Published in final edited form as:

Methods Enzymol. 2019 ; 618: 211–227. doi:10.1016/bs.mie.2018.12.027.

Approaches for investigating the extracellular signaling function of ISG15

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Abstract

ISG15 is a ubiquitin-like protein (Ubl) that is expressed in response to Type 1 Interferon (IFN- α/β) signaling. Remarkably, ISG15 has three distinct biochemical activities involved in innate immune responses to viral and/or microbial infections. The canonical function of ISG15 is as a posttranslational modifier, and protein ISGylation has been demonstrated to be antiviral. A second intracellular function, independent of conjugation activity, is attenuation of IFN- α/β signaling at the interferon receptor, which appears to be important for terminating IFN responses. The third function of ISG15, and the focus of this chapter, is as an extracellular signaling molecule that promotes the secretion of Type 2 Interferon (IFN- γ) by Natural Killer (NK) cells. This function is important for control of microbial infections, including mycobacterial infections. Here, we describe methods for purification of ISG15, preparation, and culture of primary peripheral blood mononuclear cells (PBMCs) and NK-92 cells, assays for IL-12- and ISG15-dependent cytokine (IFN- γ and IL-10) secretion, and assays for initial intracellular signaling events triggered by extracellular ISG15.

1. Introduction

ISG15 was one of the first Interferon Stimulated Genes (ISGs) to be identified (Farrell, Broeze, & Lengyel, 1979), suggesting that it was likely to play a role in innate immune responses. While ISG15 was also the first recognized ubiquitin-like protein modifier (Ubl) (Haas, Ahrens, Bright, & Ankel, 1987; Loeb & Haas, 1992; Narasimhan et al., 2005), it would be many years before a role for ISGylation in antiviral or antimicrobial responses was substantiated, as well as many years before the enzymes involved in ISGylation and de-ISGylation were identified. In addition, some of the earliest publications reported that ISG15 was a secreted cytokine-like molecule that induced Type 2 Interferon (IFN- γ) responses (D'Cunha, Knight, Haas, Truitt, & Borden, 1996; Knight & Cordova, 1991), an observation that was clearly at odds with its role as an intracellular protein modifier. It would again be many years before the significance of this observation was appreciated and at least partially understood. A key to unraveling the connection to Type 2 IFN was the identification of ISG15-deficient patients who were susceptible to mycobacterial infection (Bogunovic et al., 2012), as described further below. More recently, a second group of ISG15-deficient patients led to the finding that ISG15 has an additional intracellular function in attenuating IFN

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receptor (IFNAR) signaling, independent of its conjugation activity (Zhang et al., 2015). This group of patients exhibited pathology consistent with an “interferonopathy,” a condition in which there is inappropriate sustained expression of Type 1 IFNs. These remarkable and surprising twists in the history of ISG15 discoveries have ultimately revealed a remarkable multifunctional protein of the human innate immune system.

Human ISG15 is a 17kDa protein that consists of two ubiquitin-like domains. The N- and C-terminal domains are 31% and 33% identical, respectively, to ubiquitin. The C-terminal six residues of mature (processed) ISG15 are identical to those of ubiquitin (LRLRGG), a feature that has important practical implications for distinguishing ISG15 from ubiquitin modifications by mass spectrometry-based proteomic approaches (Narasimhan et al., 2005; Zhao, Denison, Huibregtse, Gygi, & Krug, 2005). ISG15 is found only in vertebrates and the primary sequence of ISG15 is poorly conserved across vertebrate species; for example, human ISG15 is only 66% identical to mouse ISG15.

Like Ub and other UbLs, protein ISGylation involves an E1, E2, E3 cascade. The E1 enzyme is Ube1L/UBA7 (Yuan & Krug, 2001), the E2 enzyme is UbcH8/Ube2L6 (Zhao et al., 2004), and Herc5, a HECT domain ligase, is the single major E3 for ISG15 (Dastur, Beaudenon, Kelley, Krug, & Huibregtse, 2006; Ketscher, Basters, Prinz, & Knobloch, 2012; Oudshoorn et al., 2012). Surprisingly, Herc5 mediates the ISGylation of many hundreds—if not thousands—of cellular proteins in IFN- β -stimulated cells (Durfee, Lyon, Seo, & Huibregtse, 2010; Zhao et al., 2005). The basis for this broad substrate targeting is that Herc5 associates with the 60S subunit of actively translating ribosomes and ISGylates nascent polypeptides cotranslationally, with what appears to be little substrate selectivity (Durfee et al., 2010). We proposed a model in which the primary biologically relevant targets of ISGylation are not cellular proteins, but rather newly translated viral proteins in the context of a viral infection and interferon response. Based on these findings, we proposed that ISGylation serves to interfere with the function of viral proteins, particularly oligomeric viral proteins, as shown for human papillomavirus capsids (Durfee et al., 2010) and influenza virus ribonucleoprotein (Zhao et al., 2016).

It should be noted that ISGylation does not target proteins for proteasomal degradation and poly-ISGylated chains do not appear to be formed in cells. The single de-ISGylating enzyme for ISG15 is human Usp18 (mouse Ubp43) (Malakhov, Malakhova, Kim, Ritchie, & Zhang, 2002), although several virally encoded de-ISGylating enzymes have been identified, consistent with the hypothesis that ISGylation is antiviral (Akutsu, Ye, Virdee, Chin, & Komander, 2011; Dackowski et al., 2017; Dackowski, Goodwin, Dzimianski, Farhat, & Pegan, 2017; Deaton et al., 2016; Swatek et al., 2018). Importantly, all three conjugation enzymes (UBA7, Ube2L6, and Herc5) as well as Usp18 are induced at the transcriptional level by Type 1 IFN signaling. The role of Usp18-mediated de-ISGylation in IFN-stimulated cells—when it occurs and what happens if it does not occur—remains uncertain, and these questions are complicated by the fact that Usp18 has a second function in innate immune responses unrelated to deconjugation (Malakhova et al., 2006; Tokarz et al., 2004). This second function of Usp18 interfaces with the most recently discovered function of ISG15 in attenuating IFNAR signaling (Zhao et al., 2005), as revealed by the identification of ISG15-deficient patients who presented with seizures and Idiopathic Basal Ganglia Calcification

(IBGC), typical of some other interferonopathies (Aicardi–Goutières syndrome and Spondyloenchondromatosis) (Crow & Manel, 2015; Volpi, Picco, Caorsi, Candotti, & Gattorno, 2016).

ISG15 was first reported to have an extracellular signaling function in the early 1990s, with results showing that ISG15 could be found extracellularly and that it stimulated the release of IFN- γ from peripheral blood lymphocytes (D’Cunha et al., 1996; Knight & Cordova, 1991). There was little follow-up to these studies, which were also difficult to reconcile with the emerging role of ISG15 as a ubiquitin-like protein modifier. A focus on the extracellular function was revived with the identification of a new set of Mendelian Susceptibility to Mycobacterial Disease (MSMD) patients—patients with inborn defects that lead to development of mycobacterial disease after vaccination with the live attenuated *Mycobacterium bovis* BCG vaccine strain (Bogunovic et al., 2012). MSMD patients are characterized by genetic deficiencies in one of several genes encoding components of the IFN- α/β production or response pathways (Bogunovic, Boisson-Dupuis, & Casanova, 2013). Eight genes had been previously identified as causative of this disorder—IFNGR1 and IFNGR2 (the IFN- γ receptor subunits), STAT1, IL12B, IL12RB1, IRF8, NEMO, and CYBB (Bustamante, Boisson-Dupuis, Abel, & Casanova, 2014), and whole exome sequencing of the new patients revealed that they had homozygous frame-shift and missense mutations in the ISG15 gene. These findings were consistent with the very early reports of ISG15 promoting IFN- γ secretion in peripheral blood mononuclear cells (PBMCs). Further experiments showed that ISG15 worked in synergy with IL-12 to stimulate the release of IFN- γ from PBMCs, most notably from Natural Killer (NK) cells and, to a lesser degree, T cells. However, until recently, a putative ISG15 cell surface receptor and the downstream signaling events leading to IFN- γ secretion remained unknown.

To identify the receptor for ISG15, we established a cell-based signaling system using NK-92 cells (a lymphoma cell line with an NK cell origin) and purified IL-12 and ISG15. Mutagenesis of surface residues of ISG15 identified a small patch on the C-terminal ubiquitin-like domain that was critical for induction of IFN- γ secretion (Y96, R99, T101, Q102, T103). We also showed, using a cell-based binding assay, that these same residues—now referred to as the “IFN- γ patch” of ISG15—mediated stable binding of ISG15 to the surface of NK-92 cells, strongly suggesting that ISG15 was engaging a cell surface receptor.

An ISG15 Ubiquitin-Activated Interaction Trap (UBAIT) was used to identify the ISG15 receptor (Swaim, Scott, Canadeo, & Huibregtse, 2017). UBAITs are fusion proteins of a protein of interest (in this case ISG15) to ubiquitin, with ubiquitin at the C-terminus (O’Connor et al., 2015; O’Connor, Swaim, Canadeo, & Huibregtse, 2018). Like other tagged forms of ubiquitin, UBAITs can be charged by the ubiquitin E1 enzyme and transferred to an E2 protein. The principle is that if an E2-charged UBAIT engages with an interacting protein, there is a strong possibility that a lysine of the interacting protein will react with the UBAIT ~E2. This will create a stable amide bond between the UBAIT and the interacting protein, which can then be identified by immunopurification of the UBAIT and mass spectrometry-based methods. We initially developed this method to identify targets of ubiquitin ligases by expression of E3 UBAITS in cells (O’Connor et al., 2015), but the method is applicable to almost any protein. For identification of the ISG15 receptor, we

expressed and purified a FLAG-tagged ISG15-UBAIT and then charged the UBAIT with E1, E2, and ATP in vitro; this was then added directly to intact NK-92 cells. We anticipated that the ISG15 UBAIT would form a covalent adduct with its receptor. An IFN- γ patch mutant, also expressed as a UBAIT, was a critical negative control. These experiments led to the identification of LFA-1 (a heterodimer of CD11a/ α L and CD18/ β 2 integrin) as the ISG15 receptor, with ISG15 binding directly to the CD11a subunit. The identity of LFA-1 as the ISG15 receptor was confirmed in several ways, including by showing that splenocytes from CD11a knockout mice were defective for release of IFN- γ in response to IL-12 and ISG15. Further experiments demonstrated a direct interaction of ISG15 with the purified CD11a α L domain in vitro, again dependent on the IFN- γ patch of ISG15.

LFA-1 is expressed only in cells of the immune system, including NK and T cells, and the best characterized ligands of LFA-1 are Intracellular Adhesion Molecules (ICAMs) (Abram & Lowell, 2009). LFA-1/ICAM interactions are required for formation of the immunological synapse in T cells and for arrest and extravasation of NK cells at sites of infection (i.e., the movement of circulating cells from the vasculature into tissues). Importantly, LFA-1 had previously also been shown to be necessary for the IFN- γ response to *Mycobacterium tuberculosis* (Ghosh, Chackerian, Parker, Ballantyne, & Behar, 2006), consistent with ISG15-deficiency leading to MSMD. LFA-1 is subject to both inside-out signaling and outside-in signaling. Inside-out signaling refers to the fact that other incoming signals to a T or NK cell, such as phorbol esters (PMA/PMH) or activation of G-protein signaling or the T cell receptor, trigger events in the cytoplasm that result in profound conformational changes to the extracellular domain of LFA-1, which allows LFA-1 to engage ICAM ligands (Abram & Lowell, 2009). In contrast, we have shown that ISG15 initiates LFA-1 outside-in signaling in the absence of any other cytokines or stimuli. A rapid readout of this signaling is activation of Src family kinases (SFKs), and SFK small-molecule inhibitors block IFN- γ release from NK-92 cells. LFA-1 is also expressed in Jurkat T cells, and these also respond to ISG15 and secrete IFN- γ . Interestingly, Jurkat cells lack a functional IL-12 receptor, and therefore to determine whether ISG15 could promote IFN- γ secretion by these cells we first expressed IFN- γ by plasmid transfection. This led to the finding that the basis of synergism between IL-12 and ISG15 is that IL-12 induces expression of IFN- γ (at the transcriptional level) and ISG15 induces the secretion of IFN- γ (see Fig. 1). ISG15 effects on cytokine secretion are not limited to IFN- γ , as ISG15 was shown to also induce the secretion of IL-10 from NK-92 cells. Importantly, while some of the initial ISG15 signaling events are beginning to be characterized (SFK activation), the events further downstream that mediate cytokine secretion remain undefined. Furthermore, it remains unclear how ISG15, itself, is secreted into the extracellular space. Granulocytes appear to be one source of secreted ISG15 (Bogunovic et al., 2012), but the complete spectrum of cell types capable of releasing ISG15 into the extracellular space as well as the mechanism of secretion remains unclear.

The sections below focus on methods for investigating the extracellular function of ISG15, including (1) methods for expression and purification of ISG15, (2) preparation and culture of primary PBMCs and NK-92 cells, (3) assays for IL-12- and ISG15-dependent cytokine (IFN- γ and IL-10) secretion, and (4) assays for initial ISG15-dependent signaling events.

2. Methods

2.1 Bacterial expression and purification of ISG15

This section describes the procedures for expressing and purifying ISG15 from *E. coli*. Several different ISG15 variants can be critical controls for extracellular signaling experiments. These include human and mouse wild-type ISG15, ISG15 GG (deleted of the terminal glycine residues; this is incapable of being conjugated), as well as ISG15 IFN- γ patch mutants (R99A, T101A, Y96LQ102D, and T103A). In the course of identifying the IFN- γ patch a total of 31 surface residues of ISG15 were altered individually or in groups of two, and each of these was also expressed and purified from *E. coli* (Swaim et al., 2017). Residues for alteration were initially chosen based on the X-ray crystal structure of ISG15 (PDB 1Z2M) and conservation among mammalian ISG15 proteins as well as differences between ISG15 and ubiquitin. All altered proteins were soluble when purified as described later and they were expressed at similar levels to wild-type ISG15. It should be noted that ISG15 has been crystallized with several interacting partners including Influenza B NS1 (NS1B), Usp18, and viral de-ISGylases (Akutsu et al., 2011; Basters et al., 2017; Daczkowski, Dzimianski, et al., 2017; Daczkowski, Goodwin, et al., 2017; Deaton et al., 2016; Swatek et al., 2018; Zhao et al., 2016). Residues necessary for interaction with NS1B are located within the N-terminal ubiquitin-like domain of ISG15, while the residues for interactions with the de-ISGylases are located on the C-terminal lobe. The IFN- γ patch of ISG15 is also on the C-lobe but represents a distinct surface from that required for interaction with de-ISGylases.

3X-FLAG-tagged ISG15 proteins are expressed in *E. coli* BL21-DE3 as GST (glutathione *S*-transferase) fusion proteins using the pGEX6p-1 vector, with the PreScission Protease cleavage site between the N-terminal GST tag and the 3X-FLAG epitope. In brief, the expression and purification scheme is as follows: (1) growth of an overnight culture of BL21 *E. coli* transformed with a pGEX6p1-3X-FLAG-ISG15 expression plasmid, (2) expansion of the culture and induction of protein expression, (3) harvesting and lysis of cells and clarification of the cell lysate, (4) purification of GST-ISG15 proteins on glutathione-Sepharose, (5) removal of GST with PreScission protease, and (6) collection and quantitation of purified soluble 3X-FLAG ISG15 protein.

Reagents and Equipment

1. *E. coli* BL21-DE3 transformed with pGEX6p1-3X-FLAG-ISG15 or variant.
2. LB broth with 100 μ g/mL ampicillin: 10g tryptone, 5g yeast extract, 10 g NaCl in 1 L H₂O. Autoclave, cool, and add ampicillin to 100 μ g/mL.
3. 100mM isopropyl β -D-1-thiogalactopyranoside (IPTG).
4. Sonicator cell disruptor with microtip.
5. PreScission Protease (GE Healthcare).
6. PreScission Protease Cleavage (PC) buffer: 50mM Tris-HCl, pH 8.0, 150mM NaCl, 0.01% Triton X-100, 2.5mMEDTA.

7. PBS-Triton: PBS (phosphate-buffered saline), 1% Triton X-100.
8. 500mL Erlenmeyer flasks.
9. Glutathione-Sepharose (GE Healthcare).
10. Bovine serum albumin (Thermo Scientific #23209), as a standard for quantification of purified ISG15 protein.

Expression and Purification

1. Inoculate a 25mL overnight starter culture at 37°C for 16h with shaking.
2. Dilute cultures to a final volume of 250mL and grow for 2h at 37°C.
3. Induce protein expression with 100 μ M IPTG for 3h at 30°C.
4. Collect cells by centrifugation (6000 $\times g$ for 5min), resuspend in 10mL PBS-Triton, and sonicate for 30s on ice (Branson sonicator with microtip; duty cycle 50%, output 5).
5. Clarify lysate by centrifugation at 15,000 $\times g$ for 10min at 4°C. Remove clarified supernatant to 15mL disposable conical tube.
6. Add 100 μ L of Glutathione-Sepharose (GE Healthcare), prewashed and equilibrated in PBS-Triton, and 100 μ L PMSF (100 μ M in ethanol). Incubate for 2h with rotation at 4°C.
7. Collect beads and wash 3 $\times$ with PBS-Triton and 3 $\times$ with PC buffer. The final volume of PC buffer after wash should be equivalent to the bead volume (100 μ L).
8. Add 2 μ L PreScission Protease and incubate overnight with rotation at 4°C.
9. Remove beads by brief microcentrifugation and collect supernatant containing the eluted protein and keep on ice.
10. Quantitation of the protein is accomplished by SDS-PAGE and Coomassie Blue staining. Run 2 μ L of purified protein on a 15% Tris-Glycine gel with a BSA standard (250, 500, 750, and 1000ng) to establish approximate protein concentration.
11. Store on ice. For best results use the ISG15 within 16h, without freezing. Freezing and storage severely decreases the activity of ISG15 in functional assays.

2.2 Cells and cell culture

There are several points that must be considered in designing experiments to investigate ISG15-stimulated IFN- γ secretion. It was shown that IL-12 and ISG15 act synergistically to stimulate IFN- γ release from PBMCs and, more specifically, from the NK and T cell populations within PMBCs (Bogunovic et al., 2012). NK and T cells express both the ISG15 receptor (LFA-1) and the IL-12 receptor, and IL-12 is a potent inducer of IFN- γ expression at the transcriptional level. ISG15 does not influence expression of IFN- γ at the

transcriptional level, either in the absence or presence of IL-12. The Jurkat T cell line expresses a defective IL-12 receptor and therefore does not produce IFN- γ in response to IL-12 (Showe et al., 1999). However, if IFN- γ is expressed from a transfected expression vector, Jurkat cells will secrete IFN- γ in response to ISG15 (Swaim et al., 2017). In addition, ISG15 will also drive secretion of another cytokine, IL-10, from NK-92 cells and human PBMCs and mouse splenocytes, indicating that its effects on cytokine secretion pathway are not limited to IFN- γ . Like IFN- γ , IL-10 is induced at the transcriptional level by IL-12 signaling.

These observations indicate that cells used for ISG15-induced cytokine secretion must include (1) a mechanism for transcriptional induction of the cytokine (IFN- γ or IL-10), and (2) the ISG15 receptor, LFA-1. There are several lymphocyte cell lines and models that meet these requirements. For primary cells, human or mouse PBMCs will suffice, as well as splenocytes harvested directly from mouse spleens. The PBMCs and splenocytes are complex cell mixtures, but include NK and T cells. An advantage of the mouse splenocyte system is that spleens can be obtained from genetically altered mice (e.g., mice lacking CD11a, a component of LFA-1). While mouse ISG15 functions in signaling assays with human cells, human ISG15 does not function with mouse cells (unpublished results). For the most efficient signaling, we recommend using species-matched reagents (i.e., human IL-12 and human ISG15 for human cells, and mouse IL-12 and ISG15 for mouse cells). T and NK cell lines can also be used to study the extracellular signaling function of ISG15. NK-92 cells, an immortal cell line derived from a malignant non-Hodgkin's lymphoma, is one of only a few NK cell lines available, and these respond to IL-12 and ISG15 treatment to release IFN- γ . As mentioned earlier, Jurkat cells can be used if a mechanism to express IFN- γ is provided (e.g., transient transfection).

Reagents and Equipment

1. NK-92 cells (ATCC CRL-2407).
2. Jurkat E6-1 cells (ATCC TIB-152).
3. Primary PBMCs (Immunospot, CTL-UP1).
4. Mouse spleens (Jackson Labs).
5. NK-92 medium: MEM- α supplemented with myo-Inositol, Folic Acid, β ME, HEPES, Sodium Pyruvate, Penicillin, Streptomycin, L-Glutamine, Horse serum, and 10% Fetal bovine serum (FBS). Supplemented every 2 days with IL-2 (PeproTech 200-02, 100 μ /mL).
6. Jurkat Cell medium: RPMI 1640 (Sigma, R8758) supplemented with 1 $\times$ Penicillin, Streptomycin, and 10% FBS.
7. PBMC medium: CTL Test-medium (Immunospot; CTLT-010).
8. IL-12 human (BD Pharmingen 554613) or mouse (R&D Systems 419-ML).
9. Purified ISG15 (prepared as described earlier).
10. pGEM-T IFN- γ cDNA expression vector (SINO Biological).

11. NP40 Lysis buffer (100mM Tris-HCl pH 7.5, 100mM NaCl, 1% NP40).

Cell methods—Culturing NK-92 cells

1. NK-92 cells should be maintained between 5×10^4 and 1×10^5 cells per mL. Expect doubling time to be about 3–4 days.
2. Cells should be plated at 1×10^6 cells per mL (without IL-2) 16h before use for ELISAs.

Culturing Jurkat cells

1. Jurkat cells should be maintained between 1×10^5 and 1×10^6 cells per mL. Expect doubling time to be about 1 day.
2. Cells should be used at 2×10^6 cells per mL for cell-binding assays and in vitro IFN- γ release.

Jurkat cell transfections

1. Seed cells at 5×10^5 cells/mL 2 days prior to transfection.
2. Collects and resuspend 1×10^6 Jurkat cells in 100 μ L of Lonza SE Nucleofection reagent.
3. Add 2 μ g of DNA per sample (empty vector or pGEM-T IFN- γ).
4. Transfect cells by electroporation. We use a Lonza Nucleofector 4d with program cl-120.
5. Rest freshly transfected cells for 3h in RPMI 1650 with 10% serum, then plate at a density of 1×10^6 cells/mL.
6. Treat cells with between 15nM (450ng/mL) and 235nM (7.2 μ g/mL) ISG15 for 48h and assess IFN- γ release by IFN- γ ELISA.

Culturing PBMCs

1. Thaw PBMCs from in CTL Test Media (Cellular Technology Limited).
2. Plate cells at 1×10^6 cells per mL in CTL Test Media and culture overnight at 37°C with 5% CO₂.

Mouse splenocytes

1. Harvest spleen from one mouse with desired genetic background and wash in RPMI 1640.
2. Place spleen in a 10cm sterile dish with 10mL of PBS and poke 5–10 holes in the spleen with a 16-gauge needle.
3. Using the flat end of the plunger of a 3mL syringe to crush the spleen to release the splenocytes.
4. With a pipette, transfer tissue debris and released cells to a 15mL conical tube.
5. Allow large debris to settle to the bottom of the conical tube (about 8min).

6. Transfer cells to new tube being careful to not disturb large debris.
7. Centrifuge cells at $500 \times g$ for 5min.
8. Resuspend cell pellet in 10mL RPMI 1640 and count cells using a hemocytometer.
9. Adjust cell concentration to 1×10^6 cells per mL and aliquot 300 μ L into wells of a 48-well plate in preparation for cytokine secretion assays. One spleen will yield enough cells for more than 200 cytokine secretion assays, as described later.
10. Place in incubator for 16h at 37°C with 5% CO₂ before use in cytokine secretion experiment.

2.3 ELISAs for cytokine secretion

Enzyme-linked immunosorbent assays (ELISAs) are a simple and convenient way to assay cell culture supernatant for the presence of secreted cytokines. Commercial ELISA kits are available for both human and mouse IFN- γ and IL-10. Several controls are necessary for interpreting the IL-12/ISG15 secretion assays. Combined treatment with PMA and Ionomycin will induce strong IFN- γ expression and release from lymphocytes and serves as a positive control for both the health of the cells and the sensitivity of the ELISA. The effect of ISG15 on cytokine secretion is measured as the ability to release IFN- γ above that of IL-12 alone, and therefore treatment with IL-12 alone is a necessary control. Addition of ISG15 alone is an additional negative control; there should be no secretion of either IFN- γ or IL-10 above background from NK-92 cells or PBMCs. It is generally useful to perform a titration with increasing amounts of ISG15 (30–235 nM for NK-92 cells, PBMC, and mouse splenocytes).

ISG15-mediated IFN- γ release can be blocked by interfering with either the LFA-1/ISG15 interaction or by disrupting the signaling pathway initiated by LFA-1. We have demonstrated that the LFA-1 inhibitor A-286982 and the CD11a-specific antibodies MHM24 and TS1/22 inhibit IFN- γ secretion when added to NK-92 cells prior to the addition of IL-12 and ISG15. In addition, pretreatment of NK-92 cells with PP2, a SRC family kinase inhibitor, inhibits IFN- γ release.

Reagents and Equipment

1. ELISA kits (human IL-10: Thermo Scientific EHIL10, mouse and human IFN- γ : Thermo Scientific EMIFNG, EHIFNG).
2. Phorbol myristate acetate (PMA) (InvivoGen; tlr1-pma).
3. Ionomycin (InvivoGen; Inh-ion).
4. IL-12 (BD Pharmingen; 554613).
5. A-286982 (LFA-1 Inhibitor, Tocris; 280749-17-9).
6. PP2 (SFK Inhibitor, Adipogen; AG-CR1-35630-m001).
7. LFA-1 Antibodies (Developmental Studies Hybridoma Bank; MHM24, TS1/22).

8. Cells at appropriate densities (as described in Section 2) and purified ISG15 (as described in Section 1).
9. Multichannel pipettor.
10. 96-Well plate reader capable of reading absorbance at 450 and 550nm.

Procedures

1. Prepare ISG15 using the protocol described in Section 1. ISG15 should be used within 16h of PreScission protease cleavage.
2. Seed 300 μ L of cells for each condition being tested in a 48-well plate excluding wells at the plate perimeter. Nonadherent NK-92, PBMCs, and mouse splenocytes are seeded at 1×10^6 cells per mL. Leave overnight (16h) in appropriate media in the tissue culture incubator at 37°C with 5% CO₂.
 - a. In addition to having a well for each treatment condition, there should be a well for each of the following controls: untreated, IL-12 alone, ISG15 alone, and PMA (10 μ g/mL) plus Ionomycin (33nM) (PMA and Ionomycin added together to the same well).
 - b. Do not plate cells in the wells at the perimeter of the plate; instead, add 300 μ L of PBS to each of these wells to prevent evaporation during following incubations.
3. After cells have been seeded for 16h, treat with ISG15, IL-12 (6 ng/condition), and PMA/Ionomycin for 48h as well as any inhibitors you wish to test. Gently tap plate to mix.
 - a. Treatment with inhibitors should be done prior to the addition of ISG15 and IL-12.
 - b. Use PP2 (SFK inhibitor) or A286982 (LFA-1 inhibitor) at 10 μ M. Antibodies should be used in a concentration range of 5–500ng of protein.
4. Transfer cells and cell culture supernatants to 1.5mL tubes with a P1000 micropipette. Pellet the cells by centrifugation at $1000 \times g$ for 1min.
5. Transfer 300 μ L of the supernatants to wells of a 96-well plate.
6. From the 96-well plate, transfer samples, in duplicate, using a multichannel pipette, to ELISA plates provided in the kit at volumes indicated by the manufacturer. ELISA Plates are coated with antibodies against either IFN- γ or IL-10.
7. Follow the manufacturer's protocol for IFN- γ or IL-10 ELISAs.
8. Read plate at 450 nm in a 96-well plate reader as described in the kit and quantitate IFN- γ and IL-10 release by comparison to the standards provided.

2.4. Assays for ISG15-dependent SFK activation

An early step in ISG15-mediated IFN- γ signaling is the engagement of LFA-1 (CD11a/CD18), which triggers outside-in signaling, leading to activation of SFKs. Both ISG15 and ICAMs bind to LFA-1 via the α I domain of CD11a; however, they bind to the α I domain when it is in different configurations within the LFA-1 heterodimer. For ICAM binding, LFA-1 must be in an “extended” conformation that is triggered by inside-out signaling, as stimulated by phorbol esters (PMA), activation of G-protein signaling cascades, or activation of the T cell receptor (Abram & Lowell, 2009; Binnerts, van Kooyk, Simmons, & Figdor, 1994; Ding et al., 1999; Ghosh et al., 2006; Grönholm et al., 2016).

In contrast, inside-out signaling (PMA stimulation) actually blocks binding of ISG15 to LFA-1, strongly suggesting that ISG15 binds to the “closed” form of LFA-1 (unpublished data). Consistent with differing modes of binding, ICAM binding is dependent on magnesium ion binding to the α I MIDAS site (metal ion-dependent adhesion site), while ISG15 binding is independent of magnesium ion binding. A proximal step in the ISG15 signaling pathway is LFA-1-dependent activation of SFKs, which are activated within 10min of adding ISG15 to NK-92 cells. The predominant SFKs in NK-92 cells are Lyn, Hck, and Fgr, and a small-molecule inhibitor of these enzymes blocks ISG15- and LFA-1-dependent secretion of both IFN- γ and IL-10 (Swaim et al., 2017). The methods below describe assays for detecting ISG15-dependent SFK activation.

Reagents and equipment

1. SDS-PAGE equipment.
2. Jurkat Cells.
3. NK-92 Cells.
4. 37°C CO₂ incubator.
5. PBS.
6. NP40 lysis buffer (100mM Tris-HCl pH 7.5, 100mM NaCl 1% NP-40).
7. PP2 (SFK inhibitor Adipogen; AG-CR1-35630-m001).
8. SFK antibody (Cell Signaling Technologies; CST 2101).
9. A286982 (LFA-1 Inhibitor, Tocris; 4228).
10. 4%–12% Bis-Tris Gels (Thermo Scientific NW04120BOX).
11. Phosphatase/protease inhibitor tablets (Thermo Scientific 88669).

Procedures—Assays for activation of kinases by ISG15

1. Treat 300 μ L of 2×10^6 Jurkat cells/mL or 1×10^6 NK-92 cells/mL in their respective growth media with 1.8 μ g/mL ISG15 for 10min. If Inhibitors (e.g., 10 μ M PP2 or A286982) are to be used, pretreat cells for 1h with inhibitors.
2. Transfer cells to 1.5mL tubes by pipetting, centrifuge at $500 \times g$ for 1min, and discard the supernatant.

3. Lyse in 50 μ L NP40 lysis buffer supplemented with phosphatase/protease inhibitor tablets for 10min on ice. Spin sample for 15,000 $\times g$ for 10min and remove the supernatant to a new tube (discard pellet).
4. Add SDS-PAGE loading buffer and load entire sample on an SDS 4%–12% Bis–Tris gel. Transfer to nitrocellulose and blot for phosphorylated protein of interest. ISG15 should elicit a significant increase in phosphorylated SFKs (Swaim et al., 2017).

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Fig. 1.

Overview of the extracellular ISG15 signaling pathway and the basis of synergism with IL-12 signaling in Natural Killer cells. IL-12 engages the IL-12 receptor to induce signaling that leads to transcriptional activation of *INFG*, encoding IFN- γ . The IFN- γ is packaged into secretory vesicles. Extracellular ISG15 binds to the CD11a/ α L integrin component of the LFA-1 receptor (a heterodimer of CD11a/ α L and CD18/ β 2); the interaction is mediated by determinants on the C-terminal ubiquitin-like domain of ISG15 and the α domain of CD11a. Src Family Kinases (SFks), which include Lyn, Hck, and Fgr in NK cells, are rapidly activated in response to ISG15-LFA-1 engagement, as detected by autophosphorylation of the SFks. This leads to downstream signaling events (so far uncharacterized) that lead to enhanced secretion of IFN- γ . Similarly, IL-12 and ISG15 also act synergistically to promote the expression and secretion of IL-10 (not shown).