation. Accordingly granulocytes are a major source of extracellular ISG15 *in vivo*, although many other types of cells also have the ability to secret ISG15. However, the secretory pathway for ISG15 is still unclear, and will possibly be of future interest.

Prior works have shown that free ISG15 can induce the secretion of IFN-γ by NK cells and T cells [5, 6]. The current report in *Science* provides supportive evidence to prior findings once again that extracellular ISG15 was required for the induction of IFN-γ in both T cells and NK cells. Their elegant studies also show that NK cells are the key ISG15-responsive leukocytes, in terms of IFN-γ induction. It is very interesting that a synergic effect on IFN-γ secretion was observed when the cells were co-treated with ISG15 and IL-12, a cytokine well-known to induce IFN-γ. However, the mechanisms of how ISG15 triggers IFN-γ production remain unclear. It is noteworthy that the successful attempt of using recombinant ISG15 and ISG15 antibody in regulating IFN-γ production by *in vitro* cell-based assay suggested potentially plausible strategies for ISG15-targeted drug discovery. Furthermore, the importance of ISG15 in controlling mycobacterial disease was tested in the mouse model. Consistent with enhanced susceptibility to mycobacterial disease in humans with inherited ISG15 deficiency, *ISG15* knockout mice died earlier than their WT littermates upon infection with *M. tuberculosis*, with an overall survival

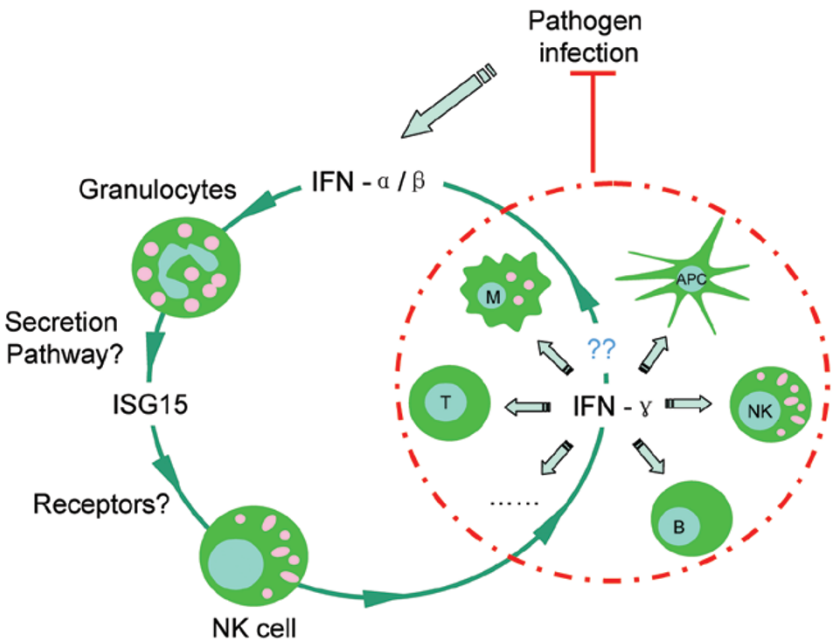

Figure 1 ISG15 regulates IFN-γ immunity against pathogen infection. Pathogen infection triggers the release of IFN-α/β and in turn upregulates ISG15 expression in the cells, including granulocytes, which are the major source for the secretion of free ISG15 into extracellular compartment. Subsequent induction of IFN-γ production mainly from NK cells by free ISG15 plays an essential role in IFN-γ immunity against the pathogen infection. Potential feedback between IFN-γ production and IFN-α/β signal pathway is unclear. M, macrophage; T, T cell; B, B cell; NK, natural killer cell; APC, antigen presenting cell.

rate of ~40% at 45 weeks post infection compared to almost no death in the control mouse group within the same period. These data strongly indicate the essential role of ISG15 in anti-mycobacterial disease.

In summary, the discovery by Bogunovic and colleagues has revealed the critical role of ISG15 in human health. It provides new evidence for ISG15-regulated IFN-γ immunity, which is summarized in Figure 1. The report also clearly shows ISG15 cytokine-related defects in *ISG15* knockout mice, the lack of which has been challenging the idea that ISG15 acts as a cytokine for over a decade. These findings may also answer the question why ISG15 is only induced by IFNs or pathogen infection but not constitutively expressed to counter potential pathogen infection. Possibly, constitutive ISG15 expression and active IFN-γ immunity is deleterious in contexts other than mycobacterial infection. For example, in certain cases of autoimmune disease, a blockade of IFN-γ immunity actually limits the diseases [12, 13]. The cytokine properties of ISG15 are reminiscent of potential cytokine properties of ubiquitin and other ubiquitin-like proteins. Several studies have suggested that extracellular ubiquitins serve as immune modulator during infectious and noninfectious inflammation [14, 15]. However, not many reports are available regarding other ubiquitin-like proteins. Meanwhile, future investigation focusing on how ISG15 is secreted from certain cells, which cell surface receptors and cellular pathways are responsible for ISG15-induced IFN-γ production, and how the components of these pathways are targeted to regulate IFN-γ immunity, will turn out to be fruitful and point to more applicable strategies for ISG15-targeted drug discovery.

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